



***In vitro* evaluation of antioxidant properties of *Chlorella vulgaris* and its derivatives for use as antioxidant supplements in animal production**

A B SIKIRU¹, A ARANGASAMY^{2✉}, I C ALEMEDE³, A T IJAIYA³, S S A EGENA³,
R BHATTA² and S B N RAO²

ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka 560 030 India

Received: 16 December 2022; Accepted: 24 November 2023

Keywords: Antioxidants, *Chlorella vulgaris*, Microalgae, Oxidative stress

Microalgae are biological resources with potential application in animal and human nutrition as supplements. *Chlorella vulgaris* is an example of such microalgae that is commercially available, but there is a need to quantify the extent of the antioxidants in the algae in order to fully explore its nutri-functional benefits for animal nutrition application, as these could eliminate high cost and improve efficiency of utilizing the algae (Kodner *et al.* 2009). The quantification can also enhance the use of the microalgae for its functional potential uses such as antioxidants, as anti-inflammatory, antiviral, antibacterial and general antimicrobial agents (Kiuru *et al.* 2014).

Oxidative stress prevention and management in animals is important because it is a stress compromise which is responsible for reproductive failures and inefficiencies, generally limiting the productivity of animals. Specifically, oxidative stress is reported to be linked with reproductive failures such as abnormal reproductive cycles, embryonic or fetal loss, neonatal mortality, delayed attainment of puberty and poor sperm production, since they are all associated with metabolic imbalance which nutritional management could ameliorate through exogenous supply of antioxidant supplements (Miller and Brzezinska-Slebodzinska 1993). *Chlorella vulgaris* is a reported source of antioxidant including astaxanthin, lutein, fucoxanthin, and carotenoids which can serve as free radical scavengers for improving performance of breeding animals via oxidative stress attenuation. This position was agreed upon by authors including Fan Vonshak and Boussiba (1994), Harker Tsavalos and Young (1996), He Duncan and Barber (2007) and Desai (2016). Therefore, in this study, an *in vitro* evaluation of the microalgae and its derivatives was carried out to determine its potential for oxidative stress attenuation potentials.

Present address: ¹Department of Animal Science, Federal University of Agriculture Zuru-872101, Nigeria. ²ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka. ³Department of Animal Production, Federal University of Technology, Minna-920262, Nigeria.
✉Corresponding author email: arangasamyars@gmail.com

The microalgae biomass used in this study was a commercial microalgae product SeaLipro™ (Seagrass Tech Pvt. Ltd, India). Two approaches were used for the preparation of the microalgae derivatives, the first approach was preparation of the derivatives using whole raw unextracted biomass of the microalgae while the second approach was preparation of derivatives using hot water extracts of the *Chlorella vulgaris* biomass. This method of hot water extraction has been reported by Konishi *et al.* (1985), Hasegawa *et al.* (1990), Tatke and Jaiswal (2011), Flórez Conde and Domínguez (2015) and Sánchez Roque *et al.* (2018). The eight derivatives prepared were labelled T1–T8; the labelled derivatives T1–T4 were derivatives prepared with raw unextracted biomass while T5–T8 were derivatives prepared with hot-water extracts of the *Chlorella vulgaris* biomass. These two forms of derivatives contained 20%, 40%, 60%, and 80% *Chlorella vulgaris* biomass and a corresponding balance of 80%, 60%, 40% and 20% whole wheat flour, respectively.

Each derivative prepared was sampled in triplicates of 10 g each and dissolved in 100 ml methanol, then kept at room temperature for 24 h after which the mixtures were filtered through a Whatman filter paper while the filtrates were stored at 4°C for downstream analysis (Bhuvana *et al.* 2019). Total phenolic content of the samples was determined using the principles of Folin Coicalteau's phenol reagent test while the Ferric Reducing Antioxidant Power (FRAP) of the samples were determined using FRAP chemical assay described by Vijayalakshmi and Ruckmani (2016). The assay uses antioxidant as reductants in a redox-linked reaction where the reducing oxidant is iron (II) and the change in colour of ferric iron (III) from colourless to blue coloured iron (II) was used for the determination of antioxidant power of the samples through absorbance readings (Benzie and Strain 1996). The procedure described by Mohamed *et al.* (2014) was adopted for the determination of the hydrogen peroxide scavenging power of the microalgae and its derivatives using the absorbance of H₂O₂ at 230 nm. The total antioxidant composition of the samples was determined using the protocol with an assay

principle based on reduction of iron (III) to iron (II) at low pH. The data analysis involved subjecting all data obtained to one-way analysis of variance, the significant differences in means were determined at $p < 0.05$, while means separations was based on Duncan test and in addition, all the parameters evaluated were ranked with Kruskal-Wallis's test for ranking them based on their antioxidant potentials using all the parameters.

The samples differed significantly in total antioxidant composition, ferric reducing antioxidant power, hydrogen peroxide scavenging capacity and total phenolic content ($p < 0.05$). The mean total antioxidants concentration was 621.83 ± 36.41 $\mu\text{mol/g}$ ascorbic acid equivalent ($p < 0.001$), while minimum and maximum total antioxidant concentrations were 280.00 and 962.18 $\mu\text{mol/g}$ ascorbic acid equivalent, respectively. The average FRAP was 1.28 ± 0.09 , while the minimum and maximum antioxidant FRAP were 0.56 and 2.65, respectively ($p < 0.001$).

The average Hydrogen peroxide scavenging potential capacity was $34.04 \pm 3.18\%$, while minimum and maximum hydrogen peroxide scavenging capacities were 4.66 and 59.91%, respectively ($p < 0.001$). The average total phenolic content of the samples was 30.54 mg/g ellagic acid equivalent, while minimum and maximum total phenolic contents were 16.20 and 45.27 mg/g ellagic acid equivalent, respectively ($p < 0.001$). These indicated that the samples differ in all the antioxidant indicators upon which they were assessed (Table 1). There was also a significant relational difference in the total antioxidant capacities, ferric reducing antioxidant power and total phenolic content ($p < 0.05$) of the samples (Fig. 1).

The study showed that *Chlorella vulgaris* and its derivatives highlighted above are potential antioxidant supplements for use in animal feeds and nutrition taking advantage bioactive antioxidant compounds they contained. The evaluations carried out in this study showed that the microalgae derivatives prepared with untreated algae biomass are better compared with the derivatives prepared with the hot-water extracts of the biomass. This observation specifically indicated that proportional addition of the microalgae in a non-antioxidant material such as wheat flour could serve as antioxidant supplements that are suitable as additives for improving animal productivity. This is in agreement with reports of Yan Lim and Kim (2012), which suggested that the microalgae *Chlorella vulgaris* is a suitable source of antioxidant additives for animal feeds production.

In agreement with the antioxidant capacities recorded in this study, *Chlorella vulgaris* was described as a feed resource that could be used for enhancement of physiological activities, growth promotion, antioxidant protection, immune modulation and protection of animals against pathogenic microorganisms in separate submissions by Guzmán *et al.* (2003), Lee *et al.* (2010), Kang *et al.* (2013) and Wan *et al.* (2019). Further to these assertions, the outcome of this present study also indicated that supplementing the microalgae in the forms evaluated

Table 1. *In vitro* total antioxidant capacity, ferric reducing antioxidant power, hydrogen peroxide scavenging power and total phenolic composition of the microalgae *Chlorella vulgaris* biomass and its derivatives

Parameter	Unextracted <i>Chlorella vulgaris</i> biomass	T1	T2	T3	T4	T5	T6	T7	T8	SEM	p-value
Total antioxidant capacity ($\mu\text{mol/g}$ ascorbic acid)	938.42 ^a	663.03 ^{bc}	670.30 ^{cd}	601.21 ^b	760.00 ^b	298.18 ^f	412.12 ^e	545.82 ^d	707.39 ^{bc}	36.41	0.001
Ferric reducing antioxidant power	2.48 ^a	1.10 ^e	1.34 ^{cd}	1.20 ^{de}	1.52 ^b	0.60 ^g	0.82 ^f	1.09 ^e	1.41 ^c	0.09	0.001
Hydrogen peroxide scavenging power (%)	41.15 ^e	7.38 ^h	47.45 ^b	23.27 ^f	59.58 ^a	27.08 ^e	13.40 ^g	43.49 ^c	43.53 ^c	3.18	0.001
Total phenolic composition (mg/g ellagic acid)	43.93 ^a	29.96 ^d	39.39 ^{bc}	36.28 ^c	41.16 ^{ab}	17.97 ^e	20.41 ^e	18.97 ^e	26.85 ^d	1.89	0.001

Means with different superscripts along the same row are significantly different from each other ($p < 0.05$). T1: Derivative prepared with 20% untreated *Chlorella vulgaris* biomass; T2: Derivative prepared with 40% untreated *Chlorella vulgaris* biomass; T3: Derivative prepared with 60% untreated *Chlorella vulgaris* biomass; T4: Derivative prepared with 80% untreated *Chlorella vulgaris* biomass; T5: Derivative prepared with 20% hot water *Chlorella vulgaris* biomass extract; T6: Derivative prepared with 40% hot water *Chlorella vulgaris* biomass extract; T7: Derivative prepared with 60% hot water *Chlorella vulgaris* biomass extract; T8: Derivative prepared with 80% hot water *Chlorella vulgaris* biomass extract.

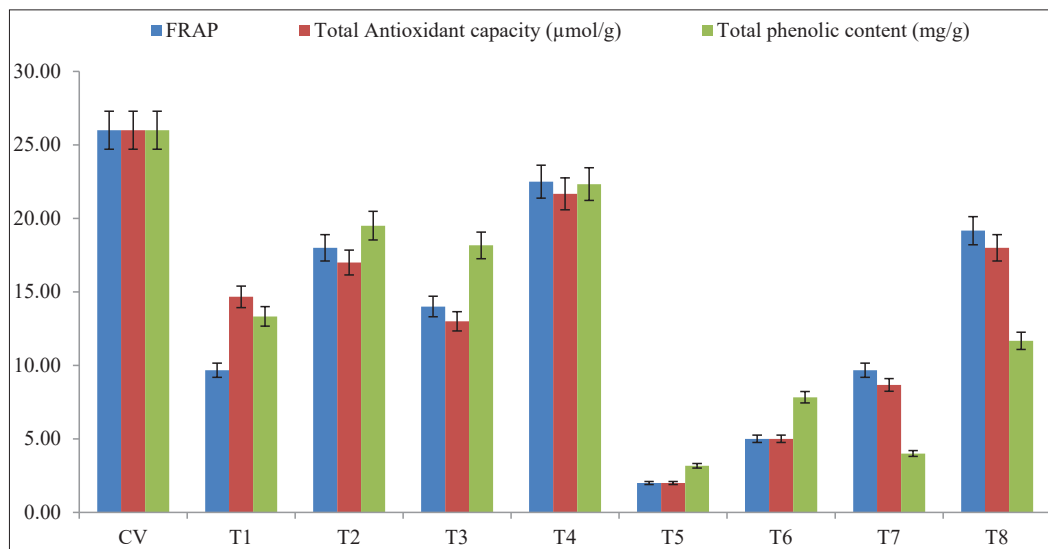


Fig. 1. Comparative relational ranking of the samples based on their ferric reducing antioxidant power (FRAP), total antioxidant capacity and total phenolic content. CV: Whole raw *Chlorella vulgaris* biomass; T1: Derivative prepared with 20% untreated *Chlorella vulgaris* biomass; T2: Derivative prepared with 40% untreated *Chlorella vulgaris* biomass; T3: Derivative prepared with 60% untreated *Chlorella vulgaris* biomass; T4: Derivative prepared with 80% untreated *Chlorella vulgaris* biomass; T5: Derivative prepared with 20% hot water *Chlorella vulgaris* biomass extract; T6: Derivative prepared with 40% hot water *Chlorella vulgaris* biomass extract; T7: Derivative prepared with 60% hot water *Chlorella vulgaris* biomass extract; T8: Derivative prepared with 80% hot water *Chlorella vulgaris* biomass extract.

could attenuate oxidative stress in *in vivo* trials in pregnant rabbits (Sikiru *et al.* 2021). Therefore, the topmost ranked samples and whole unextracted *Chlorella vulgaris* biomass are recommended for *in vivo* exploration as antioxidant supplement in animals at different physiological states.

The above recommendations were made because the *in vitro* evaluation of the prepared derivatives and the microalgae biomass suggested that the samples investigated have higher antioxidant capabilities which could serve as a source of free radical scavengers when consumed by animals. These cannot be unconnected with other promising areas using these derivatives such as their uses for the improvement of animal physiological performances and products qualities. This is because both the physiological performances and products qualities improvements are closely linked to oxidative stress and usually associated with an imbalance between free radicals and antioxidants.

SUMMARY

Bioactive compounds with varied functional properties found in microalgae such as *Chlorella vulgaris* can serve as antioxidants supplement against free radicals causing oxidative stress in food animals, but there is a need to quantify these antioxidants in the algae for effective applications. Therefore, this study quantified antioxidants in *Chlorella vulgaris* and derivatives prepared with the microalgae biomass for enhancing its application in animal nutrition. Eight derivatives prepared using the *Chlorella vulgaris* biomass were subjected to *in vitro* antioxidant supplements in animals for attenuation of oxidative stress

to improve performances and productivity because of their antioxidant potentials. Antioxidant assessments including ferric antioxidant power, hydrogen peroxide scavenging potential, total antioxidant capacity, and total phenolic content. The results showed that the mean total capacities of the preparations were 621.83 ± 36.41 μmol/g ascorbic acid equivalent, ferric reducing antioxidant power (FRAP) was 1.28 ± 0.09 , while the hydrogen peroxide scavenging (HPS) potential was $34.04 \pm 3.18\%$, and the total phenolic content was 30.54 mg/g ellagic acid equivalent. It was concluded from these observations that the microalgae *Chlorella vulgaris* and its derivatives as prepared in this study could be used as antioxidant supplements in animals for attenuation of oxidative stress to improve performances and productivity because of their antioxidant potentials.

ACKNOWLEDGEMENTS

The author thanks to The World Academy of Sciences (TWAS), Italy; Department of Biotechnology (DBT), Government of India for joint award of doctoral fellowship to Sikiru A B at ICAR-NIANP, Bengaluru where this study was conducted. We are grateful to authorities and management of Federal University of Technology, Minna, Nigeria, the Director of ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India for supporting this research and providing enabling environment for studies. We are also grateful to Centre for Co-operation in Science and Technology among Developing Societies – CCSTDS (formerly CICS), Chennai, India for coordination of fellowship under which this study was conducted.

REFERENCES

- Benzie I F F and Strain J J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. *Analytical Biochemistry* **239**: 70–76.
- Bhuvana P, Sangeetha P, Anuradha V and Ali M S. 2019. Spectral characterization of bioactive compounds from microalgae: *N. oculata* and *C. vulgaris*. *Biocatalysis and Agricultural Biotechnology* **19**: 101094.
- Desai R K. 2016. ‘Ionic liquid pre-treatment of microalgae and extraction of biomolecules.’ Ph.D. Thesis, Wageningen University, Netherlands.
- Fan L, Vonshak A and Boussiba S. 1994. Effect of temperature and irradiance on growth of *Haematococcus pluvialis* (Chlorophyceae). *Journal of Phycology* **30**: 829–33.
- Flórez N, Conde E and Domínguez H. 2015. Microwave assisted water extraction of plant compounds. *Journal of Chemical Technology & Biotechnology* **90**: 590–07.
- Guzmán S, Gato A, Lamela M, Freire-Garabal M and Calleja J M. 2003. Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytotherapy Research* **17**: 665–70.
- Harker M, Tsavalos A J and Young A J. 1996. Autotrophic growth and carotenoid production of *Haematococcus pluvialis* in a 30 liter air-lift photobioreactor. *Journal of Fermentation and Bioengineering* **82**: 113–18.
- Hasegawa T, Yoshikai Y, Okuda M and Nomoto K. 1990. Accelerated restoration of the leukocyte number and augmented resistance against *Escherichia coli* in cyclophosphamide-treated rats orally administered with a hot water extract of *Chlorella vulgaris*. *International Journal of Immunopharmacology* **12**: 883–91.
- He P, Duncan J and Barber J. 2007. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*: Effects of cultivation parameters. *Journal of Integrative Plant Biology* **49**: 447–51.
- Kang H K, Salim H M, Akter N, Kim D W, Kim, J H, Bang H T, Kim M J, Na J C, Hwangbo J, Choi H C and Suh O S. 2013. Effect of various forms of dietary *Chlorella* supplementation on growth performance, immune characteristics, and intestinal microflora population of broiler chickens. *Journal of Applied Poultry Research* **22**: 100–08
- Kiuru P D, Auria M, Muller C, Tammela P, Vuorela H and Yli-Kauhaluoma J. 2014. Exploring Marine Resources for Bioactive Compounds. *Planta Medica* **80**: 1234–46.
- Kodner, R B, Summons R E and Knoll A H. 2009. Phylogenetic investigation of the aliphatic, non-hydrolyzable biopolymer algaenan, with a focus on green algae. *Organic Geochemistry* **40**: 854–62.
- Konishi F, Tanaka K, Himeno K, Taniguchi K and Nomoto K. 1985. Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): Resistance to meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. *Cancer Immunology Immunotherapy* **19**: 73–78
- Lee S H, Kang H J, Lee H J, Kang M H and Park Y K. 2010. Six-week supplementation with *Chlorella* has favorable impact on antioxidant status in Korean male smokers. *Nutrition* **26**:175–83.
- Miller J and Brzezinska-Slebodzinska E. 1993. Oxidative stress, antioxidants, and animal function. *Journal of Dairy Science* **76**: 2812–23.
- Mohamed R M A, Fageer A S M, Eltayeb M M and Mohamed Ahmed I A. 2014. Chemical composition, antioxidant capacity, and mineral extractability of Sudanese date palm (*Phoenix dactylifera* L.) fruits. *Food Science and Nutrition* **2**: 478–89.
- Sánchez Roque Y, Pérez-Luna Y, Moreira Acosta J, Farrera Vázquez N, Berrones Hernández R, Saldaña Trinidad S and Pathiyamattom J. 2018. Evaluation of the population dynamics of microalgae isolated from the state of Chiapas, Mexico with respect to the nutritional quality of water. *Biodiversity Data Journal* **6**: e28496
- Sikiru A B, Arangasamy A, Alemede I, Egena S, Ippala J and Bhatta R. 2021. Effects of dietary supplementation of *Chlorella vulgaris* on oxidative stress attenuation and serum biochemical profile of pregnant New Zealand White rabbits. *Indian Journal of Animal Sciences* **90**: 88–91.
- Tatke P and Jaiswal Y. 2011. An overview of microwave assisted extraction and its applications in herbal drug research. *Research Journal of Medicinal Plant* **5**: 21–31.
- Vijayalakshmi M and Ruckmani K. 2016. Ferric reducing antioxidant power assay in plant extract. *Bangladesh Journal of Pharmacology* **11**: 3.27663
- Wan X, Li T, Zhong R, Chen H, Xia X, Gao L, Gao X, Liu B, Zhang H and Zhao C. 2019. Anti-diabetic activity of PUFAs-rich extracts of *Chlorella pyrenoidosa* and *Spirulina platensis* in rats. *Food and Chemical Toxicology* **128**: 233–39.
- Yan L, Lim S U and Kim I H. 2012. Effect of fermented *Chlorella* supplementation on growth performance, nutrient digestibility, blood characteristics, fecal microbial and fecal noxious gas content in growing Pigs. *Asian-Australasian Journal of Animal Sciences* **25**: 1742–47.