

Inhibition of lipase and inflammatory mediators by *Chlorella* lipid extracts for antiacne treatment

G. Sibi

Department of Biotechnology, Indian Academy Degree College, Centre for Research and Post Graduate Studies, Bengaluru, Karnataka, India

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ABSTRACT

Acne vulgaris is a chronic inflammatory disease, and its treatment is challenging due to the multifactorial etiology and emergence of antibiotic-resistant *Propionibacterium acnes* strains. This study was focused to reduce antibiotics usage and find an alternate therapeutic source for treating acne. Lipid extracts of six *Chlorella* species were tested for inhibition of lipase, reactive oxygen species (ROS) production, cytokine production using *P. acnes* (Microbial Type Culture Collection 1951). Lipase inhibitory assay was determined by dimercaprol Tributyrates - 5, 5'-dithiobis 2-nitrobenzoic acid method and ROS production assay was performed using nitro-blue tetrazolium test. The anti-inflammatory activity of algal lipid extracts was determined by *in vitro* screening method based on inhibition of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF- α) produced by human peripheral blood mononuclear cells. Minimum inhibitory concentration (MIC) values of lipid extracts were determined by microdilution method, and the fatty acid methyl esters (FAME) were analyzed by gas chromatography-mass spectroscopy. *Chlorella ellipsoidea* has the highest lipase inhibitory activity with 61.73% inhibition, followed by *Chlorella vulgaris* (60.31%) and *Chlorella protothecoides* (58.9%). Lipid extracts from *C. protothecoides* and *C. ellipsoidea* has significantly reduced the ROS production by 61.27% and 58.34% respectively. Inhibition of pro-inflammatory cytokines TNF- α showed the inhibition ranging from 58.39% to 78.67%. *C. vulgaris* has exhibited the MIC value of 10 μ g/ml followed by *C. ellipsoidea*, *C. protothecoides* and *Chlorella pyrenoidosa* (20 μ g/ml). FAME analysis detected 19 fatty acids of which 5 were saturated fatty acids, and 14 were unsaturated fatty acids ranging from C14 to C24. The results suggest that lipid extracts of *Chlorella* species has significant inhibitory activity on *P. acnes* by inhibiting lipase activity. Further, anti-inflammatory reaction caused by the pathogen could be reduced by the inhibiting the production of ROS and inflammatory mediators TNF- α and exposes new frontiers on the antiacne activities of *Chlorella* lipid extracts.

Key words: Antiacne, antiinflammatory, *Chlorella*, lipase inhibition, reactive oxygen species

Address for correspondence:

Dr. G. Sibi,
Department of Biotechnology, Indian Academy Degree College, Centre for Research and Post Graduate Studies, Bengaluru - 560 043, Karnataka, India.
E-mail: gsibii@gmail.com

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INTRODUCTION

Acne is a chronic inflammatory disease characterized by seborrhea, the formation of open and closed comedones, erythematous papules, pustules and in more severe cases nodules, deep pustules and pseudocysts.^[1] It affects approximately 85% of the individuals aged between 12 and 24 years at some time.^[2] Excess sebum production, hyperkeratinization of the hair follicle, oxidative stress and the release of inflammatory mediators are the common pathways involved in acne development.^[3,4] Colonization of the skin by *Propionibacterium acnes* is one factor involved in the etiology of acne vulgaris.^[5] *P. acnes* is the dominant isolate from acne lesion,^[6] which is a Gram-positive

anaerobe and has been implicated in inflammatory phase of acne.^[7] It induces inflammation of sebaceous glands in human face, neck, chest or back.^[8]

It is challenging to treat acne vulgaris due to the multifactorial etiology.^[9] Triclosan, benzoyl peroxide, azelaic acid, retinoid, tetracycline, erythromycin, macrolide, levofloxacin and clindamycin are the most commonly prescribed antibiotics to treat acne vulgaris.^[10-14] However, these antibiotics are associated with several side-effects when used for a long period.^[15] Combination therapy with a topical retinoid and an antibiotic can normalize follicular epithelial desquamation and reduce bacterial proliferation.^[2] Antimicrobial therapy for acne has also been complicated by the emergence of antibiotic-resistant strains of *P. acnes*.^[16,17] The widespread and long-term use of antibiotics in the treatment of acne has resulted in the spread of resistant bacterial strains and treatment failure.^[18,19] The inevitable emergence of antibiotic-resistant strains of *P. acnes* has created some serious health care implication.^[5] Therefore, there is a need to develop new medicines or therapies for acne treatment and this study was focused to reduce antibiotics usage and find an alternate therapeutic source for treating acne. In this regard, lipid extracts of *Chlorella* species were tested for inhibition of lipase and inflammatory mediators as novel therapeutic agents for effective acne therapy.

MATERIALS AND METHODS

Chemicals and reagents

Tetracycline hydrochloride, isopropyl methylphenol, dimercaprol tributyrates (BALB), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich (Bengaluru), India and all other chemicals of highest purity grade were purchased from SD Fine Chemicals, Bengaluru. Brain heart infusion (BHI) broth was obtained from HiMedia Laboratories. For the quantification of cytokines, tumor necrosis factor alpha (TNF- α) ELISA kit was purchased from Sigma-Aldrich (Mumbai).

Algal lipid extraction

Six *Chlorella* species namely *Chlorella ellipsoidea*, *Chlorella emersonii*, *Chlorella protothecoides*, *Chlorella pyrenoidosa*, *Chlorella sorokiniana* and *Chlorella vulgaris* were used in this study. The algae were isolated from Bangalore freshwater habitats (13°04'N and 77°58'E), identified^[20,21] and cultivated in Bold's basal medium. Algal lipids were extracted according to the method of Folch *et al.*^[22] Briefly, the cells were centrifuged at 10,000 rpm for 10 min and the pellet was homogenized with chloroform-methanol (2:1 v/v) solution. The sample was centrifuged and to the supernatant, 0.73% NaCl water was added to produce a final solvent system of 2:1:0.8 chloroform: Methanol: Water (v/v/v). The mixture was shaken for 5 min and centrifuged for 15 min at 2000 rpm to separate the phases. The lower organic phase was

collected, and the chloroform-methanol solution was evaporated under a steam of nitrogen for further studies.

Test organism

Propionibacterium acnes (Microbial Type Culture Collection [MTCC] 1951) was procured from MTCC, India.

Lipase inhibitory assay

Crude lipase was prepared by centrifuging cell suspension of *P. acnes* (rabbit blood agar) at 900 \times g for 10 min at 4°C. The precipitate was diluted in phosphate buffer saline (PBS) (pH 6.8). The cells were homogenized and centrifuged at 5000 \times g for 1 min. The filtrate was collected and dialyzed for 6 days, followed by lyophilization of the crude extract.^[23,24]

Lipase inhibitory assay was determined by BALB-DTNB method described by Furukawa *et al.*^[25] using tetracycline hydrochloride and isopropyl methylphenol as the positive controls.

Reactive oxygen species production inhibition assay

Propionibacterium acnes cultivated in BHI and glucose with and without algal extracts (100 μ g/ml) for 72 h at 37°C in anaerobic conditions were used as stimulant for reactive oxygen species (ROS) activity. ROS production assay was performed using nitro-blue tetrazolium (NBT) test according to the method of Park *et al.*^[26] Briefly, 500 μ l of venous blood of healthy Sprague-Dawley (SD) rats, 50 μ l of stimulants (*P. acnes* with and without algal extracts), positive control (polymorphonuclear leucocytes with zymosan) and negative control (culture media) were mixed and incubated at 25°C for 15 min. This was followed by the addition of 100 μ l of NBT solution in 1 mg/ml of PBS and incubated at 37°C for 30 min and then again at 25°C for 20 min. Finally, smears were prepared and stained by Leishman's stain for differential counting of formazan deposits in polymorphonuclear leukocytes.

Cytokine production inhibition assay

The anti-inflammatory activity of algal extracts was determined by *in vitro* screening method based on inhibition of pro-inflammatory cytokines (TNF- α) produced by human peripheral blood mononuclear cells (PBMC).^[27] *P. acnes* was grown in 1% glucose BHI for 72 h at 37°C in an anaerobic atmosphere. The log phase bacterial culture was harvested, washed thrice in PBS (pH 7.2), and incubated at 80°C for 30 min to heat-kill the bacteria.

Isolation of PBMC was prepared from venous blood of healthy SD rats. Blood was diluted 1:2 with phosphate-buffered saline (pH-7.2), layered on Histopaque, washed thrice with PBS and resuspended in complete RPMI-1640 supplemented with 10% fetal calf serum (FCS). The cells were counted and resuspended at a concentration of 1×10^6 cells/ml in RPMI supplemented with 10% FCS. Cell viability was determined using the trypan blue dye exclusion test.

Quantification of cytokines

A 1-ml culture of PBMC (1×10^6 cells) was setup in 24 well tissue culture plates and stimulated with heat-killed *P. acnes* (1×10^8 cells/ml) in the presence or absence of algal extracts at a concentration of 40 $\mu\text{g/ml}$. Cultures were incubated at 37°C for 18 h in a humidified the atmosphere containing 5% CO_2 . Cultures without stimulants were set up as controls. The cultures were centrifuged to collect cell-free supernatant containing secreted cytokines and analyzed for TNF- α using sandwich ELISA (Sigma, India).

The ratio (%) of inhibition of the cytokine release was calculated using the following equation:

$$\text{Degree of inhibition (\%)} = 100 \times (1 - T/C)$$

T: Concentration of cytokines in culture supernatant with the test compound.

C: Concentration of cytokines in culture supernatant with the solvent.

Antiacne assay

The antibacterial activity of algal lipid extract was determined by microdilution method in 96 well plates. Lipid extracts of 5, 10, 20, 40 and 80 $\mu\text{g/ml}$ were used to determine the minimum inhibitory concentration (MIC) values. *P. acnes* was incubated in BHI medium for 48 h under anaerobic conditions and 100 μL of bacterial inoculum contained approximately 1×10^8 CFU/ml was inoculated into the wells. This was followed by incubation at 37°C for 72 h under anaerobic conditions in an anaerobic bag with gas pack and indicator tablets. All tests were performed in triplicates using clindamycin as a positive control.

Fatty acid methyl ester preparation and analysis

The fatty acid methyl esters (FAME) were converted from lipids and free fatty acids according to protocol of Lepage and Roy.^[28] Algal cultures were centrifuged, and 0.1 g of pellet was homogenized with 1.5 ml of acetyl chloride and methanol (20:1, v/v) in reaction vessels. Subsequently, 1 ml of hexane was added to the mixture and heated to 100°C for 1 h for derivatization. The mixture was cooled, and 1 ml of distilled water was added and the organic phase was separated by centrifugation and dried with anhydrous sodium sulfate. The extracts were filtered and FAME was analyzed on gas chromatography-mass spectroscopy by following conditions described earlier.^[29]

RESULTS

Lipase inhibitory assay using BALB-DTNB method revealed that *C. ellipsoidea* has the highest activity with 61.73% inhibition, followed by *C. vulgaris* (60.31%) and *C. protothecoides* (58.9%). Superoxide radical production by measuring polymorphonuclear leucocytes containing

formazan deposit in the presence of the algal extract was done using NBT assay. The results showed that *Chlorella* extracts significantly reduced the ROS production with the inhibitory ratio of 61.27% and 58.34% by *C. protothecoides* and *C. ellipsoidea* respectively [Table 1]. Inhibition of pro-inflammatory cytokines (TNF- α) by the algal extracts were performed along with stimulant and positive control to determine the stimulatory role of *P. acnes*. Heat killed *P. acnes* have increased the production of TNF- α at 89.34 $\mu\text{g/ml}$, which was 19.8% higher than the positive control (71.58 $\mu\text{g/ml}$). Inhibitory effects of algal extracts on TNF- α showed the inhibition with 78.67% by *C. ellipsoidea*. Lipid extracts of *Chlorella* species were tested for *in vitro* anti acne activity by micro dilution method and the MIC was observed as 10 $\mu\text{g/ml}$ for *C. vulgaris* and 20 $\mu\text{g/ml}$ for *C. ellipsoidea*, *C. protothecoides* and *C. pyrenoidosa* [Table 2].

Fatty acid analysis of *Chlorella* extracts detected 19 fatty acids altogether [Table 3], including 5 saturated fatty acids (SFA) and 14 unsaturated fatty acids. The unsaturated fatty acids comprised of 8 mono-unsaturated fatty acids (MUFA), 6 polyunsaturated fatty acids (PUFAs). The SFA ranged from C14 to C18 and the unsaturated fatty acids were from C14 to C24. The most abundant fatty acids were pentadecyclic (C15:0), palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids. All the species analyzed in this study presented considerably higher amounts of unsaturated fatty acids (73.6%). In *C. vulgaris*, the content of palmitic and linoleic acid were

Table 1: Inhibitory activities of *Chlorella* lipid extracts on lipase, ROS and pro-inflammatory cytokines production

Organism	Lipase inhibition (%)	ROS inhibition (%)	TNF- α inhibition (%)
<i>C. ellipsoidea</i>	61.73 \pm 0.06	58.34 \pm 1.05	78.67 \pm 0.62
<i>C. emersonii</i>	39.67 \pm 0.19	49.20 \pm 0.27	58.39 \pm 0.05
<i>C. protothecoides</i>	58.90 \pm 0.74	61.27 \pm 0.21	71.02 \pm 0.15
<i>C. pyrenoidosa</i>	47.16 \pm 1.37	55.09 \pm 0.54	69.17 \pm 1.94
<i>C. sorokiniana</i>	46.91 \pm 0.28	46.38 \pm 0.02	63.75 \pm 1.16
<i>C. vulgaris</i>	60.31 \pm 0.10	57.95 \pm 0.10	73.46 \pm 1.00

ROS: Reactive oxygen species, TNF- α : Tumor necrosis factor-alpha. $P \leq 0.05$

Table 2: Anti-acne activity of *Chlorella* lipid extracts

Organism	MIC values ($\mu\text{g/ml}$)
<i>C. ellipsoidea</i>	20
<i>C. emersonii</i>	40
<i>C. protothecoides</i>	20
<i>C. pyrenoidosa</i>	20
<i>C. sorokiniana</i>	≥ 80
<i>C. vulgaris</i>	10
<i>Clindamycin</i>	5

MIC: Minimum inhibitory concentration

Table 3: FAME analysis of *Chlorella* lipid extracts (%)

Fatty acids	<i>C. ellipsoidea</i>	<i>C. emersonii</i>	<i>C. protothecoides</i>	<i>C. pyrenoidosa</i>	<i>C. sorokiniana</i>	<i>C. vulgaris</i>
SFAs						
C14:0	0.17	0.09	ND	0.11	0.08	0.10
C15:0	0.48	0.01	0.51	0.09	0.22	0.37
C16:0	4.21	7.02	8.37	8.04	3.96	11.31
C17:0	ND	ND	ND	ND	ND	0.06
C18:0	0.07	0.09	ND	0.17	ND	0.26
MUFA						
C14:1	ND	0.12	0.26	0.05	0.17	ND
C15:1	ND	0.06	ND	ND	ND	ND
C16:1	0.27	0.05	0.14	ND	0.11	0.16
C17:1	0.03	ND	0.14	0.11	ND	ND
C18:1	2.37	2.09	4.38	1.84	0.98	3.07
C20:1	1.05	ND	0.96	ND	1.47	0.63
C22:1	ND	0.05	0.33	ND	ND	ND
C24:1	1.67	1.25	ND	0.37	0.66	0.39
PUFA						
C14:2	0.02	ND	0.05	ND	ND	0.09
C15:3	ND	ND	ND	0.08	0.03	ND
C16:2	0.10	ND	ND	0.09	ND	ND
C17:2	0.16	0.08	ND	ND	ND	ND
C18:2	4.37	6.24	7.35	5.47	2.06	8.29
C23:6	0.20	ND	ND	0.45	0.44	0.26

*ND: Not detected, FAME: Fatty acid methyl esters, SFA: Saturated fatty acid, MUFA: Mono unsaturated fatty acid, PUFA: Poly unsaturated fatty acid

higher (11.31% and 8.29%) while oleic acid was higher in *C. protothecoides* (4.38%). Optimum fatty acid levels were observed with *C. ellipsoidea* while it exhibited the largest fatty acid profile as it contained 14 different fatty acids and the next diverse were *C. emersonii*, *C. pyrenoidosa* and *C. vulgaris* with 12 different fatty acids.

DISCUSSION

The major factors to cause acne vulgaris include follicular hyperkeratosis, sebum secretion, *P. acnes* and inflammation.^[30] *P. acnes* produce enzymes such as lipases, proteases and hyaluronidases leading to subsequent inflammatory reactions in the surrounding dermis.^[31] Formation of free fatty acids as a result of *P. acnes* lipases on sebaceous triglycerides induces severe inflammation.^[32] One of the objectives of this study was to determine the lipase inhibition by algal extracts thereby reducing the pathogenicity of *P. acnes*. The use of BALB-DTNB method revealed that the lipase activity was inhibited up to 61.73% by *C. ellipsoidea*, followed by *C. vulgaris* and *C. protothecoides*. Lipase might play an important role in facilitating bacterial colonization in nutrient-limited environments such as the human skin. Compounds targeting acne should inhibit *P. acnes* lipase activity^[23] and the present findings suggests that algal inhibitory action may contribute to the eradication of *P. acnes* colonization on human skin through lipase inhibition and at the same time it could be used as a nonantibiotic source for skin care.

Propionibacterium acnes can evoke local inflammation by producing neutrophil chemotactic factors and the attracted neutrophils release inflammatory mediators such as ROS.^[33] Though ROS perform a useful function in the skin barrier against acne microbes^[34] excess formation affects skin condition by activating neutrophil infiltration leads to irritation and disruption of the integrity of the follicular epithelium and are responsible for the progression of inflammatory acne. Removal of the ROS can significantly reduce cell damage that may occur during acne inflammation.^[35] Inhibition of ROS production using the lipid extracts revealed that *Chlorella* species has significant inhibitory activity thereby reducing inflammatory cell damage.

In addition, free fatty acids released from lipase activity and ROS can also act as second messengers in the induction of several biological responses like the generation of cytokines.^[36] Inflammation acts as a central executor in the pathogenesis of acne where TNF- α and interleukin-1b are the cytokines that act as signaling molecules for immune cells and co-ordinate the inflammatory responses.^[37] In this study, the stimulatory role of *P. acnes* on pro-inflammatory cytokine (TNF- α) production was demonstrated followed by inhibitory action by the algal extracts where 78.67% inhibition was observed. The results suggest that the anti-inflammatory activity of the microalgal extract may be used in down-regulation of the inflammatory mediator's production by *P. acnes* in acne vulgaris. Antiacne

compounds from marine algae were reported in earlier studies^[38-40] and in this study, antiacne activity of lipid extracts from fresh water *Chlorella* species were determined. It was hypothesized that lipids kill microorganisms by disruption of the cellular membrane.^[41] Antimicrobial susceptibility of *P. acnes* to microalgal extract was performed using micro dilution method in which the MIC values were from 10 to 40 µg/ml except *C. sorokiniana* (≥80 µg/ml). In the previous report, MIC of glycolipid extract of macroalgae against *P. acnes* was observed at 50 µg/ml.^[42] Clindamycin and erythromycin are the most common antibiotics used against *P. acnes*^[14] hence clindamycin was used as positive control.

The data about the detailed composition of *Chlorella* lipids are available but no reports exist in literature about anti acne activity of their lipids. In this work, the six *Chlorella* species lipids were investigated as a natural source of functional bio-actives to control acne. In addition, inhibitors of bacterial lipase, ROS and inflammatory mediators from *Chlorella* lipids were also studied. For this, FAME were prepared and analyzed. MUFA and PUFA were the main FAME detected in the profile among various *Chlorella* species. Regarding the size of the carbon chain, the species displayed a FAME profile ranging from C14 to C23. The presence of oleic and linoleic acid in *Chlorella* species was reported earlier.^[43]

CONCLUSION

This study analyzed whether algal lipid extract could reduce the pathogenicity of *P. acnes* with regard to acne development. Further, anti-inflammatory reaction caused by the pathogen could be reduced by the inhibiting the production of ROS and inflammatory mediators (TNF-α) which exposes new frontiers on the anti-acne activities of *Chlorella* lipid extracts.

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