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Research Article

Effects of vegetable amino acids and oils supplementation on *Chlorella vulgaris* amino acid profile

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Abstract

The aim of the present research is to investigate the profile of amino acid of *Chlorella vulgaris* supplemented with some plant amino acid and oil extracts. The bioactive oil, and amino acid from four Iranian medicinal plants, namely *Oliveria decumbens*, *Thymus kotschyanus*, *Trachyspermum ammi* and *Zataria multiflora* were obtained. The LC-MS / MS analysis demonstrated that the major amino acids component of *Chlorella vulgaris* were alanine (30.4 mg/g), glycine (23.2 g/g), glutamic acid (22.7 mg/g), aspartic acid (21.1 mg/g), arginine (20.9 mg/g), leucine (18.9 mg/g), lysine (16.5 mg/g), phenylalanine (14.0 mg/), serine (13.0 mg/g), asparagine (12.7 mg/g), valine (12.6 mg/g), isoleucine (11.5 mg/g), and tyrosine (11.0 mg/g). The major fatty acids of the plants detected are 9, 12, 15-octadecatrienoic acid, 9, 12-octadecadienoic acid, 9-octadecenoic acid, and hexadecanoic acid but with different concentrations. The major non-fat components (lipophilic monoterpenoid) detected in the oils were thymol and carvacrol with different concentrations. Vegetable oils mainly composed of polyunsaturated fatty acids and reduced amino acid contents in *Chlorella vulgaris*. The main amino acids from plant-derived protein hydrolysate were aspartic acid, glycine, glutamic acid, leucine, alanine, lysine, arginine, serine, phenylalanine, valine, proline, and histidine but with different levels. Vegetable amino acids mainly composed of functional amino acid and significantly enhanced amino acids production in *Chlorella vulgaris*. *Zataria multiflora*, *Trachyspermum ammi* and *Thymus kotschyanus* amino acid supplementations as nitrogen and carbon sources also exhibited the same and better effects on the amino acid profile compared to the *Oliveria decumbens* amino acid supplementation.

Keywords: *Chlorella vulgaris;* Amino acid profile; Fatty acid supplementation; Amino acid supplementation

Introduction

Presently, the universal resources of omega-3 fatty acid especially α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are fish oil. However, due to the depletion of fish oil supplies, high costs of polyunsaturated fatty acid (PUFA) purification and pollution of marine environments, other alternative raw materials are being evaluated to replace fish oils with microalgae which are the natural primary producers of PUFA to fulfill the consumers need for a more sustainable food supply. These microorganisms are rich in many nutrient compounds such as carbohydrates, vitamins, pigments, lipids, proteins, minerals, and other nutraceuticals (Koyande *et al*., 2019). *Chlorella vulgaris* is a unicellular, freshwater microalgae with high growth rate, high protein and lipid contents, and resistant to climatic variations that makes it a very interesting microalgae from the health benefits point of view (Liang *et al*., 2019). Omega-3 fatty acids (ALA, EPA and DHA) are important in reducing blood cholesterol, preventing cardiovascular diseases and obesity (Prasad *et al*., 2020). Essential amino acids, play an important role in the formation and function of enzymes, food digestion, and molecular transport, which could be used to improve human health (Amorim *et al*., 2020).

Some reports have indicated that some nutritional disorders such as nitrogen and iron deficiency, salt stress and also physical environments (temperature and light intensity) and physiological factors (growth phase, physiological status) affect the lipid contents, fatty acids, and amino acids compositions in microalgae (Aratboni *et al*., 2019). The possibility of modulating lipid and protein concentrations through changes in culture medium has increased ongoing researches since the mid-twentieth century, either for biomass synthesis or to meet the human/animal nutritional demands (Lohri *et al*., 2017). Considerable information on microalgae has been accumulated about the microalgae potential in the utilization of various substrates such as organic acids, amino acids, sugars, and perilla seed meal as carbon and nitrogen sources for growth and lipid and protein production (Dave and Routray, 2018). Besides, the presence of carrier systems for amino acid uptake and transport that allow algae to grow and survive under certain environmental conditions has also been reported (Marchand *et al*., 2018).

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The relationship between human health and marine foods including microalgae, is progressively becoming topics of interest. The process of food breakdown in the gastrointestinal tract is important in educating food quality. The microalgae must pass through the digestive system and decompose under the influence of gastric acid in order to release nutritional substances. Therefore, a digestion model similar to the one present in gastric and intestinal tracts can provide a more accurate estimate of microalgae nutritional quality. In vitro digestion models have been widely used to overcome the limitations associated with *in vivo* methodology (Mulet-Cabero *et al*., 2020). Algae nutrition starvation or limitation is thought to be a feasible approach to induce lipid and amino acid productions. Many studies have reported that the composition and quantity of lipid production can be influenced by organic carbon sources (Choi, 2020) including sugar substrates, especially glucose (Miao and Wu, 2006) and acetate as other carbon sources (Liang *et al*., 2009).

However, the effects of medicinal plant oils and amino acids on the amino acid profile of microalgae have not been investigated extensively. In the present study, plant oils and amino acids from four medicinal plants, namely *Oliveria decumbens*, *Thymus kotschyanus*, *Trachyspermum ammi* and *Zataria multiflora*, were added at the end of the logarithmic phase to the culture medium and their effects on *Chlorella vulgaris* amino acid profiles were examined. The overall aim of this research was to determine and improve the amino acids profile of *Chlorella vulgaris* mixotrophically supplemented with plant oils and amino acids in a mixotrophically condition.

Materials and Methods

Plant materials: The aerial part of *O. decumbens* (herbarium number: 55078) were collected from the mountainous areas of kazeroon (Fars, Iran). The seeds of *T. ammi* were prepared from local perfumeries of Shiraz city (Iran). *Z. multiflora* (herbarium number: 24985) was collected from the mountainous areas of Marvdasht (Fars, Iran). The aerial parts of *T. kotschyanus* (herbarium number: 65110) were prepared from mountainous areas of Marvdasht (Fars, Iran). The taxonomy of plants was confirmed kindly by Professor A.R. Khosravi, a plant taxonomist in Shiraz University (Iran).

Preparation of oil and amino acid extracts from plants: Plant material powders (100 grams) were suspended in 1000 ml hydrolysis buffer containing normal saline: methanol: hydrochloric acid (1:1:2), mixed carefully, and incubated at 70 °C for three days to hydrolyze plant biomass. In this condition proteins, lipids and carbohydrates digested to amino acid, fatty acid and monosaccharide, respectively. To remove fatty acids, 500 ml hexane was added to normal saline: methanol: hydrochloric acid hydrolysate and vortexed for 10 min. The fatty acid was separated from plant biomass overnight at room temperature. The fatty acid in the upper phase (hexane phase) was separated and used for chemical analysis by gas chromatography-mass spectrometry (GC-MS). The hexane evaporated with rotary evaporator at room temperature to concentrate the oil. The remaining oil were dissolved in dimethyl sulfoxide solution (10 µg/ml) containing free fatty acid bovine serum albumin (100 µg/ml) and incubated at 70 °C for 60 min then at ambient temperature for two days to complete emulsification. Separated amino acids in the lower phase were used for chemical analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Amino acid extracts lyophilized and the powder was dissolved in phosphate buffered saline (PBS). The concentration of amino acids was determined by ninhydrin using glycine as standard. The amino acids were adjusted to 10 mg/ml using PBS for further experiments (Mulet-Cabero *et al*., 2020).

Microalgae culture and treatment: Freshwater strain of microalgae *Chlorella vulgaris* was isolated from water and soil of Northern provinces of Iran by agar plate method. *Chlorella* was cultured in bold basal medium (BBM) for purification and then transferred to same liquid medium. Cultures were bubbled and illuminated continuously. Light was prepared via fluorescent lamps (3000 lux). Temperature was regulated at 28 °C and pH was adjusted at 7. The algae were transferred to semi-large scale media (1.0 L Erlenmeyer flasks) for enhancement of biomass. Culture was aerated by a mechanical pump passing through a 0.22 µm filter. Algal cells at the end of the logarithmic phase (10-12 days) supplemented with 5 ml/1000 ml of plant oil extracts and amino acid extracts (5 g/1000 ml) and the cultures were incubated for 4-5 days. Biomasses from supplemented algal cultures were harvested at the end of the stationary phase by centrifugation, washed twice with deionized water to remove attached residuals, and dried by lyophilisation (Gaffney *et al*., 2014). Dried biomass was used for amino acid profiling using LC-MS/MS.

Preparation of amino acid and fatty acid from microalgae: An in vitro digestion method containing normal saline: methanol: hydrochloric acid (1:1:2) was used to hydrolyze proteins to amino acids while simultaneously preparing fatty acids methyl ester from microalgae as mentioned above. For the separation of fatty acid methyl esters from amino acids, hexane was added to the mixture of normal saline: methanol: hydrochloric acid (1:1:2) hydrolysate. Briefly, freeze-dried microalgae mass (1000 mg) was suspended in 10 ml hydrolyzing buffer containing normal saline: methanol: hydrochloric acid (1:1:2), and incubated at 70°C for two days to completely hydrolyze proteins to amino acids and also to prepare fatty acids methylesters. To the hydrolysate, 5.0 ml hexane were added in the test tubes and vortexed for 10 min. The amino acid and fatty acid methyl esters were separated in normal saline: methanol: hydrochloric acid: hexane mixture over night at room temperature. The fatty acid methyl ester in the

upper phase (hexane phase) was separated and used for chemical characterization by GC-MS. Amino acids in the lower phase were separated and used for chemical characterization by LC-MS/MS (Mulet-Cabero *et al*., 2020).

Fatty acids profiling by GC-MS: The separated fatty acid methyl esters were used for compound analysis by GC-MS. GC-MS analysis was done using an Agilent gas chromatogram (7890B GC 7955AMSD) equipped with a silica capillary column (HP-5MS, 30 m × 0.25 mm × 0.25 µm) coupled with a quadrupole mass spectrometer. Helium (99.99%) was applied as the carrier gas. The temperature of the ion source and fixed interface was set to 230 and 290 °C, respectively. The oven temperature was programmed as follows: 80 °C for 4 min, rising at 20 °C/min to 140 °C, rising at 10 °C/min to 250 °C and kept for 10 min at this temperature. Fatty acids were identified by comparing their fragmentation arrangements of the related peaks with those informed in the libraries of NIST05 and Wiley 7n (Tammekivi *et al*., 2019).

Amino acids profiling by LC-MS/MS: An Agilent 1100 L/MS series (G1313) mass spectrometer was used to carry out LC-MS/MS analyzes. The LC separation was performed on a 5MS column with an injection volume of 10 µl and a temperature column of 40 °C. The mobile phases included A (0.1 % formic acid in water) and B (0.1% formic acid in acetonitrile) running at a flow rate of 250 µl/min. Elution was programmed as a linear gradient that began by increasing from 5 to 100% B solution for 15 min, held at 100 % B for 3 min, decreasing from 100 to 5% in 1 min, and then maintained at 5% B for 2 min. Mass spectra were generated in the positive ion mode electrospray ionization (ESI) source. Nitrogen was used as the drying gas, The ESI spray voltage was 5500v. Full scan mass spectra were obtained over an m/z range of 30 to 3000. The amino acids were identified by the LC-MS/MS apparatus by comparing both their retention times and fragmentation patterns of the corresponding peaks with those reported in the literature. For the identification of amino acids, various biochemical databases and standard libraries, including the human metabolome database (HMDB) and the NIST mass spectral library (NIST 14) were used (Young *et al*., 2014).

Statistical analysis: The significant differences of amino acids concentrations in the treatments were statistically determined by the analysis of variance (Univariate) using the Tukey test in SPSS ver.22. Significant levels were recorded at $P< 0.05$. Each test was run in the three independent experiments.

Results and Discussion

Fatty acid compositions of the plant oil extracts: The main fatty acids detected in the prepared oil extracts from the studied plants are shown in Table S1 (In supplemental file). The most abundant compounds in *O. decumbens* oil were thymol (26.7%), octadecadienoic acid (23%), octadecatrienoic acid (19.8%), and hexadecanoic acid (15.5%). The main components in the *T. kotschyanus* oil were octadecatrienoic acid (35.4%), thymol (29.7%), hexadecanoic acid (14.8%), octadecadienoic acid (8.9%), as well as carvacrol (5.7%). The main components in the oil of *T. ammi* were octadecenoic (43.1%), thymol (23.8%), octadecadienoic acid (19.3%), hexadecanoic acid (5.7%) and cuminaldehyde (5.2%). Major components in the *Z. multiflora* oil were; carvacrol (48.5%), octadecatrienoic acid (15.5%), thymol (10.3%), hexadecanoic acid (8.7%), octadecadienoic acid (7.6%), and octadecenoic acid (6.6%). The medicinal plants analyzed in our study are important not only as sources of essential fatty acids but also are superior in terms of ω6/ω3 balance that is regarded as a priority in dietary supplements. For that reason, the plant extracts that are rich in essential fatty acids including omega-3 and omega-6 can have multiple biological actions. Data on the chemical compositions and nutritional values of the plants are highly variable which are due to structural characteristics, type of plant, growth cultures, geographical regions, processing technologies required to extract oil and fractionation of oil constituents. The major fatty acids of the plants considered are 9, 12, 15-octadecatrienoic acid, 9, 12-octadecadienoic acid, 9-octadecenoic acid, and hexadecanoic acid but with different concentrations. The major non-fat components (lipophilic monoterpenoid) detected in the oils were thymol and carvacrol with different concentrations.

Amino acid compositions of the plant protein extracts: The main amino acid compositions of *O. decumbens*, *T. kotschyanus*, *T. ammi*, and *Z. multiflora* along with essential amino acids, non-essential amino acids, and non-protein amino acids obtained from *in vitro* digestion are summarized in Table S2 (In supplemental file). The yield of protein amino acid was between 23-34%. The main amino acids in *O. decumbens* protein consisted of aspartic acid (2.22 g/100g), glutamic acid (2.04 g/100g), leucine (1.96 g/100g), alanine (1.82 g/100g), lysine (1.77 g/100g), glycine (1.64 g/100g), serine (1.59 g/100g), arginine (1.51 g/100g), proline (1.25 g/100g), and phenylalanine (1.20 g/100g). The major amino acids in *T. ammi* protein were glycine (4.25 g/100g), glutamic acid (3.93 g/100g), aspartic acid (4.04 g/100g), arginine (2.97 g/100g), lysine (2.45 g/100g), alanine (2.41 g/100g), leucine (2.30 g/100g), serine (1.79 g/100g), phenylalanine (1.54 g/100g), histidine (1.46 g/100g), valine (1.33 g/100g), proline (1.09), and tyrosine (1.05 g/100g). *T. kotschyanus* protein contained mainly aspartic acid (5.39 g/100g), glycine (3.51 g/100g), leucine (2.92 g/100g), glutamic acid (2.90 g/100g), alanine (2.79 g/100g), lysine (2.67 g/100g), arginine (2.14 g/100g), phenylalanine (1.96 g/100g), serine (1.90 g/100g), valine (1.47 g/100g), histidine (1.18 g/100g), proline (1.10 g/100g), as well as tyrosine (1.00 g/100g). The most abundant amino acids in *Z. multiflora* proteins were glycine (1.93 g/100g), aspartic acid (1.64 g/100g),

serine (1.47 g/100g), alanine (1.45 g/100g), lysine (1.38 g/100g), glutamic acid (1.37 g/100g), arginine (1.33 g/100g), leucine (1.35 g/100g), histidine (0.94 g/100g), and proline (0.84 g/100g). The main amino acids from plant-derived protein hydrolysate were aspartic acid, glycine, glutamic acid, leucine, alanine, lysine, arginine, serine, phenylalanine, valine, proline, and histidine but with a different level. The differences in the chemical composition of amino acid may be due to structural characteristics, type of plants, growth cultures, geographical regions, and genetic parameters of plants.

Amino acid profile of *Chlorella* in response to vegetable oils: The effects of oil supplements on the yield and amino acid compositions are shown in Table 1. The LC-MS / MS analysis demonstrated that the major amino acids component of *C. vulgaris* are alanine (30.4 mg/g), glycine (23.2 g/g), glutamic acid (22.7 mg/g), aspartic acid (21.1 mg/g), arginine (20.9 mg/g), leucine (18.9 mg/g), lysine (16.5 mg/g), phenylalanine (14.0 mg/), serine (13.0 mg/g), asparagine (12.7 mg/g), valine (12.6 mg/g), isoleucine (11.5 mg/g), and tyrosine (11.0 mg/g). The major amino acid components of *C. vulgaris* supplemented with *O. decumbens* oil were glycine (19.35 mg/g), arginine (17.10 mg/g), lysine (16.47 mg/g), leucine (16.20 mg/g), serine (12.15 mg/g), and threonine (9.27 mg/g). The major amino acid components of *C. vulgaris* supplemented with *T. kotschyanus* oil were alanine (24.75 mg/g), glycine (19.71 mg/g), glutamic acid (18.09 mg/g), arginine (17.10 mg/g), aspartic acid (17.01 mg/g), lysine (16.47 mg/g), leucine (15.21 mg/g), serine (12.15 mg/g), phenylalanine (11.16 mg/g), and valine (10.89 mg/g). The major amino acid components of *C. vulgaris* supplemented with *T. ammi* oil were alanine (26.10 mg/g), glycine (20.79 mg/g), glutamic acid (18.72 mg/g), arginine (18.09 mg/g), aspartic acid (18.09 mg/g), lysine (16.92 mg/g), leucine (15.75 mg/g), serine (12.78 mg/g), phenylalanine (11.70 mg/g), valine (11.43 mg/g), as well as threonine (9.36 mg/g). The major amino acid components of *C. vulgaris* supplemented with *Z. multiflora* oil were alanine (25.02 mg/g), glycine (19.62 mg/g), glutamic acid (18.45 mg/g), arginine (17.28 mg/g), aspartic acid (17.28 mg/g), lysine (15.57 mg/g), leucine (15.48 mg/g), serine (11.79 mg/g), phenylalanine (11.34 mg/g), and valine (10.80 mg/g).

The amounts of non-essential amino acids were much higher than the essential amino acids in all supplementation studies. The highest concentration of amino acids, essential amino acids, and non-essential amino acids were found in *Chlorella* cultivated under the *Trachyspermum* oil supplementation. Regarding amino acid profile, *Chlorella* supplemented with *Trachyspermum* oil had higher concentrations of non-essential amino acids, such as alanine, arginine, aspartic acid, glutamic acid, and glutamine (Table 1). Alanine was the most prevalent amino acid ranging from 24.30 mg/g in *Oliveria* to 30.46 mg/g in control. Glutamic and glycine were in the second position. Aspartic and arginine occupied the third position in terms of quantity after alanine, glutamic acid, and glycine in *Chlorella*, accounting, 17.82 to 23.20; 19.35 to 20.90; 16.47 to 22.70 and 17.10 to 21.10 mg/g, respectively (Table 1).

The quantity of total essential amino acids found in *Chlorella* showed little variation and ranged from 78.39 to 90.80 mg/g (Table 1). The lowest concentration of essential amino acids was found in *Chlorella* supplemented with *Zataria* oil, while the highest was in *Chlorella* supplemented with *Trachyspermum* oil. Considering the essential amino acid, the amount of leucine in the culture supplemented with *Oliveria* oil was the highest, whereas, the amounts of threonine, valine, phenylalanine, and lysine were higher in cultures supplemented with *Trachyspermum oil* (Table 1). Leucine (Yang *et al*., 2015) and Isoleucine (Newmire *et al*., 2019) are considered functional amino acids because they can stimulate the pancreas to produce insulin and can be considered as a natural remedy for type 1 diabetes. Lysine improves both stress tolerance and lipids metabolism and also enhances the formation of antibodies, hormones, and enzymes (Sulochana *et al*., 1998).

With respect to the effect of vegetable oils on the amino acid profile, it can be concluded that, nitrogen sources are necessary and are the most important factors for in protein synthesis. In this study, at the end of the growth phase due to nitrogen depletion in the culture media, the amino acid profile in *Chlorella* has undergone many changes both in quantity and quality terms. In addition, supplementing media with plant oils as carbon sources were not effective in amino acid production, so that, total amino acids, essential and non-essential decreased as compared to control (Pleissner *et al*., 2017). Plant oils are broken down by the β-oxidation pathway yielding acetyl-CoA. Acetyl-CoA trough tricarboxylic acid cycle converts to energy and ketoacids like citrate in mitochondria. Citrate can transfer from mitochondria to cytosol and again convert to acetyl-CoA and participate in the synthesis of fatty acids (Figure 1). However, the amount of the proline and histidine amino acids increased compared to the other individual amino acids. The reason may be that, glutamic acid transferred from mitochondria to cytosol through glutamate carrier and converted to proline. Regarding the histidine, phosphoribosyl pyrophosphate is a precursor for the production of histidine. The increase of the histidine content may be due to the increased pentose phosphate pathway activity followed by an increase in the contents of ribose-5-phosphate and phosphoribosyl pyrophosphate. Whereas, ornithine decreased noticeably. Because it is an intermediate compound in the metabolic pathways, it can converted to proline, arginine and puterscine. Puterscine converted to gama-aminobutyric acid and transferred to mitochondria and converted to the succinate and participated to the tricarboxylic acid (citrate), finally produced energy and acetyl CoA. Totally, it can be found that, oil treatments mostly influence β-oxidation, fatty acid synthesis and glutamic acid and ornithine

Table 1. Amino acid composition (mg/g) of *Chlorella* treated with fatty acid (FA) from Oliveria *decumbens* (OD), *Zataria multiflora* (ZM), *Trachyspermum ammi* (TA), *and Thymus kotschyanus* (TK).

Amino acid	Chlorella	Chlorella-OD - FA	Chlorella-TK - FA	Chlorella-TA-FA	Chlorella-ZM- FA
Alanine	30.40(1.65)[b]	24.30(1.46)[a]	24.75(1.44)[a]	26.10(1.31)[a]	25.02(1.30)[a]
Glycine	20.90(1.12)[a]	19.35(1.16)[a]	19.71(1.14)[a]	20.79(1.04)[a]	19.62(1.02)[a]
Glutamic acid	23.20(1.10)[b]	17.82(1.07)[a]	18.09(1.05)[a]	18.72(0.94)[a]	18.45(0.96)[a]
Arginine	21.10(1.0)	17.10(1.03)	17.10(0.99)	18.09(0.90)	17.28(0.90)
Aspartic acid	22.70(1.11)[b]	16.74(1.0)[a]	17.01(0.99)[a]	18.09(0.90)[a]	17.28(0.90)[a]
Lysine	18.90(1.0)	16.47(0.99)[a]	16.47(0.96)[a]	16.92(0.85)[a]	15.57(0.81)[a]
Leucine	16.50(0.83)[a]	16.20(0.97)[a]	15.21(0.88)[a]	15.75(0.79)[a]	15.48(0.80)[a]
Serine	12.60(1.00)[a]	12.15(0.73)[a]	12.15(0.70)[a]	12.78(0.64)[a]	11.79(0.61)[a]
Phenylalanine	13.00(1.12)[b]	10.80(0.65)[a]	11.16(0.65)[a]	11.70(0.59)[a]	11.34(0.59)[a]
Valine	14.00(1.15)[b]	10.89(0.65)[a]	10.8990.65)[a]	11.43(0.57)[a]	10.80(0.56)[a]
Tyrosine	8.20(0.43)[a]	8.37(0.50)[a]	8.73(0.51)[a]	9.00(0.45)[a]	8.91(0.46)[a]
Isoleucine	11.50(0.92)[b]	9.18(0.55)[a]	8.10(0.47)[a]	8.46(0.42)[a]	8.64(0.45)[a]
Threonine	11.00(0.85)[b]	9.27(0.56)[a]	9.18(0.51)[a]	9.36(0.42)[a]	8.37(0.44)[a]
Proline	3.30(0.14)[a]	5.13(0.31)[b]	5.31(0.31)[b]	5.40(0.27)[b]	5.13(0.27)[b]
Methionine	5.90(0.22)[b]	4.41(0.26)[a]	4.41(0.26)[a]	4.59(0.23)[a]	4.59(0.24)[a]
Asparagine	5.60(0.20)[c]	0.99(0.06)[a]	0.90(0.05)[a]	2.16(0.11)[b]	4.32(0.22)[c]
Histidine	1.10(0.10)[a]	4.05(0.24)[b]	4.05(0.23)[b]	4.32(0.22)[b]	3.60(0.19)[b]
Ornithine	12.70(1.10)[b]	0.54(0.03)[a]	0.90(0.05)[a]	1.26(0.06)[a]	0.90(0.05)[a]
Cystine	1.20(0.08)[b]	0.81(0.05)[a]	0.90(0.05)[a]	0.81(0.04)[a]	0.81(0.04)[a]
Beta-alanine	0.70(0.05)[a]	0.54(0.03)[a]	0.54(0.03)[a]	0.72(0.04)[a]	0.72(0.04)[a]
Total	254.6(8.5)[c]	205.1(10.31)[a]	205.8(9.94)[a]	216.72(8.84)[b]	208.8(7.86)[a]
∑NEAA	160.60(7.7)[b]	121.86(7.31)[a]	123.75(7.18)[a]	131.13(6.56)[a]	127.71(6.64)[a]
∑EAA	90.80(4.5)[b]	81.18(4.87)[a]	79.47(4.61)[a]	82.53(4.13)[a]	78.39(4.08)[a]
∑NPAA	3.20(0.17)[b]	2.07(0.12)[a]	2.61(0.15)[a]	3.06(0.15)[a]	2.61(0.14)[a]
EAA/NEAA	0.57(0.12)[a]	0.67(0.04)[a]	0.64(0.04)[a]	0.63(0.03)[a]	0.61(0.03)[a]

The values are expressed as means (SD) for three replicate experiments. Mean values with different letters within a column are significantly different by Tukey test at (P < 0.05).

degradation pathways (Li and Liao, 2013; Hildebrandt *et al.*, 2015).

Amino acid profile of *Chlorella* in response to vegetable amino acids: The effects of amino acid supplements on the yield and amino acid compositions are shown in Table 2. The major amino acids component of *C. vulgaris* included; alanine (30.4 mg/g), glycine (23.2 mg/g), glutamic acid (22.7 mg/g), aspartic acid (21.1 mg/g), arginine (20.9 mg/g), leucine (18.9 mg/g), lysine (16.5 mg/), phenylalanine (14.0 mg/g), serine (13.0 mg/g), asparagine (12.7 mg/g), valine (12.6 mg/g), isoleucine (11.5 mg/g), and tyrosine (11.0 mg/g). The major amino acid components of *C. vulgaris* supplemented with *Oliveria* amino acid were; alanine (41.15 mg/g), arginine (33.37 mg/g), glycine (28.73 mg/g), glutamic acid (27.11 mg/g), aspartic acid (26.89 mg/g), leucine (24.41 mg/g), lysine (22.46 mg/g), serine (17.39 mg/g), valine (17.17 mg/g), phenylalanine (17.06 mg/g), tyrosine (13.61 mg/g), isoleucine (13.39 mg/g), and threonine (11.88 mg/g). The major amino acid components of *C. vulgaris* supplemented with *Thymus* amino acid included; alanine (46.66 mg/g), arginine (34.88 mg/g), glycine (31.21 mg/g), aspartic acid (28.84 mg/g), glutamic acid (27.11 mg/g), leucine (26.89 mg/g), lysine (26.78 mg/g), valine (20.20 mg/g), serine (19.66 mg/g), phenylalanine (17.71 mg/g), threonine (15.01 mg/g), isoleucine (14.26 mg/g), and tyrosine (13.50 mg/g). The major amino acid components of *C. vulgaris* supplemented with *Trachyspermum* amino acid were; alanine (45.58 mg/g), arginine (34.99 mg/g), glycine (31.00 mg/g), aspartic acid (28.62 mg/g), glutamic acid (27.54 mg/g), leucine (26.46 mg/g), lysine (25.81 mg/g), valine (19.44 mg/g), serine (19.22 mg/g), phenylalanine (17.82 mg/g), isoleucine (14.26 mg/g), threonine (14.15 mg/g), and tyrosine (13.72 mg/g). The major amino acid components of *C. vulgaris* supplemented with *Zataria* amino acid were; alanine (46.76 mg/g), arginine (35.10 mg/g), glycine (31.43 mg/g), aspartic acid (28.94 mg/g), glutamic acid (27.43 mg/g), leucine (27.00 mg/g), lysine (26.78 mg/g), valine (20.09 mg/g), serine (19.76 mg/g), phenylalanine (17.82 mg/g), threonine (14.90 mg/g), isoleucine (14.36 mg/g), and tyrosine (13.61 mg/g).

The green microalgae *Chlorella* presented in this study was evaluated as rich sources of essential and non essential amino. It is known that most reactions of amino acids catabolic pathways are localized in the mitochondria or cytosol. Neutral non-polar and acidic polar amino acids are catabolized via extracellular amino acid oxidases. Whereas, the basic polar amino acids are transported to the cell. Inside the cell, amino acids are converted to nitrogen supply and alpha-ketoacids by the activity of enzymes amino acid oxidase and dehydrogenase (Zuo *et al.*, 2012). The carbon skeletons of amino acids are generally converted to precursors or intermediates of the tricarboxylic acid

Table 2. Amino acid composition (mg/g) of *Chlorella* treated with amino acid (AA) from Oliveria *decumbens* (OD), *Zataria multiflora* (ZM), *Trachyspermum ammi* (TA), *and Thymus kotschyanus* (TK).

Amino acid	Chlorella	Chlorella-OD - AA	Chlorella-TK - AA	Chlorella-TA-AA	Chlorella-ZM-AA
Alanine	30.40(1.65)^{a}	41.15(2.32)^{b}	46.66(2.30)^{b}	45.58(2.42)^{b}	46.76(2.50)^{b}
Arginine	20.90(1.12)^{a}	33.37(1.45)^{b}	34.88(1.54)^{b}	34.99(1.60)^{b}	35.10(1.67)^{b}
Glycine	23.20(1.10)^{a}	28.73(1.35)^{b}	31.21(1.44)^{b}	31.00(1.50)^{b}	31.43(1.62)^{b}
Aspartic acid	21.10(1.0)^{a}	26.89(1.25)^{b}	28.84(1.47)^{b}	28.62(1.45)^{b}	28.94(1.50)^{b}
Glutamic acid	22.70(1.11)^{a}	27.11(1.33)^{b}	27.11(1.30)^{b}	27.54(1.48)^{b}	27.43(1.50)^{b}
Leucine	18.90(1.0)^{a}	24.41(1.2)^{b}	26.89(1.15)^{b}	26.46(1.4)^{b}	27.00(1.37)^{b}
Lysine	16.50(0.83)^{a}	22.46(1.32)^{b}	26.78(1.37)^{c}	25.81(1.30)^{c}	26.78(1.44)^{c}
Valine	12.60(1.00)^{a}	17.17)1.45)^{b}	20.20(1.65)^{b}	19.44(1.52)^{b}	20.09(1.50)^{b}
Serine	13.00(1.12)^{a}	17.39(1.22)^{b}	19.66(1.66)^{b}	19.22(1.60)^{b}	19.76(1.47)^{b}
Phenylalanine	14.00(1.15)^{a}	17.06(1.55)^{b}	17.71(1.58)^{b}	17.82(1.62)^{b}	17.82(1.58)^{b}
Threonine	8.20(0.43)^{a}	11.88(0.65)^{b}	15.01(1.13)^{c}	14.15(1,16)^{c}	14.90(1.20)^{c}
Isoleucine	11.50(0.92)^{a}	13.39(1.27)^{ab}	14.26(1.35)^{b}	14.26(1.28)^{b}	14.36(1,36)^{b}
Tyrosine	11.00(0.85)^{a}	13.61(1..11)^{b}	13.50(1.33)^{b}	13.72(1.20)^{b}	13.61(1.00)^{b}
Histidine	3.30(0.14)^{a}	5.94(0.24)^{b}	8.10(0.37)^{c}	7.56(0.30)^{c}	7.99(0.42)^{c}
Methionine	5.90(0.22)^{a}	7.13(0.35)^{b}	7.04(0.28)^{b}	7.14(0.30)^{b}	7.54(0.37)^{b}
Proline	5.60(0.20)^{a}	6.80(0.33)^{ab}	7.34(0.30)^{b}	7.24(0.36)^{b}	7.34(0.32)^{b}
Ornithine	1.10(0.10)^{a}	2.27(0.12)^{b}	4.10(0,16)^{b}	3.56(0.13)^{b}	4.00(0.15)^{b}
Asparagine	12.70(1.10)^{d}	7.78(0.45)^{c}	1.51(0.11)^{a}	3.67(0.22)^{b}	2.05(0.14)^{a}
Beta-alanine	1.20(0.08)^{a}	1.19(0.09)^{a}	1.62(0.12)^{a}	1.51(0.10)^{a}	1.75(0.13)^{a}
Cystine	0.70(0.05)^{a}	0.86(0.06)^{a}	1.19(0.56)^{a}	1.08(0.43)^{a}	1.19(0.33)^{a}
Total	254.60(8.5)^{a}	327.24(9.7)^{b}	354.13(10)^{c}	351.00(8.8)^{c}	356.08(11)^{c}
∑NEAA	160.60(7.7)^{a}	202.93(7.0)^{b}	210.60(8.4)^{b}	211.57(9.0)^{b}	212.44(9.5)^{b}
∑EAA	90.80(4.5)	119.45(6.3)	136.19(7.0)	132.84(7.5)	136.40(8.2)
∑NPAA	3.20(0.17)^{a}	4.40(0.18)^{b}	6.90(0.20)^{b}	6.20(0.23)^{b}	6.70(0.21)^{b}
EAA/NEAA	0.57(0.12)^{a}	0.59(0.15)^{a}	0.65(0.17)^{a}	0.63(0.16)^{a}	0.64(0.18)^{a}

The values are expressed as means (SD) for three replicate experiments. Mean values with different letters within a column are significantly different by Tukey test at (P < 0.05).

cycle which can contribute to ATP production and also amino acid synthesis (Figure 1). Intermediates of the tricarboxylic acid cycle including α-ketoglutarate, are is able to convert to glutamic acid that is the precursor for proline and arginine synthesis. Succinyl-CoA and fumarate are precursors for the synthesis of threonine, methionine, valine, isoleucine, tyrosine, and phenylalanine. Also, oxaloacetate can be converted to aspartic acid, asparagine, lysine, methionine, and threonine. Moreover, other components such as pyruvate and phosphoenolpyruvate can also be converted to valine, leucine, alanine, tyrosine and phenylalanine, respectively (Figure 1). It can be interpreted from the results that Asparagine decreased compared to the other individual amino acids. This may be because; Asparagine can participate in two different metabolic pathways under the plant oil and amino acid supplementations. Asparagine converted to aspartic acid. Under oil treatments, aspartic acid converted to the oxaloacetic acid and entered to the tricarboxylic acid cycle and produced energy and acetyl-CoA. Whereas, under the condition of amino acid supplementations, the hypothesis is strengthened that aspartic acid transferred to the cytosol by the malate/aspartate shuttle and converted to threonine, methionine and lysine (Li and Liao, 2013). As can be seen from the table 2, the levels of these amino acids have increased compared to that in control.

Protein nutritional quality is determined by its amino acids profile. According to WHO, the concentrations of amino acids valine, isoleucine, leucine, lysine and threonine for an ideal protein are 5.0, 4.0, 7.0, 5.5 and 4.0 g/16g nitrogen, respectively (Safi *et al*., 2014). The amino acid profiles of the *Chlorella* supplemented with *Trachyspermum* and *Thymus* oils in this study was favorable or a little lower than what has been recommended by WHO. While, lysine content was lower in cultures supplemented with *Oliveria* amino acids. In addition, the amount of amino acid phenylalanine determined in this study was slightly lower than WHO recommendations.

Conclusion

In conclusion, one of the main objectives of some studies is the improvement of the quality and quantity of amino acids. The amino acids profile in the genus of *C. vulgaris* were tested under various plant oil and amino acid supplements. Data from this research confirm the direct correlation between the presence of carbon and nitrogen sources to the contents of amino acids and ultimately optimizing the proportion of amino acids in the diet. Results have indicated that the profile of amino acid undergone many changes in quantitative terms in *C. vulgaris* supplemented with plant oils. Total amino acids, essential amino acids, non-essential amino acids and non-protein amino acids decreased considerably in

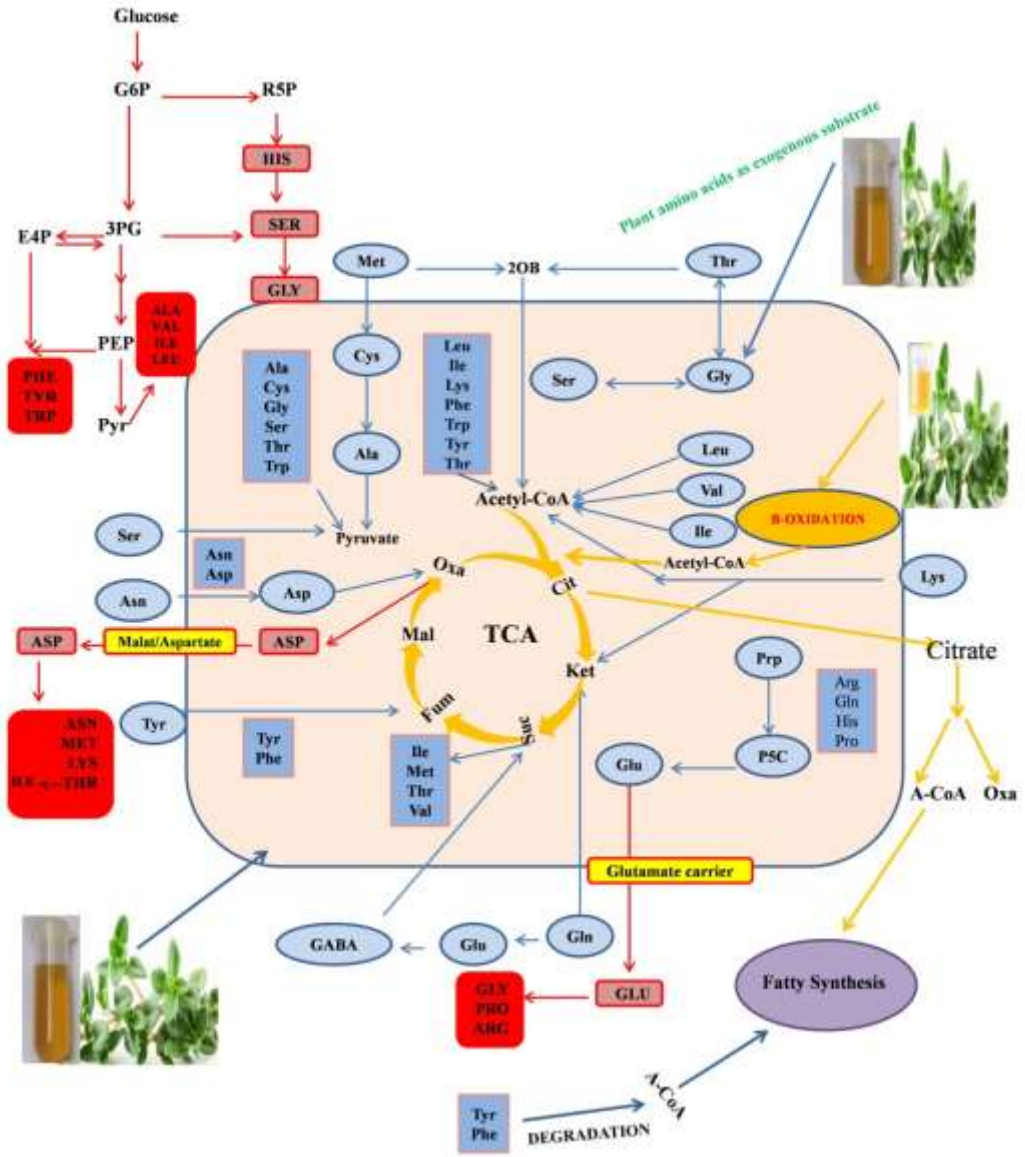

Figure 1. Representation of the glycolytic, pentose phosphate and tricarboxylic acid cycles in amino acid synthesis in red arrows, catabolism of amino acids pathway in blue arrows, and fatty acid catabolism in yellow arrows are shown (Adopted from Li and Liao, 2013; Hildebrandt *et al.*, 2015).

the *C. vulgaris* supplemented with plant oils. Whereas, amino acids as nitrogen and carbon sources had an impact on the biochemical composition of the *C. vulgaris*. In the presence of the plant amino acids, total amino acids, essential amino acids, non-essential amino acids and non-protein amino acids increased. The present study showed that amino acids as a carbon and nitrogen sources have great influence on the quality and quantity of the amino acid profile of *Chlorella*.

Conflicts of interest

The authors confirm that they have no conflicts of interest.

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Author contribution

Roghayeh Siahbalaei and Gholamreza Kavoosi conceived and designed research and conducted experiments and provide reagents and analyzed the data and performed the statistical analysis. They participated in writing the manuscript and its revisions.

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