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Research Article
Enhancement of Pharmaceutical and Bioactive Components of *Scenedesmus obliquus* Grown Using Different Concentrations of KNO_3

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Abstract

Background and Objective: The growth rate, primary and secondary metabolites are influenced by nitrogen concentration of media. This study aimed to determine the effect of different concentrations of KNO_3 as a source of nitrogen on the growth, phytochemical components, cholesterol reduction, antioxidant and antibacterial activities of the green alga, *Scenedesmus obliquus* (*S. obliquus*). **Materials and Methods:** *Scenedesmus obliquus* was cultured in Kuhl's medium and KNO_3 was added with different concentrations in the medium at different concentration (0.12, 0.75, 1.5, 2.25 and 3 g L^{-1}). The effect of different five concentrations of nitrogen on protein and carbohydrates was determined. Antioxidant activity, total phenolic content (TPC) of alga extracts, cholesterol reduction and antibacterial activity were evaluated. The data analyses were carried out using SPSS software version 16. **Results:** The results revealed that the best KNO_3 concentration for algal growth and carbohydrate content is1.5 g L^{-1}. Meanwhile, the high lipid content was obtained with KNO_3 starvation. Medium containing 0.75 g L^{-1} of KNO_3 has the highest effect on the protein production of *S. obliquus*. The contents of dry alga extracted with chloroform: methanol and the extracted contents were determined by GC/MS chromatogram; the major contents were 5-Hydroxymethylfurfural followed by hexadecanoic acid (palmitic acid), cis-9-octadecenoic acid (oleic acid) and hexadecanoic acid and methyl ester, respectively. **Conclusion:** Potassium nitrate limitation caused the highest effect on the total phenol content (TPC), increased antioxidant capacity, cholesterol reduction activity and also antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Key words: Potassium concentrations, green algae, lipids, primary metabolites, secondary metabolites, antibacterial

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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

Microalgae are good producers of a broad variety of valuable compounds like lipids, carbohydrates, proteins, vitamins, amino acids and Beta-carotene that are used as feedstock for energy production, feed additives, food, cosmetics and medicine[1,2]. Numerous applications of microalgae such as aquaculture feeding, manufacture of active ingredients for cosmetics and food formulations due to promising sources of fine chemicals[3]. *Scenedesmus* sp. contains high nutritional and bioactive metabolite contents. So, it is used in numerous biotechnological applications[4]. Hamouda *et al.*[5] reported that *S. obliquus* produce high amount of carbohydrates over short periods. Also contains protein, all the essential amino acids, a good amount of minerals and lipid[6]. Antiproliferation and antioxidants agents could be used as ingredients that helps in health promotion and disease prevention had been extracted from green microalgae *S. obliquus*[7]. The favorable antioxidant compounds have been extracted from *S. obliquus* and used in food additives and active ingredients for therapeutics[3]. Green alga *Scenedesmus* showed antibacterial effect against *Staphylococcus aureus* and *Bacillus subtilis*[8]. Carotenoids and phenolic compounds are good potential source of natural antioxidants that had been extracted from microalgae *Chlorella* sp. and *S. obliquus*[9]. A major variety of microalgae commercialized for human nutrition such as *Spirulina*, *Chlorella*, *Dunaliella salina* and *Aphanizomenon flos-aquae*[10].

The present research aimed to study the influence of KNO_3 concentrations on *S. obliquus* growth, tannins, flavonoids, phenolic contents, cholesterol reduction effect, antibacterial and antioxidant activities those can be used in many biotechnological applications.

MATERIALS AND METHODS

The studying was carried out in the Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City; Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt and Department of Botany, Faculty of Science, Menoufia University, Egypt (2016) and all chemicals were used from Sigma Aldrich.

Alga: *Scenedesmus obliquus* was collected from river Nile and then purified and identified according to the method of Prescott[11]. The axenic culture was maintained on Kuhl agar slants[12] at 4°C.

***S. obliquus* cultivation using different KNO_3 (nitrogen) concentrations:** Standard Kuhl's medium was prepared and used for growth of the microalgae[12]. KNO_3 was added with different concentrations (0.12, 0.75, 1.5, 2.25 and 3 g L^{-1}) to the medium.

Determination of algal growth parameters: Algal growth was followed by measuring optical density of growth using Unico UV-2000 spectrophotometer[13]. Cell numbers were estimated using Neubour Haemocytometer. Growth rate (μ) and doubling time (T_2) were calculated by the following Eq:

$$\mu = \frac{\ln\left(N_2 - N_1\right)}{T_2 - T_1} \times 100$$

where, N_1, N_2 cell number at Time T_1 and T_2 while $T_2 = 0.6391/\mu$. Total carbohydrate content, total soluble proteins and lipids were estimated after 18 days of incubation period[14-16].

Analysis of extracted alga by GC/MS analysis: Oil content of alga was extracted by chloroform: methanol (1:1) was analyzed by GC/MS analysis[17].

Influence of different KNO_3 concentrations on antioxidant activity and total phenolic contents (TPC) of alga extracts: Half gram of each treatment of fine grind dried alga (*S. obliquus*) was soaked in 10 mL methanol for 48 h. The extracts were filtered and used for determination of total phenol content.

Cholesterol reduction effect: The cholesterol reduction by algal extracts was determined by using enzymatic colorimetric kit[18].

Antibacterial activity of algal methanol extracts that grown under different nitrogen concentrations: The antibacterial activity of methanol extracts of alga in comparison with Vancomycin as positive control was assessed against both *Escherichia coli* and *Staphylococcus aureus* using agar well diffusion method according to Perez *et al.*[19]. Algal methanol extract was dissolved in dimethyl sulfoxide (DMSO) which also used as negative control. Exactly 200 μL from algal methanol extract (1 mg mL^{-1}) was used for each well. The inhibition zones diameters were measured in mm after 24 h of incubation.

Statistical analysis: Results of the study were expressed as ±standard error of the mean. Significant differences between the means of parameters (LSD) were estimated using Duncan's multiple range tests ($p \leq 0.05$). All the above mentioned data analyses were carried out with SPSS software version 16[20].

RESULTS AND DISCUSSION

Influence of KNO_3 concentrations on *S. obliquus* growth: The effect of different concentrations of KNO_3 on *S. obliquus* growth is shown in Fig. 1. The best growth of alga was at 1.5 g L^{-1} KNO_3 after 13 days of cultivation. The lowest biomass was obtained in medium containing 0.75 g L^{-1} KNO_3. The best biomass was obtained when alga cultivated in concentration of 1.5 g L^{-1} KNO_3 followed by 2.25, 3, 0.75 and 0.12 g L^{-1} KNO_3, respectively. The minimum amount of *S. obliquus* biomass was obtained with KNO_3 limitation in Kuhl's medium. Table 1 shows the specific growth rate and doubling time of alga that cultivated under various KNO_3 concentrations. The exponential growth phase of alga grown in Kuhl's medium was achieved at concentrations of 1.5 g L^{-1} of KNO_3 at 10 days with specific growth rate of 0.427 and doubling time of 1.620. The green microalga *Parietochloris incisa* that grown on (+N) was possessed higher final biomass than the nitrogen-forbid (-N) cultures[21].

Effect of different KNO_3 concentrations on primary metabolites of *S. obliquus*: Table 1 shows the impact of KNO_3 concentrations on total carbohydrates, protein and lipids contents of *S. obliquus*. The total carbohydrates and protein of alga are significantly reduced at low KNO_3 concentrations, lead to an increase in lipids. The carbohydrate contents were 18.3, 17.49, 17.2, 15.31 and 9.65% of dry weight at 1.5, 2.25, 3.0, 0.75 and 0.12 g L^{-1} KNO_3 of medium, respectively. The protein contents of *S. obliquus* under different concentrations of KNO_3 were increased to 30.56 and 30.06 with 0.75 and 2.25 g L^{-1} of medium, respectively compared to control (1.5 g L^{-1} KNO_3). The lowest concentration of KNO_3 (0.12 g L^{-1}) significantly increased the lipid content to double compared with control, the lipid content was 26% with 0.12 g L^{-1} KNO_3 medium while it was 12% with control (1.5 g L^{-1} KNO_3 of medium). Lipids content of *S. obliquus* was increased when KNO_3 contents in media decrease, meanwhile total carbohydrate and protein contents were decreased. The lowest amount of protein was achieved with nitrogen limitation[22] reported that nitrogen starvation is essentially characterized by a large reduce in the protein pool. Thompson[23] reported that lipid accumulation of green algae increase of up to 2-3 folds might be expected under conditions of nitrogen deprivation. Nigam *et al.*[24] demonstrated that lipid content rises as nitrogen concentration decrease in the medium. Nitrogen is the highest

Table 1: Effect of KNO_3 concentrations on specific growth rate, doubling time carbohydrate, protein and lipids contents of *S. obliquus*

KNO_3 concentrations (g L^{-1} of medium)	0.12	0.75	1.5	2.25	3.0
Specific growth (μ/day)	0.391202	0.401638	0.427667	0.422926	0.415888
Doubling time (T_2 days)	1.771718	1.725682	1.620655	1.638819	1.666553
Carbohydrate (%)	9.65	15.31	18.13	17.49	17.2
Protein (%)	14.87	30.56	25.18	30.06	26.62
Lipids (%)	26	11.5	12	13	15.33

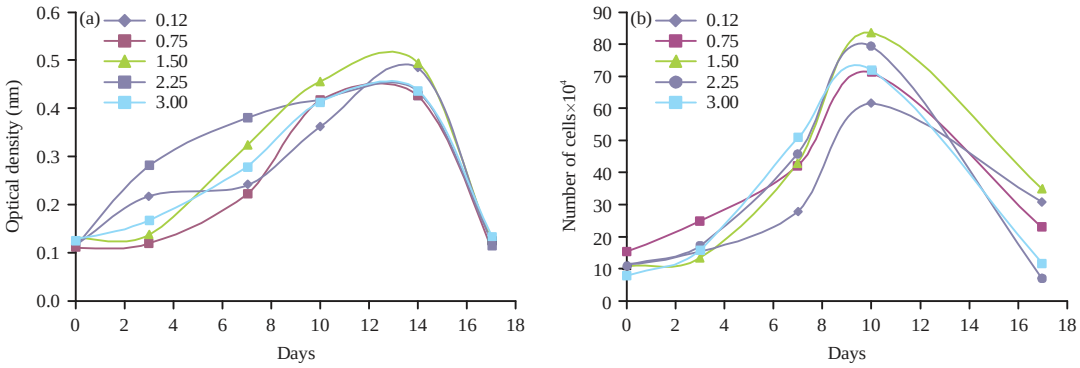

Fig. 1(a-b): Influence of KNO_3 concentrations on *S. obliquus* growth measured by (a) Optical density and (b) Cell numbers
Values were taken as SE

Table 2: GC/MS chromatogram of chloroform: methanol extraction from *S. obliquus*

Compounds	Formula and structure	Area (%)
5-Hydroxymethylfurfural	$C_6H_6O_3$	39.92
1-Octadecene	$C_{18}H_{36}$	1.28
Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	10.07
Hexadecanoic acid (palmitic acid)	$C_{16}H_{32}O_2$	16.32
Octadeconic acid methyl ester	$C_{19}H_{38}O_2$	10.90
Cis-9-octadecenoic acid (oleic acid)	$C_{18}H_{34}O_2$	9.54
Octadecanoic acid (stearic acid)	$C_{18}H_{32}O_2$	2.35
Octadecadienoic acid (linoleic acid)	$C_{19}H_{38}O_4$	1.16
Acetohydrazide 2-(3-Hydroxy-2-pentyl cyclopentyl)	$C_{24}H_{38}O_4$	1.27
Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl)	$C_{24}H_{38}O_4$	1.47
1, 2-1, 2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	0.84
Dioctyl phthalate	$C_{24}H_{38}O_4$	4.87

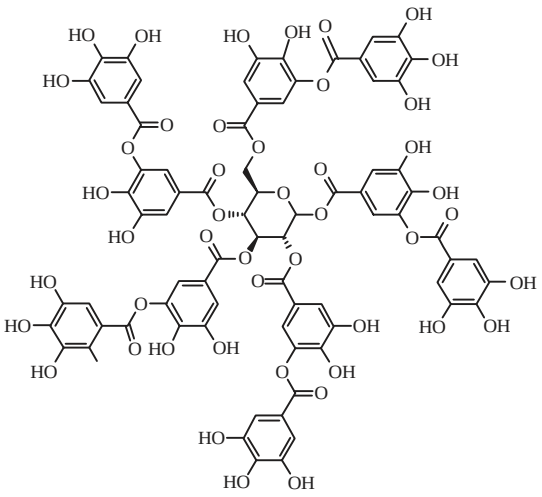

Fig. 2: Type of tannins (tannic acid, polyphenol compounds)

critical nutrient affecting in algae lipid metabolism. The accumulation of lipids, particularly TAG, related to nitrogen limitation has been noticed in a large number of species or strains of various microalgae[25]. In various microalgae, nitrogen starvation or limitation conditions are shown to enhance the biosynthesis and accumulation of lipids or carbohydrates or both[26].

GC/MS chromatogram of chloroform: methanol extraction of *S. obliquus*: The results obtained by GC/MS chromatogram of methanol: chloroform extraction of *S. obliquus* indicate the presence of twelve components of extract. These components are 5-Hydroxymethylfurfural, 1-Octadecene,hexadecanoic acid methyl ester, hexadecanoic acid (palmitic acid), octadeconic acid methyl ester, cis-9-octadecenoic acid (oleic acid), octadecanoic acid (stearic acid), octadecadienoic acid (linoleic acid), acetohydrazide 2-(3-Hydroxy-2-pentyl cyclopentyl), hexadecanoic acid 2-Hydroxy-1-(hydroxymethyl), 1,2-Benzenedicarboxylic acid, diisooctyl ester and dioctyl phthalate (Table 2). Chloroform: methanol extract of dry *S. obliquus* biomass showed many compounds that had many biotechnological applications. 5-Hydroxymethylfurfural (HMF) used for the production of biofuels and plastics[27]. Also it has multi-functional compounds such as intermediate for polymers, pharmaceuticals, fine chemicals and for the synthesis of other organic derivatives[28]. Li *et al.*[29] reported that 5-HMF has new marine natural antioxidant and prospective precursor for practical applications in the food, cosmetic and pharmaceutical fields. Hexadecanoic acid methyl ester has also been observed to cause autolysis of membranous structures, inhibit phagocytic activity, stimulate significant aortic dilation and nitric oxide production of various cells, diminish levels of tumor necrosis factor-alpha (TNF) and prostaglandin E2 (PGE2)[30]. Palmitic acid, oleic acid and linoleic acid were the three main compounds in the high-acid oil-biodiesel[31]. 1, 2-Benzenedicarboxylic acid, di-isooctyl ester has antimicrobial and antifouling[32]. Linton *et al.*[33] stated that the octadecanoic acid (OA) methyl ester had antiviral activity against measles disease virus. Stearic acid is used in the manufacture of pharmaceutical products[34]. Also used for a cyclosporine-A drug carrier system[35] and used for vanishing the bitter taste of pharmaceutical compounds[36].

Influence of different KNO_3 concentrations on tannins, flavonoids and phenolic contents of *S. obliquus*: Free radicals are controlled by natural products before attack cells and causes many diseases, these natural products are antioxidant. Tannins, phenolic compounds (Fig. 2) and flavonoids are accumulated by plants as secondary metabolites and considered antioxidant substances that advantage in the pharmaceutical industry.

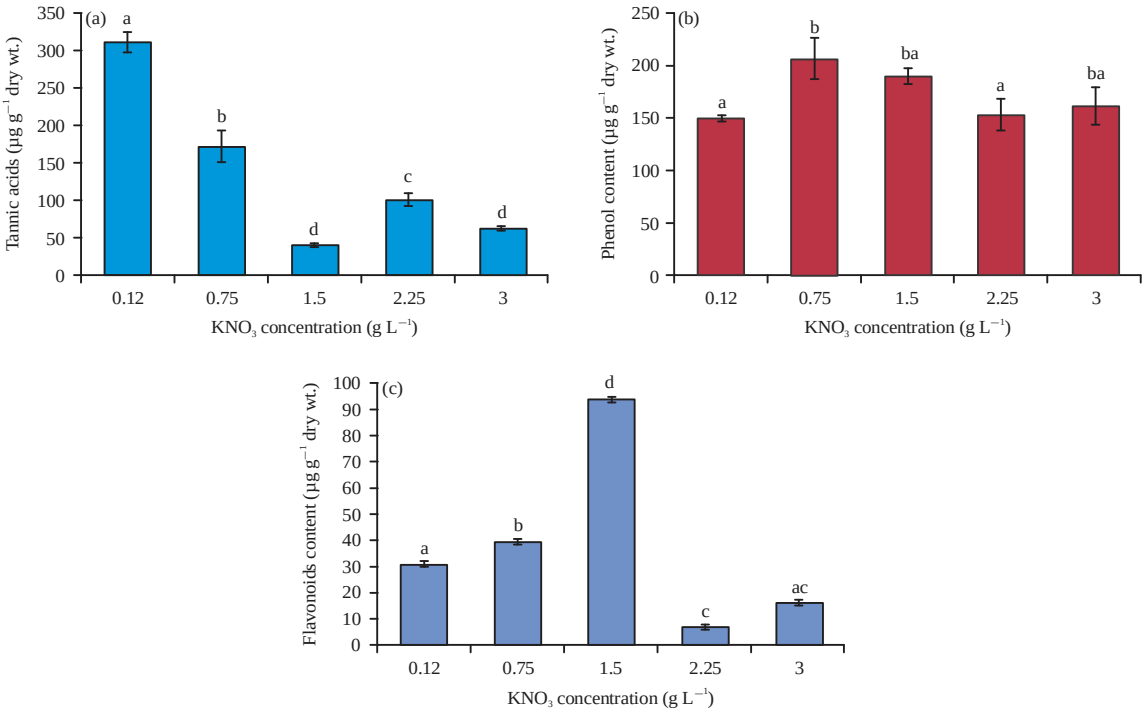

Fig. 3(a-c): Influence of different KNO_3 concentrations on bioactive components (a) Tannins, (b) Phenolic and (c) Flavonoids contents produced by of *S. obliquus*

Different small letter(s) on the bars indicate significant differences (p>0.05) between KNO_3 concentrations according to Duncan's multiple range test. Values were taken as SE

The effect of KNO_3 concentrations on tannins contents of *S .obliquus* was clear in Fig. 3. Potassium nitrate limitation enhances tannic acid content of *S. obliquus*. The highest phenolic contents of *S. obliquus* were recorded when grown at 0.75 g L^{-1} KNO_3 of Kuhl's medium followed by 1.5, 3.0, 2.25 and 0.12 g L^{-1} KNO_3, respectively. Figure 3 shows that the flavonoids content of *S. obliquus* was highest when alga cultivated with 1.5 g L^{-1} KNO_3 medium and the stress conditions had no effect on flavonoids content. Flavonoids are secondary metabolites and have the ability to act as antioxidant, antibacterial, anti-inflammatory and anti-cancer agent[37].

Antioxidant activity (DPPH), cholesterol reduction and antibacterial activity of methanol extract of *S. obliquus* grown under different concentrations of KNO_3: Figure 4 represents influence of KNO_3 concentrations on the antioxidant activity, cholesterol reduction and antibacterial activity of *S. obliquus*. The results revealed that the maximum amounts of antioxidant activity were present in alga grown under low amount of KNO_3 (0.12 and 0.75 g L^{-1}) followed by alga grown under a high amount of KNO_3 (3.0 g L^{-1}). Results clear that the high antioxidant activity present in *S. obliquus* cultivated under stress conditions with low and high concentrations of KNO_3. The antioxidant activities of *S. obliquus* extracts around (59.8-64.3%) was recorded by Ali *et al.*[9]. Biochemical content of *Scenedesmus* sp. possessed antioxidant properties and used in the neutraceutical industry[38].

The results clear that under KNO_3 deprivation, *Scenedesmus* secondary metabolites have been affected and hence affect the cholesterol reduction. The high levels of cholesterol reduction (80.12 and 77.9%) were observed with culture grown under KNO_3 concentrations at 0.12 and 0.75 g L^{-1} KNO_3, respectively. The low concentrations of KNO_3 in Kuhl's medium were caused high lowering of cholesterol. The *Scenedesmus acutus*-enriched diet prevented an excessive deposition of cholesterol in the liver[39].

The methanol extract of *S. obliquus* showed significant inhibition activities against *Staphylococcus aureus* and *Escherichia coli*. The highest zone of inhibition was observed at 0.75 g L^{-1} KNO_3. Both concentrations of nitrogen (0.75 and 3.0 g L^{-1} KNO_3) showed highest algal growth and also highest antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Salem *et al.*[8] reported that *Scenedesmus* sp. can serve as a potential antibacterial agent against food-borne pathogen of *S. aureus*.

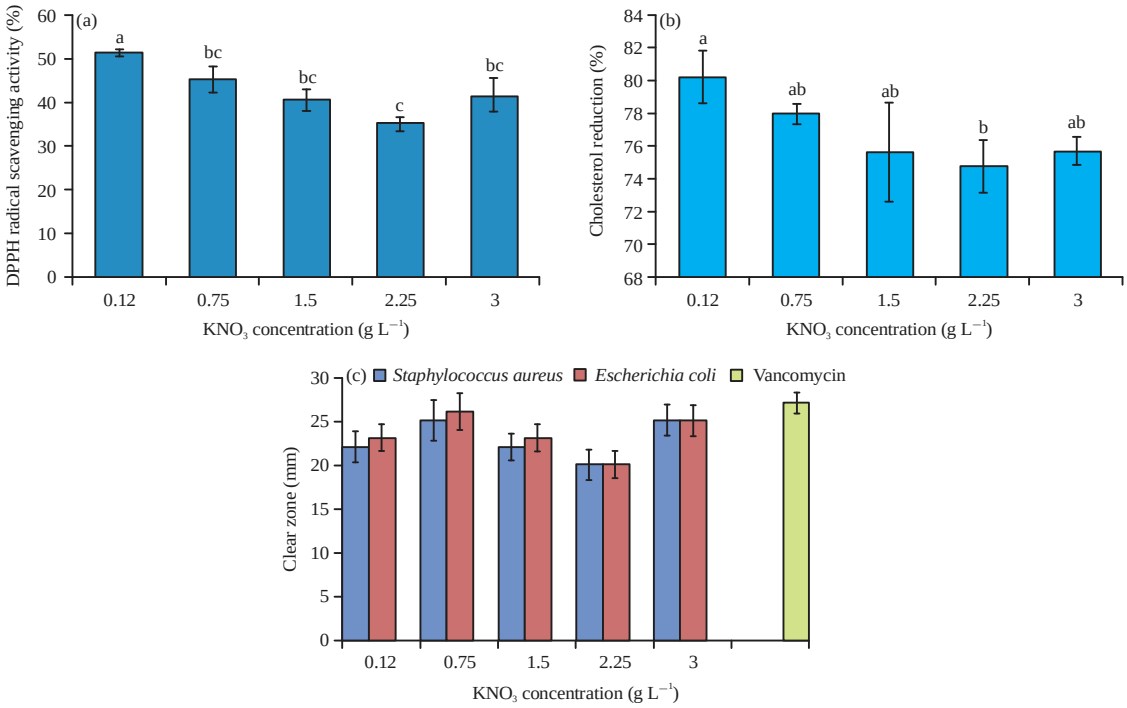

Fig. 4(a-c): (a) DPPH radical scavenging activity (%), (b) Cholesterol reduction (%) and (c) Antibacterial activity (mm) of *S. obliquus* grown with different KNO_3 concentrations

Different small letter(s) on the bars indicate significant differences ($p>0.05$) between KNO_3 concentrations according to Duncan's multiple range test. Values were taken as SE

CONCLUSION AND FUTURE RECOMMENDATIONS

KNO_3 concentrations effect on the growth, primary metabolites (lipids, proteins and carbohydrates)and secondary metabolites (phenolic, tannins and flavonoids) of algae. Antibacterial activity, antioxidant activity and lowering cholesterol activity of *S. obliquus* varied with KNO_3 concentration in the medium. The tannins content, antioxidant activity and also cholesterol reduction of alga are significantly increased at low KNO_3 concentrations. The best concentration of KNO_3 (0.75 g L^{-1} nitrogen medium) significantly increases production of phenol and antibacterial activity. 1.5 g L^{-1} of KNO_3 medium) was best for flavonoids content. The extraction contents of *S. obliquus* were determined by GC/MS chromatogram and the major content present is 5-Hydroxymethylfurfural (HMF) that used for the production of various high-volume plastics, foods and treatments followed by palmitic acid (C16:0) that is display antioxidant.

That different concentration of nitrogen can enhance primary and secondary metabolites of *Scenedesmus obliquus* and significantly increases the production of bioactive compounds. Much study still needs to be done in such area.

SIGNIFICANCE STATEMENT

This study discovers the effect of various concentrations of KNO_3 on the growth, production of primary and secondary metabolites of the micro green alga, *Scenedesmus obliquus* and also the effect of total phenolic content (TPC), antioxidant activities, cholesterol reduction and antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* were studied that can be beneficial for studying the contents of *S. obliquus* extract that was determined by GC/MS analysis. This study will help the researchers to uncover the critical areas of that the potassium nitrate limitation effect on the contents of *S. obliquus* extract, that many researchers were not able to explore. Thus a new theory on the effect of potassium nitrate on the production of primary and secondary metabolites of the green alga, *Scenedesmus obliquus* may be arrived.

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