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Preliminary Evaluation of the Antibacterial Activity of an Ethanolic Extract from Commercial *Chlorella vulgaris* Powder Against Two Reference Bacterial Strains

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Abstract

Antibiotic resistance is a growing clinical challenge, particularly among *Klebsiella pneumoniae* and *Staphylococcus aureus*, prompting interest in natural antimicrobial agents such as microalgae. To evaluate the in vitro antibacterial activity of an ethanolic extract prepared from commercial *Chlorella vulgaris* powder against reference strains of *K. pneumoniae* (ATCC 13883) and *S. aureus* (ATCC 25923). Three extract concentrations (100, 50, and 10 mg/mL) were tested using the disk diffusion method alongside standard antibiotics, in triplicate. Total inhibition diameters were recorded, and the 6 mm disk diameter was subtracted to obtain net inhibition zones. Data were analysed by two-way ANOVA with Tukey's HSD post-hoc test. Treatment significantly affected inhibition zone size ($F(6,28) = 262.58$, $P < 0.001$), while bacterial species alone had no significant effect ($P = 0.456$) and the treatment × species interaction was not significant ($P = 0.114$). A dose-dependent increase was observed; at 100 mg/mL, mean net inhibition zones reached 12.33 ± 2.52 mm for *K. pneumoniae* and 10.33 ± 1.53 mm for *S. aureus*. The ethanolic extract of commercial *C. vulgaris* powder exhibited detectable, dose-dependent antibacterial activity against both reference strains, warranting further MIC, MBC, and chemical characterisation studies.

Keywords: antibacterial activity, *Chlorella vulgaris*, disk diffusion, ethanolic extract, microalgae, preliminary screening.

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الملخص

تُعدّ مقاومة المضادات الحيوية تحديًا سريريًا متزايدًا، لا سيما بين بكتيريا كليبسيلا الرئوية والمكورات العنقودية الذهبية، مما يُثير الاهتمام بالعوامل الطبيعية المضادة للميكروبات مثل الطحالب الدقيقة. هدفت هذه الدراسة إلى تقييم النشاط المضاد للبكتيريا في المختبر لمستخلص إيثانولي مُحضّر من مسحوق طحلب الكلوريلا الشائع التجاري ضد سلالات مرجعية من كليبسيلا الرئوية (ATCC 13883)والمكورات العنقودية الذهبية (ATCC 25923).تم اختبار ثلاثة تراكيز من المستخلص (100، 50، و10 ملغم/مل) باستخدام طريقة انتشار القرص جنبًا إلى جنب مع المضادات الحيوية القياسية، في ثلاث نسخ. سُجّلت أقطار التثبيط الكلية، وطُرح قطر القرص البالغ 6 مم للحصول على مناطق التثبيط الصافية. تم تحليل البيانات باستخدام تحليل التباين ثنائي الاتجاه مع اختبار توكي HSD اللاحق. أثر العلاج بشكلٍ ملحوظ على حجم منطقة التثبيط ($F(6,28) = 262.58$، $P < 0.001$)، بينما لم يكن لنوع البكتيريا وحده تأثيرٌ يُذكر ($P = 0.456$)، ولم يكن التفاعل بين العلاج ونوع البكتيريا ذا دلالة إحصائية ($P = 0.114$).ولوحظ ازديادٌ في حجم منطقة التثبيط تبعًا للجرعة؛ فعند تركيز

100 ملغم/مل، بلغ متوسط حجم منطقة التثبيط الصافي 12.33 ± 2.52 مم لبكتيريا *Klebsiella pneumoniae* و10.33 ± 1.53 مم لبكتيريا *Staphylococcus aureus*. وأظهر المستخلص الإيثانولي لمسحوق C. vulgaris التجاري نشاطًا مضادًا للبكتيريا قابلًا للكشف، ويعتمد على الجرعة، ضد كلا السلالتين المرجعتين، مما يستدعي إجراء المزيد من الدراسات لتحديد الحد الأدنى للتركيز المثبط (MIC)والحد الأدنى للتركيز القاتل (MBC)والخصائص الكيميائية).

الكلمات المفتاحية: النشاط المضاد للبكتيريا، كلوريلا فولغاريس، انتشار القرص، المستخلص الإيثانولي، الطحالب الدقيقة، الفحص الأولي.

Introduction

Antibiotic resistance remains a growing problem, particularly in opportunistic pathogens such as *Klebsiella pneumoniae* and *Staphylococcus aureus* [1,3]. Some microalgae, including *Chlorella vulgaris*, produce metabolites with antimicrobial properties. *C. vulgaris* contains phenolics, flavonoids, unsaturated fatty acids, carotenoids, and bioactive peptides [4,5]. Pradhan et al. [6] also reported antibacterial activity of *C. vulgaris* methanolic extract against various pathogenic bacteria. Previous studies suggest that these compounds may contribute to membrane disruption, enzyme interference, and oxidative damage [7,8].

Most previous studies have evaluated laboratory-cultured *Chlorella* biomass. In contrast, commercially available *Chlorella* products are widely used as nutraceutical supplements and are readily accessible to consumers. Evaluating their biological activity is therefore relevant from an applied perspective because it reflects products currently available in the marketplace. The present study, therefore, evaluated an ethanolic extract prepared from commercial *C. vulgaris* powder against *K. pneumoniae* and *S. aureus*.

Material and Methods

Extract preparation

Commercial organic *Chlorella vulgaris* powder (Dragon Superfoods, Smart Organic Ltd., Sofia, Bulgaria; 200 g package, raw organic, EU certified; best before: 11 December 2025; batch code: 2416) was used. Ten grams were mixed with 100 mL of 70% ethanol and shaken at room temperature (25 ± 2 °C) for 24 hours [9]. A lipid layer formed and was reincorporated by vortexing before filtering through Whatman No. 1 paper. The filtrate was dried at 45 °C and then dissolved in 10% DMSO to obtain concentrations of 100, 50, and 10 mg/mL. Extraction yield was calculated as (weight of dried extract / dry powder weight) × 100, yielding approximately 12.5% (w/w). The dried extract was dark green and semi-viscous.

Bacterial strains

ATCC reference strains *Klebsiella pneumoniae* (ATCC 13883) and *Staphylococcus aureus* (ATCC 25923) were used. Both were maintained on nutrient agar at 4 °C and subcultured before each experiment.

Disk diffusion assay

The CLSI guidelines were followed [10,11]. Bacterial suspensions were adjusted to 0.5 McFarland and swabbed onto Mueller-Hinton agar. Sterile blank paper disks (6 mm diameter, Whatman No. 1) were loaded with 10 µL of each extract. Positive controls included amoxicillin-clavulanate (AMC, 30 µg), gentamicin (CN, 10 µg), and cefuroxime (CXM, 30 µg). DMSO-loaded disks served as a negative control. After 24 hours at 37 °C, inhibition zones were measured to the nearest 0.1 mm. All procedures were done aseptically. Total inhibition diameters (including the 6 mm disk) were recorded. The disk diameter (6 mm) was then

subtracted to obtain the net inhibition zone, ensuring consistent comparison across treatments. Extract amounts per disk were 1 mg (100 mg/mL), 0.5 mg (50 mg/mL), and 0.1 mg (10 mg/mL).

Statistical analysis

All experiments were performed in triplicate (n = 3). Data are presented as means ± SD. Two-way ANOVA was run with treatment (7 levels) and bacterial species (2 levels) as fixed factors. Pairwise comparisons used Tukey's HSD post-hoc test. A P value < 0.05 was considered significant. SPSS version 26 was used (IBM Corp., Armonk, NY, USA).

Results

Antibacterial activity

Table 1 summarises the net inhibition zones. A dose-dependent increase was observed. At 100 mg/mL, mean net inhibition zones reached 12.33 ± 2.52 mm for *K. pneumoniae* and 10.33 ± 1.53 mm for *S. aureus*. The extraction yield from the commercial powder was approximately 12.5% (w/w).

ANOVA results

Two-way ANOVA results are summarised in Table 2. Treatment significantly affected inhibition zone size ($F(6,28) = 262.58$, $P < 0.001$). Bacterial species alone had no significant effect ($F(1,28) = 0.57$, $P = 0.456$), and the treatment × species interaction was also not significant ($F(6,28) = 1.91$, $P = 0.114$). $R^2 = 0.983$, Adjusted $R^2 = 0.975$.

Dose-dependent antibacterial activity

Tukey HSD post-hoc analysis separated the treatments into six homogeneous subsets. Cefuroxime (CXM) was most effective (mean 24.00 mm, group a). Gentamicin (CN) and amoxicillin-clavulanate (AMC) formed group b. The extract showed a clear dose-response pattern: 100 mg/mL (11.33 mm, group c) > 50 mg/mL (8.67 mm, group d) > 10 mg/mL (6.75 mm, group e). DMSO (group f) produced minimal inhibition (2.42 mm). The negligible inhibition produced by the DMSO control (<3 mm) indicates that the observed antibacterial activity was primarily attributable to the Chlorella extract.

Table 1. Mean net inhibition zones (mm ± SD) excluding the 6 mm disk diameter for each treatment and bacterial species with Tukey HSD groupings (n = 3).

Treatment	Concentration	K. pneumoniae (mm ± SD)	S. aureus (mm ± SD)	Tukey Group
DMSO	Control	2.17 ± 0.29	2.67 ± 0.29	f
Extract	10 mg/mL	6.67 ± 1.15	6.83 ± 0.76	e
Extract	50 mg/mL	8.17 ± 1.61	9.17 ± 1.04	d
Extract	100 mg/mL	12.33 ± 2.52	10.33 ± 1.53	c
CN	10 µg	20.00 ± 1.00	19.00 ± 1.00	b
AMC	30 µg	20.00 ± 0.50	21.33 ± 1.53	b
CXM	30 µg	25.00 ± 1.00	23.00 ± 1.00	a

Treatments sharing the same letter are not significantly different (Tukey HSD, $P < 0.05$).
AMC = amoxicillin-clavulanate; CN = gentamicin; CXM = cefuroxime; DMSO = dimethyl sulfoxide.
Note: Net inhibition zone = total diameter (including the 6 mm disk) minus the 6 mm disk diameter.

Table 2. Two-way ANOVA summary for net inhibition zone diameters.

Source	df	Sum of Squares	Mean Square	F	P
Corrected Model	13	2381.333	183.179	122.12	< 0.001
Intercept	1	7466.667	7466.667	4977.78	< 0.001
Treatment	6	2363.250	393.875	262.58	< 0.001
Bacteria	1	0.857	0.857	0.57	0.456
Treatment × Bacteria	6	17.226	2.871	1.91	0.114
Error	28	42.000	1.500		
Total	42	9890.000			
Corrected Total	41	2423.333			

$R^2 = 0.983$, Adjusted $R^2 = 0.975$.

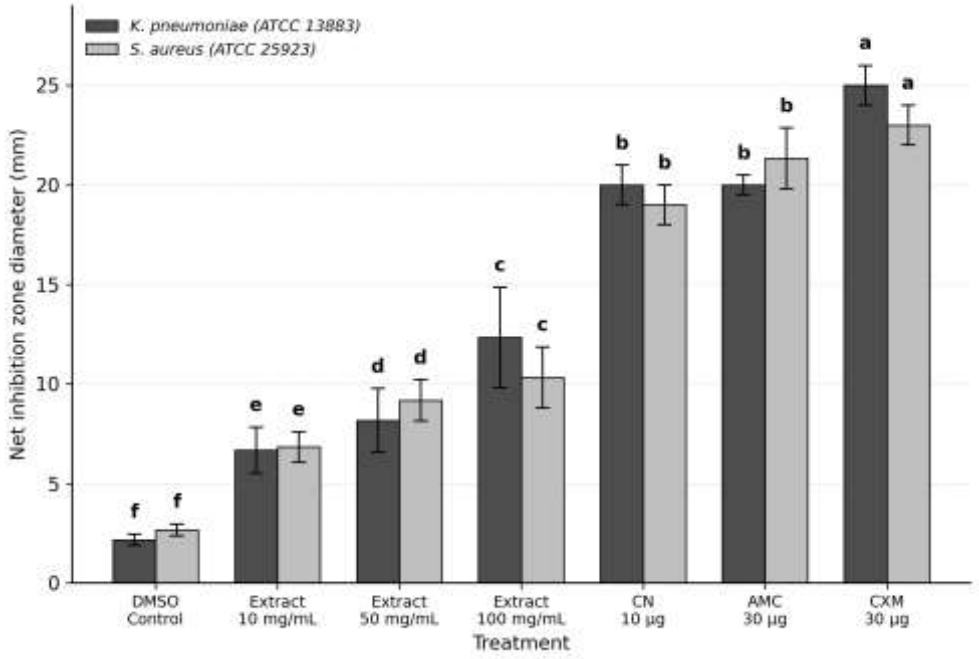

Figure 1. Inhibition zone diameters (net, excluding 6 mm disk) of C. vulgaris ethanolic extract and antibiotic controls against *K. pneumoniae and S. aureus*. Bars = mean ± SD (n = 3). Different letters above bars indicate significant differences (Tukey HSD, P < 0.05).

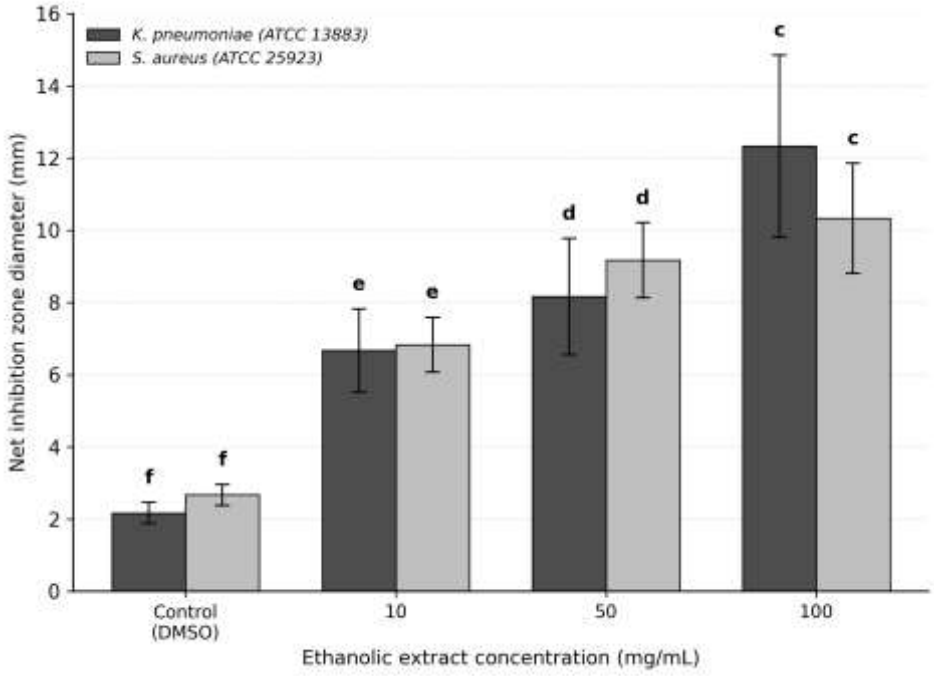

Figure 2. Dose-response relationship of *C. vulgaris* ethanolic extract at three concentrations against both test bacteria. Bars = mean ± SD (n = 3). Different letters indicate significant differences (Tukey HSD, P < 0.05).

Discussion

The statistical analysis confirmed a strong treatment-dependent effect on bacterial growth inhibition ($F(6,28) = 262.58$, $P < 0.001$). Importantly, the lack of significance for bacterial species ($P = 0.456$) and the interaction term ($P = 0.114$) indicates that, under the conditions tested, the ethanolic extract exhibited a comparable antibacterial response against the two reference strains rather than a clearly species-specific effect.

At the highest tested concentration (100 mg/mL), we recorded net inhibition zones of 12.33 mm for *K. pneumoniae* and 10.33 mm for *S. aureus* (corresponding to total diameters of 18.33 mm and 16.33 mm, respectively). These values generally fall within the range reported for other crude algal extracts in the literature [12,13]. Of particular relevance to our context, a previous investigation conducted in Libya on freshwater algae isolated from the Derna waterfall region, which included *C. vulgaris*, reported similar inhibitory effects against these same pathogens [14].

Direct comparison with previous studies should be interpreted with caution because of methodological differences in inhibition zone measurements. Our net zones are smaller than the 30 mm diameters documented for chloroform extracts by Mashhadinejad et al and Alshintari et al. 2026. [13;26]. We attribute the moderate activity observed in our ethanolic extract to the differential solubility of bioactive compounds [25]. While nonpolar solvents like chloroform efficiently extract lipophilic constituents (fatty acids and carotenoids), our choice of 70% ethanol was specifically aimed at recovering moderately polar phenolic and flavonoid compounds, which are established antibacterial agents [7,8]. Moreover, the aqueous component in our solvent may have co-extracted hydrophilic polysaccharides and proteins, potentially interfering with the diffusion of active metabolites in the agar.

Our findings are consistent with previous reports demonstrating dose-dependent antibacterial activity for *C. vulgaris* extracts against both Gram-positive and Gram-negative bacteria, including notable synergistic effects with essential oils against MRSA and promising activity against clinically isolated *K. pneumoniae* [15,16,6,17]. Since only evaluated ATCC reference strains, we acknowledge that future work should incorporate multidrug-resistant clinical isolates to better assess real-world therapeutic applicability.

An important consideration is that the present study utilised commercially processed powder rather than freshly cultured biomass. Commercial processing may involve high-temperature drying and extended storage, which could potentially degrade thermolabile bioactive constituents. This could partially explain our observed zones were modest compared to some laboratory-scale studies. Nevertheless, commercial products are the most accessible form for nutraceutical or food-industry applications, making their evaluation practically relevant, especially given that global Chlorella production is estimated to exceed 2000 tons annually [19,20].

Previous FTIR analyses by Sukhikh et al. [18] identified key functional groups in *C. vulgaris*, including methylenic and carbonyl groups, ester bonds, phosphate groups, and unsaturated fatty acids such as γ-linolenic acid, which collectively underpin the antibacterial potential we observed. As expected, standard antibiotics produced larger inhibition zones than our crude extract; however, the reproducible dose-dependent activity observed in the present study supports the presence of antibacterial metabolites in the extract [22;24;25].

Although our study is preliminary, the findings suggest potential applications as natural antibacterial agents in food preservation or as adjunctive nutraceuticals, provided appropriate toxicological evaluations are conducted. Recommend that further studies assess stability, cytotoxicity, and synergistic effects, particularly against multidrug-resistant isolates, building on work such as that of Morowvat et al. [21;23], who demonstrated the efficacy of *C. vulgaris*-based nanoparticles against MRSA.

Conclusions

The ethanolic extract from commercial *C. vulgaris* powder demonstrated measurable, dose-dependent antibacterial activity against both tested species. Although preliminary, these findings support further investigation of commercial *Chlorella* preparations as potential antimicrobial sources, particularly after appropriate toxicological evaluation and bioactive compound characterisation. These findings provide a foundation for future bioassay-guided isolation of antibacterial compounds from commercially available *Chlorella vulgaris* products.

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