



Spirulina consumption effectively reduces anti-inflammatory and pain related infectious diseases

Gasem Mohammad Abu-Taweel^a, Al-Mutary Mohsen G^a, Paulrayer Antonisamy^b, Selvaraj Arokiyaraj^c, Hak-Jae Kim^{d,*}, Sun-Ju Kim^e, Kyeong Hun Park^f, Young Ock Kim^e

^a Department of Basic Sciences, College of Education, Imam Abdulrahman Bin Faisal University, P.O. Box 2375, Dammam - 31451, Saudi Arabia

^b Department of Korean Physiology, Wonkwang University School of Korean Medicine, 460 Iksan-daero, Iksan City, Jeonbuk, 570-749, Republic of Korea

^c Department of Food Science and Technology, Sejong University, Republic of Korea

^d Department of Clinical Pharmacology, College of Medicine, Soonchunhyang University, Cheonan, Republic of Korea

^e Department of Bio-Environmental Chemistry, College of Agriculture and Life Sciences, Chungnam National University, 99 Daehak-Ro, Yuseung-Gu, Daejeon, 34134, Republic of Korea

^f Rural Development Administration, Jeonju, 54875, Republic of Korea

ARTICLE INFO

Article history:

Received 24 February 2019

Received in revised form 18 April 2019

Accepted 28 April 2019

Keywords:

Arthrospira platensis

Spirulina extract

Phytochemicals

Anti-inflammatory

Analgesic

Oedema

ABSTRACT

Background: Inflammation and pain triggers several pathological illnesses. Synthetic drugs used for the controlling of inflammatory conditions convey significant toxic effects. Global scientific community continually attempt to improve effective, economic and harmless naturally derived remedies against inflammation and pain. The present study aimed to quantify the phytochemical constituents of the freshly cultivated *Spirulina* and targeted to examining the anti-inflammatory and analgesic activity of *Spirulina* extract (SE) derived from *Arthrospira platensis*.

Methods: The anti-inflammatory effect of SE was evaluated in animal models including carrageenan-induced rat hind paw oedema, and cotton pellet-induced granuloma formation. Analgesic effects of SE were evaluated by acetic acid induced writhing response and hot plate test.

Results: Phytochemical quantification guided to identify seven carbohydrates, thirteen amino acids, eleven fatty acids and polyphenolic compounds respectively. The results indicated that SE significantly attenuated carrageenan-induced hind paw oedema, and cotton pellet-induced granuloma. Preliminary molecular mechanistic studies established that SE decreased the productions of TNF- α , IL-1 β , IL-6, PGE2 and NO, and suppressed the activities of COX-2 and iNOS.

Conclusion: These results provide a strong scientific foundation for the anti-inflammatory and analgesic activities of SE against different studies in animal models.

© 2019 The Authors. Published by Elsevier Limited on behalf of King Saud Bin Abdulaziz University for Health Sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Cyanobacterium (microalgae) or *Spirulina* belonged to photosynthetic group of prokaryotic bacteria are commonly occurred as filamentous and multicellular microorganisms are typically grown in water and commonly cultivated in lakes and ponds with sufficient amount of sunlight. *Spirulina* required minimal nutrients for its growth and also grow under high salinity, and increased level of carbonates. The genus of the *Spirulina* namely *Arthrospira platensis*, *Arthrospira fusiformis* and *Arthrospira maxima* were commercially produced in Central Africa, Asia and America. Because of its wide level of nutrient contents and beneficial effects to humans

it has been used as a stable food and important ingredients in many biologically important food products and pharmaceutical components. *Spirulina* contains pharmaceutically important primary and secondary metabolites in its composition. In addition, the wide level of nutrients such as carbohydrates (30%), protein (55–70%), fat (8%) [linoleic, oleic, γ -linolenic, eicosapentaenoic, stearidonic, arachidonic acids, and docosahexaenoi], essential amino acids (lysine methionine and cysteine), vitamins (<1%) [vitamin B, vitamin D, vitamin E, ascorbic acid], sugars (3%), minerals (potassium, iron, zinc, calcium, selenium, magnesium, chromium, copper, phosphorus, manganese, and sodium), dietary fibers (3%), and phytochemicals (chlorophyll-a, diatoxanthin, xanthophyll, zeaxanthin, echinenone, 3'-hydroxyechinenone, beta-carotene, canthaxanthin, beta-cryptoxanthin, *Spirulina* extract, myxoxanthophyll, phycobiliproteins oscillaxanthin and allophycocyanin) respectively [1–3]. Food and Drug Administration (FDA) authenti-

* Corresponding author.

E-mail addresses: hak3962@sch.ac.kr, hak3962@gmail.com (H.-J. Kim).

cated and issued GRAS certificate to spirulina products when grown under approved conditions. Due to the presence of enriched phytochemicals, Spirulina is widely known for its various biomedical applications such as antigenotoxic, anti-inflammatory, antidiabetic, antioxidant, anti-cancer, hepatoprotective neuroprotective activities [4–8]. Deng and Chow claimed that regular consumption of *Spirulina* tablets effectively decreased the antiinflammatory related diseases [9]. Pham and Lee reported that intake of *Spirulina* rich products reduces the risk of inflammatory and obesity related diseases by enhancing the metabolic process of macrophage infiltration [10]. The organic extracts obtained from the *Spirulina* samples suppressed the proinflammatory cytokine expression and retarding the translocation of NF- κ B thereby minimizing the chances of spreading inflammatory disorders [11]. Recent report claimed that the consumption of *Spirulina* regulates the hormonal imbalances in women especially in premenstrual periods. Considering the multilevel advantages of the spirulina, many companies were produced wide level of spirulina products such as tablets, pastries, health drinks and fermented foods. However, the nutritional content of each spirulina products were varied. In the present study, the nutritional profile of the spirulina was characterized and investigated the anti-inflammatory and analgesic effects in mice model.

Materials and methods

Cultivation and phytochemical composition

Spirulina was cultivated in a 3-L glass bioreactor using F/2 Guillard medium under controlled conditions (pH 9 and temperature 25°C). 3.5 L/min carbon dioxide was sparged to maintain the cultivation conditions and light sources were provided using LED lamps (12 V/24 W) under an irradiance of 108 mmol. photons.m⁻²s⁻¹. After 10 days of incubation, the cells were harvested and air-dried for the determination of the nutritional compositions. Carbohydrates were quantified by calorimetric methods [12], free amino acids extracted and quantified by HPLC method [13], polyphenols present in the *Spirulina* samples were quantified by the method of Lin and Tang [14]; and lipids composition were determined by the method of Mossoba et al. [15]. The pure powdered *Spirulina* samples (250 g) was mixed with 1000 ml of ethyl acetate and kept in the orbital shaker for 2 days. After that the dry materials were separated by centrifugation and the crude extracts were obtained by vacume evaporator. The crude organic extracts were used for the animal studies.

Animals

Adult Wistar albino rats (200–220 g) and mice (24–28 g) of either sex were used for the experiments. The animals were accustomed for at least two weeks before the experiments. All experiments were carried with six animals in each group. All the animal experiments were conducted according to the ethical norms approved by Ministry of Social Justice and Empowerment, Government and Institutional Animal Ethics Committee guidelines.

Carrageenan-induced paw oedema in rats

SE (200 mg/kg) and indomethacin (10 mg/kg) were treated orally 1 h before carrageenan application. After that, 0.1 ml of carrageenan (1%) injected subcutaneously into the rat right hind paw. Hind paw thickness was tested initially (0 h) and then at 3 and 5 h after the carrageenan injection with digital vernier caliper [16].

End of the experiment, animals were sacrificed, and the right hind paws were collected. The right hind paws were immediately placed in cold PBS and homogenized. The homogenate was incubated on ice for 15 min and centrifuged at 10000 ×g for 15 min at

4°C. Then the supernatants were collected and stored at –80°C for the analysis of NO, TNF- α , IL-1 β , IL-6, and PGE2 levels.

Nitric oxide assay

The nitrite content is an indicator of NO production. Nitrite level in supernatant was measured based on the Griess reaction method described by Xian et al. [17]. Concisely, supernatant was mixed with the same volume of Griess reagents and incubated at room temperature for 10 min in dark place; the absorbance was measured at 540 nm using a microplate spectrophotometer.

TNF- α , IL-1 β , IL-6, COX-2 and PGE2 assay

The levels of TNF- α , IL-1 β , IL-6, COX-2 and PGE2 in the hind paw tissue supernatants were measured using enzyme-linked immune sorbent assay (ELISA) kits (R&D Co. Ltd., Abingdon, UK) according to the manufacturer's instructions.

Quantification of iNOS, COX-2, TNF- α , and NF- κ B (p50) mRNA expression in rat hind paw tissue via RT-PCR

Trizol reagent (Gibco BRL, Gaithersburg, MD) was used for the extraction of total RNA from the thawed hind paw tissues. Briefly, 1 g of the tissues was homogenized under ice-cold conditions using the liquid nitrogen. After extraction of the total RNA, complimentary DNA was prepared for the gene expression studies. Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA with cDNA synthesis kit (TAKARA, Shiga, Japan). The qRT-PCR was conducted using an ABI Step One Real-time PCR System (Applied Biosystems, Warrington, UK) and a reaction mixture comprised of SYBR Green 2 × PCR Master Mix, a cDNA template, and forward and reverse primers. The PCR protocol enclosed of 40 cycles of denaturation at 95°C for 15 s, followed by 60°C for 30 s for extension and amplification of the target sequence. The relative expression levels of iNOS, COX-2, TNF- α , and NF- κ B (p50) were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the SQ means method. The primer sequences used in this study are shown in Table 1.

Cotton pellet-induced granuloma in rats

Cotton pellets (35 ± 1 mg) induced granuloma was created on the axilla region of the rats after anaesthetized using organic ether [18]. Organic ether solvents were administered by mouth using the 1 ml syringe. After administration, SE (200 mg/kg) and indomethacin (10 mg/kg) was treated once a day upto seven days from the day of cotton pellet implantation. On the eighth day, cotton pellets were removed and granuloma level was measured.

Analgesic tests

Acetic acid-induced abdominal constriction response in mice

Mice weighing 28–30 g were used for this experiment. Each mouse was injected with 0.75% acetic acid aqueous solution (0.1 ml/10 g body weight) into the peritoneal cavity and mice were located in a transparent box. The number of abdominal constrictions was counted. Test drugs SE (200 mg/kg) and indomethacin (10 mg/kg) were administered 1 h before the acetic acid injection [19].

Hot-plate test in mice

Hot-plate test was conducted according to previous method of Antonisamy et al. [20]. Briefly, the mice were located on a hot plate at the temperature of 55 ± 5°C. The time that elapsed until either licking of hind paw or a jump off from the surface was noted as the

Table 1
Nucleotide sequences of primers used in real-time qRT-PCR.

Gene	Forward (5' ± 3')	Reverse (5' ± 3')
iNOS	5'-GGAATCTTGGAGCGAGTTGT-3'	5'-CCTCTTGTCTTTCACCCAGTAG-3'
COX-2	5'- GAAGATTCCCTCCGGTGT-3'	5'-CCCTTCTCACTGGCTTATGTAG-3'
TNF- α	5' - TGCTCAGAAACACACGAGACGC-3'	5'-TTCAGCAGCCTTGTGAGCCAGA-3'
NF- κ B (p50)	5' - AACGCATCCCAAGTGTCTGGAA-3'	5' - GCAGCTGAAAAAGCTCAAGCCA-3'
GAPDH	5'-AGGAGCGAGACCCCACTAAACA-3'	5'-AGGGGGGCTAAGCAGTTGGT-3'

**Fig. 1.** Macroscopic images of carrageenan-induced hind paw oedema and the effects of SE (200 mg/kg) and indomethacin (10 mg/kg). (A) Normal; (B) control; (C) SE (200 mg/kg); (D) Indomethacin (10 mg/kg b.w.).

hot plate latency. After the initial reaction time (0–10 min), mice were treated with SE (45 mg/kg p.o.), and morphine (05 mg/kg s.c.) followed by final reaction time measurement at 30 min.

Result and discussion

Quantification of nutrient composition

The nutritional and phytochemical compositions of the cultivated *Spirulina* samples were quantified by HPLC analysis. The results indicated that the *Spirulina* extracts contained different sugars such as glucose (170 mg/100 g), fructose (35 mg/100 g), xylose (21 mg/100 g), galactose (19 mg/100 g), ribose (12 mg/100 g), sucrose (54 mg/100 g) and rhamnose (21 mg/100 g) respectively. Amino acids profiles documented as aspartate (1.37 mg/100 g fresh weight), asparagines (0.25), serine (1.7), glutamine (0.84), glycine (2.53), threonine (1.54), arginine (2.81), GABA (0.38), tyrosine (2.98), valine (2.34), isoleucine (1.08), leucine (1.77) and lysine (1.58) respectively. The poly phenols contents of the *Spirulina* were determined by quantification of the gallic acid level. The results indicated that average 22 mg/g of gallic acid equivalents were noted in the spirulina samples. Quantification results confirmed that the spirulina samples contained different fatty acids namely myristic acid (0.01 g/100 g), sapienic acid (1.97 g/100 g), palmitoleic acid (0.11 g/100 g), stearic acid (0.34 g/100 g), elaidic acid (0.01 g/100 g), oleic acid (0.11 g/100 g), vaccenic acid (0.05 g/100 g), linolelaidic acid (0.04 g/100 g), linoleic acid (0.75 g/100 g), eicosenoic acid (0.01 g/100 g) and γ , linolenic acid (0.67 g/100 g) respectively.

Carrageenan-induced hind paw oedema

Carrageenan-induced hind paw oedema is a conventional preliminary screening examination for anti-inflammatory activity of natural and synthetic drugs [19]. Carrageenan able to induced hind paw oedema through bi-phasic manner. The initial phase starting from 0–2.5 h, releases histamine, serotonin and bradykinin. Conversely, the later phase of inflammation is mediated by COX enzyme by converting arachidonic acid into prostaglandins. COX is considered as a target enzyme for a variety of NSAIDs, including indomethacin, aspirin and diclofenac sodium, which inhibit rat hind paw oedema at the later phase following carrageenan injection [20–24]. In the present study, intraplantar injection of carrageenan in rats headed to a significant increases of hind paw thickness in a time-dependent manner, and this increase was maximal at 5 h after carrageenan injection (Fig. 1). The administration with SE (200 mg/kg) and indomethacin (10 mg/kg) significantly inhibited the carrageenan induced hind paw oedema in all phases of the test when compared with the vehicle group. Paw edema was suppressed by SE and indomethacin, with inhibitory rates of 54.04% and 57.53%, respectively (Fig. 2).

Further examination into the activity of SE on the productions of inflammatory cytokines was carried out. As shown in Fig. 3, the levels of pro-inflammatory cytokines such as TNF- α (Fig. 3A), IL-6 (Fig. 3B), and IL-1 β (Fig. 3C) were significantly ($p < 0.05$) increased in the vehicle group to 1.90-, 3.22-, and 2.75-fold, respectively, when compared to the normal group. However, treatment with SE (200 mg/kg) and indomethacin (10 mg/kg) significantly ($p < 0.05$) reduced the levels of TNF- α , IL-1 β , and IL-6.

As presented in Fig. 4A, compared with the normal group, the PGE2 level was increased markedly ($p < 0.05$) in the vehicle

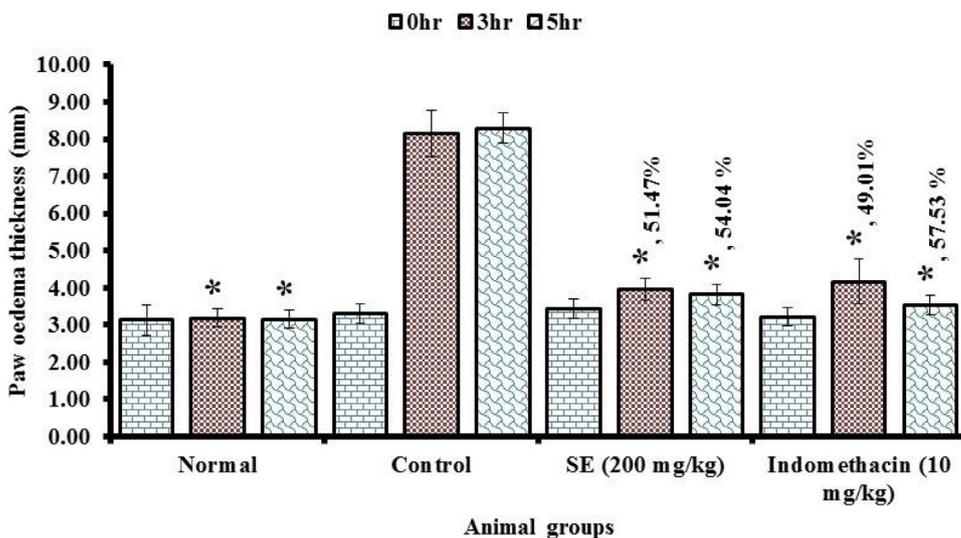


Fig. 2. Effect of SE (200 mg/kg) and indomethacin (10 mg/kg) on carrageenan induced hind paw oedema in rats.

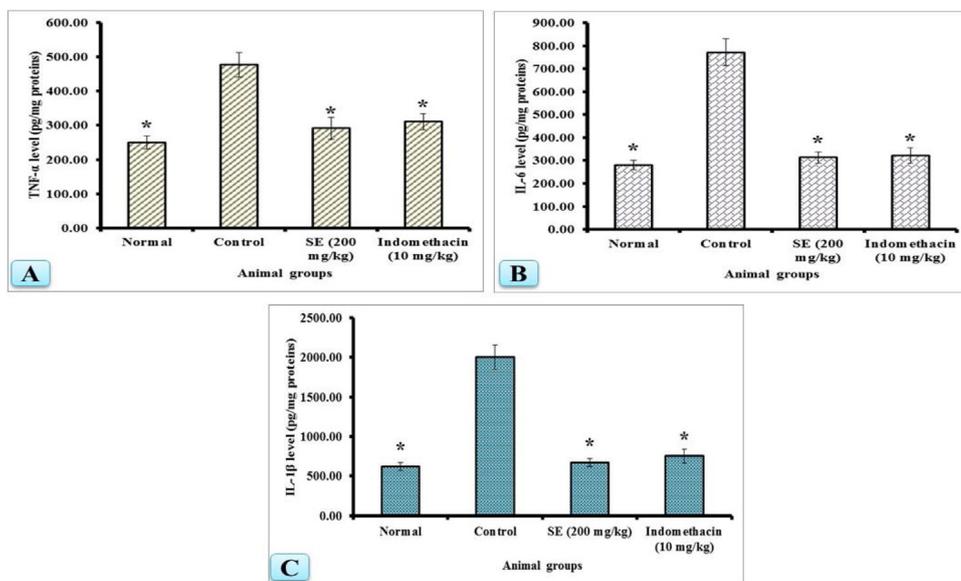


Fig. 3. Effect of SE (200 mg/kg b.w.) and indomethacin (10 mg/kg b.w.) on TNF-α, IL-6, and IL-1β levels.

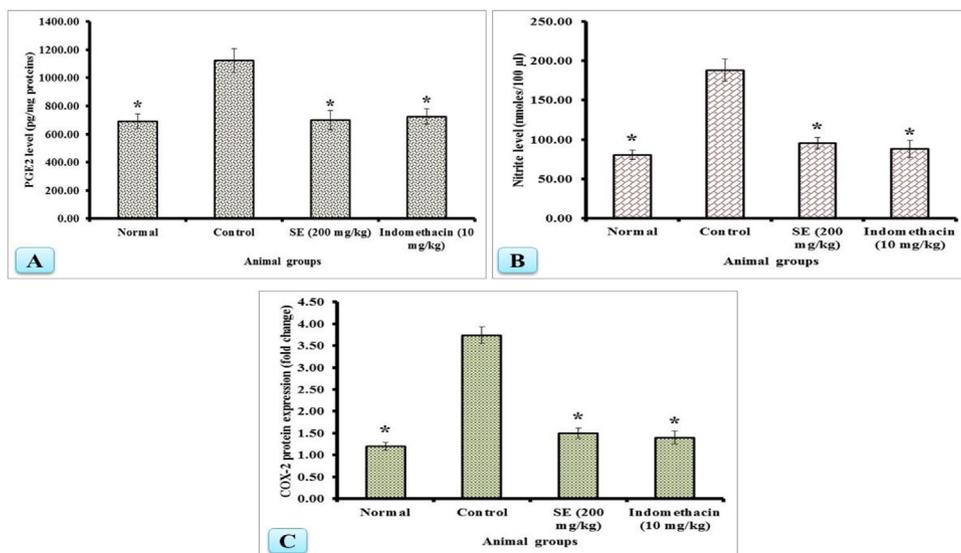


Fig. 4. Effect of SE (200 mg/kg b.w.) and indomethacin (10 mg/kg b.w.) on PGE2, nitrite and COX-2 levels.

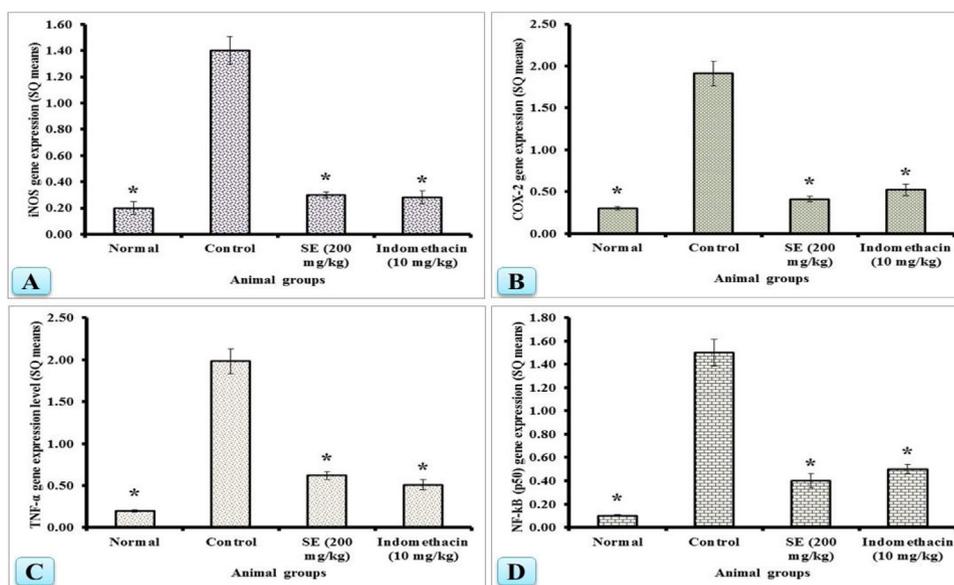


Fig. 5. Effect of SE (200 mg/kg b.w.) and indomethacin (10 mg/kg b.w.) on iNOS, COX-2, TNF- α and NF- κ B (p50) levels.

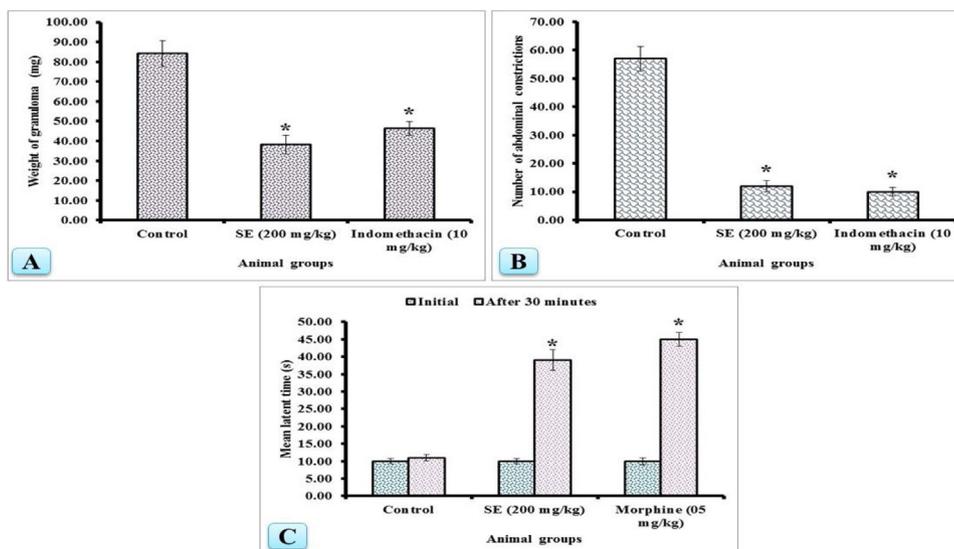


Fig. 6. Anti-inflammatory and analgesic activity of SE (200 mg/kg b.w.) and indomethacin (10 mg/kg b.w.) against cotton pellet induced granuloma, acetic acid induced writhing response and hot plate test.

group. However, the treatment of SE (200 mg/kg) and indomethacin (10 mg/kg) significantly decreased the PGE₂ level, with inhibitory rates of 1.6-fold ($p < 0.05$) and 1.54-fold ($p < 0.05$), respectively. As displayed in Fig. 4B, nitrite production was also significantly ($p < 0.05$) increased in the vehicle group when compared with the normal group. By contrast SE (200 mg/kg) significantly suppressed the nitrite production with inhibitory rate of 7.26-fold ($p < 0.05$). Similarly, indomethacin also significantly suppressed the releases of nitrite, with inhibitory rate of 11.00-fold ($p < 0.05$). COX-2 level significantly inhibited by SE (1.5 fold) compared to carrageenan-induced control paw oedema group (Fig. 4C).

As shown in Fig. 5, induction of inflammation by carrageenan intensely ($p < 0.05$) increased iNOS, COX-2, TNF- α and NF- κ B (p50) mRNA expression as compared to the normal group. However, this effect was significantly ($p < 0.05$) suppressed by SE (200 mg/kg) and indomethacin (10 mg/kg).

Investigation of the activity of SE (200 mg/kg) on the proliferative phase of inflammation indicated that significant inhibition of

granuloma tissue formation. The effects of SE and positive control (indomethacin (10 mg/kg) against cotton pellet induced granuloma in rats are shown in Fig. 6A. Both SE (200 mg/kg) and indomethacin (10 mg/kg) significantly inhibited the formation of granuloma compared with control group.

Results showed that SE (200 mg/kg) significantly ($p < 0.05$) reduced the number of abdominal constriction response induced by administration of acetic acid solution. The inhibitory effect of indomethacin (10 mg/kg) was non-significantly different from the activity revealed by SE (200 mg/kg) (Fig. 6B). In hot plate assay, SE (200 mg/kg) showed significant increases of latency time and inhibited sensation of pain in a pattern similar to standard drug (morphine 5 mg/kg) (Fig. 6C).

Conclusion

To conclude, that the *Spirulina* extract (SE) derived from *A. platensis* was supported as a natural harmless remedy for the treat-

ment of inflammation and pain. Interestingly the outcomes that SE displayed significant anti-inflammatory effect along with both peripheral as well as central analgesic effect provide a foundation for developing opioids alternative drugs, which lead to nausea and vomiting.

References

- [1] Arkadiusz C, Kaławaj K, Sławińska-Brych A, Lemieszek MK, Bartnik M, Wojtanowski KK, et al. Anticancer effect of the water extract of a commercial *Spirulina* (*Arthrospira platensis*) product on the human lung cancer A549 cell line. *Biomed Pharmacother* 2018;106:292–302.
- [2] Hosseini S, Shahbazizadeh S, Khosravi-Darani K, Mozafari M. *Spirulina paltensis*: food and function. *Curr Nutr Food Sci* 2013;9(3):189–93, <http://dx.doi.org/10.2174/1573401311309030003>.
- [3] Habib MA, Parvin M, Huntington T, Hasan M. A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish. Food and Agriculture Organization of the United Nations; 2008. ISBN 978-92-5-106106-0.
- [4] Aissaoui O, Amiali M, Bouzid N, Belkacemi K, Bitam A. Effect of *Spirulina platensis* ingestion on the abnormal biochemical and oxidative stress parameters in the pancreas and liver of alloxan-induced diabetic rats. *Pharm Biol* 2017;55(1):1304–12.
- [5] Shabana EF, Gabr MA, Moussa HR, El-Shaer EA, Ismaiel MM. Biochemical composition and antioxidant activities of *Arthrospira* (*Spirulina*) *platensis* in response to gamma irradiation. *Food Chem* 2017;214:550–5, <http://dx.doi.org/10.1016/j.foodchem.2016.07.109>.
- [6] Abdel-Daim MM, Farouk SM, Madkour FF, Azab SS. Anti-inflammatory and immunomodulatory effects of *Spirulina platensis* in comparison to *Dunaliella salina* in acetic acid-induced rat experimental colitis. *Immunopharmacol Immunotoxicol* 2015;4(2):126–39.
- [7] Gargouri M, Magne C, El Feki A. Hyperglycemia, oxidative stress, liver damage and dysfunction in alloxan-induced diabetic rat are prevented by *Spirulina* supplementation. *Nut Res* 2016;36(11):1255–68.
- [8] Arokiyaraj S, Saravanan M, Badathala V. Green synthesis of silver nanoparticles using aqueous extract of *Taraxacum officinale* and its antimicrobial activity. *South Indian J Biol Sci* 2015;2:115–8.
- [9] Deng R, Chow T-J. Hypolipidemic, antioxidant and antiinflammatory activities of microalgae spirulina. *Cardiovasc Ther* 2010;28(4):33–45.
- [10] Pham TX, Lee J-Y. Anti-inflammatory effect of *Spirulina platensis* in macrophages is beneficial for adipocyte differentiation and maturation by inhibiting nuclear factor- κ B pathway in 3T3-L1 adipocytes. *J Med Food* 2016;19(June (6)):535–42.
- [11] Park YK, Rasmussen HE, Ehlers SJ, Blobaum KR, Lu F, Schlegel VL, Carr TP, Lee JY. Repression of proinflammatory gene expression by lipid extract of *Nostoc commune* var *sphaeroides* Kützing, a blue-green alga, via inhibition of nuclear factor- κ B in RAW 264.7 macrophages. *Nutr Res* 2008;28:83–91.
- [12] Arasu MV, Jung MW, Kim DH, Park HS, Ilavenil S, Al-Dhabi NA, et al. Identification and phylogenetic characterization of novel *Lactobacillus plantarum* species and their metabolite profiles in grass silage. *Ann Microbiol* 2015;65(1):15–25.
- [13] Park S, Arasu MV, Lee M-K, Chun J-H, Seo JM, Al-Dhabi NA, et al. Analysis and metabolite profiling of glucosinolates, anthocyanins and free amino acids in inbred lines of green and red cabbage (*Brassica oleracea* L.). *LWT Food Sci Technol* 2014;58(1):203–13.
- [14] Lin JY, Tang CY. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. *Food Chem* 2007;101(1):140–7.
- [15] Mossoba M, Kramer J, Delmonte P, Yurawecz M, Rader J. AOAC official method 996.06, fat (total, saturated, and unsaturated in foods), hydrolytic extraction gas chromatographic method. Urbana: AOCS Press; 2003.
- [16] Winter CA, Risley EA, Nuss GW. Carrageenan-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proc Soc Exp Biol Med* 1962;111(3):544–7.
- [17] Xian YF, Mao QQ, Ip SP, Lin ZX, Che CT. Comparison on the anti-inflammatory effect of Cortex *Phellodendri Chinensis* and Cortex *Phellodendri Amurensis* in 12-O-tetradecanoyl-phorbol-13-acetate-induced ear edema in mice. *J Ethnopharmacol* 2011;137(3):1425–30.
- [18] Winter CA, Porter CS. Effect of alterations in side chain upon anti-inflammatory and liver glycogen activities of hydrocortisone esters. *J Am Pharm Assoc* 1957;46(9):515–9.
- [19] Mungantiwar AA, Nair AM, Shinde UA, Dikshit VJ, Saraf MN, Thakur VS, et al. Studies on the immunomodulatory effects of *Boerhaavia diffusa* alkaloidal fraction. *J Ethnopharmacol* 1999;65(2):125–31.
- [20] Antonisamy P, Subash-Babu P, Alshatwi A, Aravinthan A, Savarimuthu A, Ignacimuthu S, et al. Gastroprotective effect of nymphaeol isolated from *Nymphaea stellata* (Willd.) flowers: contribution of antioxidant, anti-inflammatory and anti-apoptotic activities. *Chem Biol Interact* 2014;224:157–63.
- [21] Tian M, Row K. Separation of four bioactive compounds from *Herba artemisiae scopariae* by HPLC with ionic liquid-based silica column. *Am J Analyt Chem* 2011;66(6):580–5.
- [22] Gilani AH, Janbaz KH. Protective effect of *Artemisia scoparia* extract against acetaminophen-induced hepatotoxicity. *Gen Pharmacol* 1993;24(6):1455–8.
- [23] Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot* 2003;91(2):179–94.
- [24] Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. *World Allergy Organ J* 2012;5(1):9–19.