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Phytochemical Screening and FTIR Characterisation of Ultrasonic Assisted Methanolic Extract of *Chlorococcum humicola*: Evaluation of its Antioxidant and Anti-inflammatory Activities

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This study investigates the phytochemical composition and evaluates the antioxidant and anti-inflammatory potential of an ultrasonic-assisted methanolic extract (UAME) obtained from the double mutant *Chlorococcum humicola* MCH4 cultivated in ultrasonically pretreated municipal wastewater (UPMWW). Qualitative phytochemical screening revealed a broader spectrum of bioactive compounds in the mutant strain compared with the wild strain *C. humicola* KMS2, in which glycosides, tannins and phytosterols were absent. Quantitative analysis showed high total phenolic content (59.47 ± 0.09 mg GAE/g DW), total flavonoid content (62.47 ± 0.12 mg QE/g DW) and total carotenoids (4.11 mg/g), confirming the enriched phytochemical profile of *C. humicola* MCH4. Based on these results, the UAME of the mutant strain was selected for further biological evaluation. The extract exhibited notable radical-scavenging activity in DPPH and ABTS assays with IC_{50} values of approximately 50 µg/mL. *In vitro* anti-inflammatory assays demonstrated concentration-dependent inhibition of albumin denaturation ($IC_{50} \approx 59.4$ µg/mL), proteinase activity ($IC_{50} \approx 57.0$ µg/mL) and lipoxygenase activity ($IC_{50} \approx 55.2$ µg/mL), indicating promising preliminary activity. FTIR analysis confirmed the presence of hydroxyl, carbonyl and aliphatic functional groups associated with phenolics, proteins, lipids, carotenoids and carbohydrates. The results indicate that the double mutant *C. humicola* MCH4 cultivated in UPMWW serves as a sustainable and promising source of bioactive compounds with potential applications in pharmaceutical, nutraceutical and cosmeceutical fields.

Keywords: *Chlorococcum humicola*, Ultrasonic pretreatment, Total phenolic content, Flavonoid, Antioxidant, Anti-inflammatory.

INTRODUCTION

Synthetic drugs are commonly using to counteract the harmful effects of free radicals (FR) and inflammatory disorders [1]. However, their safety remains debated, as studies have reported that the synthetic antioxidant potential carcinogenic effects on the liver and kidneys in experimental animals, while others suggest minimal risk or even beneficial effects in humans. Due to these conflicting findings and variations in regulatory limits across countries, the use of synthetic antioxidants has become controversial [2]. Similarly, continued usage of steroidal or non-steroidal anti-inflammatory drugs are connected with gastrointestinal ulcers, liver and kidney toxicity, high blood pressure and an increased risk of heart attack [3,4]. These adverse effects have driven interest toward safer alternatives, especially natural products and plant derived compounds with antioxidant and anti-inflammatory properties.

Plant based secondary metabolites provide the structural foundation for numerous modern pharmaceuticals and herbal remedies [5]. Several medicinal plants have demonstrated significant antioxidant and anti-inflammatory potential [6,7]. However, large-scale cultivation of medicinal plants is often time-consuming, resource-intensive and costly, which limits their scalability for widespread therapeutic applications. In this context, microalgae are increasingly recognised as promising next-generation biofactories for antioxidant and anti-inflammatory compounds. They are rich in bioactive metabolites like phenolic compounds, carotenoids, polyunsaturated fatty acids, phycobiliproteins and polysaccharides [8]. Unlike medicinal plants, microalgae offer higher yields and faster growth under controlled conditions, independent of soil, climate or season. Their cultivation is sustainable, requiring no arable land and allowing growth in saline or wastewater [9]. A single algal strain can generate a variety of bioactive

compounds that may act synergistically, highlighting microalgae as a sustainable and scalable source of novel antioxidant and anti-inflammatory agents [10,11].

To the best of our understanding, this study is the first to present the FTIR characterisation along with the evaluation of *in vitro* antioxidant and anti-inflammatory activities of the double mutant *Chlorococcum humicola* MCH4 cultivated in ultrasonic pretreated municipal wastewater (UPMWW). Therefore, the present study was undertaken to explore freshwater microalgae as a potential alternative source for managing various oxidative stress-related disorders. Specifically, this work aimed to evaluate the phytochemical composition, FTIR characterisation, *in vitro* antioxidant and anti-inflammatory activity of an ultrasonic-assisted methanolic extract derived from ultrasonically pretreated municipal wastewater-grown double mutant *C. humicola* MCH4, thereby promoting its utilisation as a sustainable natural antioxidants and anti-inflammatory agents for nutraceutical and functional food applications.

EXPERIMENTAL

All chemicals, including nutrient media components, buffers and reagents, were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India) and SRL Pvt. Ltd., (Mumbai, India). All reagents were used without further purification unless otherwise specified. Ascorbic acid was used as a standard reference drug for the comparative evaluation in *in vitro* antioxidant assays. Similarly, ibuprofen (a nonsteroidal anti-inflammatory drug) and prednisolone (a corticosteroid) were used as a standard reference drug for the comparative evaluation in *in vitro* anti-inflammatory assays.

Characterization: Ultrasonicator equipped with a 6.5 mm diameter metal probe (Lark Innovative Fine Teknowledge, Chennai, India) was used for pretreatment of 75% municipal wastewater (MWW) and methanolic extraction. Remi Model R-8C BL bench top centrifuge was used to separate the biomass from MWW. UV-Visible spectrophotometer Model 2206 (Systronics India Ltd., Gujarat, India) was used to estimate the total phenolic content (TPC), total flavonoid content (TFC), total carotenoids (TC), antioxidant and anti-inflammatory activity. Fourier-transform infrared (FTIR) spectroscopy (FT/IR-4700 type A, JASCO, Tokyo, Japan) was used to fine the major functional groups of ultrasonic assisted methanolic extract (UAME).

Development of double mutant microalga: A wild green microalga, *C. humicola* KMS2 (GenBank Accession No.: PQ650940), was originally isolated from an ancient freshwater lake (Mamandur, India) and it was subjected to sequential mutagenesis using UV-C irradiation followed by treatment with 1.5 M ethyl methanesulfonate and the resulting strain was designated as double mutant *C. humicola* MCH4, which was used in this study.

Biomass production from ultrasonically pretreated municipal wastewater: Raw MWW was collected from the MWW treatment plant located in Kanchipuram district, India and subjected to ultrasonic pretreatment at 0.35 W/mL for 20 min [12]. From this UPMWW biomass was produced using wild *C. humicola* KMS2 and double mutant *C. humicola* MCH4. Biomass production process was carried out in 1000 mL Erlenmeyer conical flask containing 500 mL of UPMWW. The flask was inoculated with 8% (v/v) of an actively growing strain KMS2 and MCH4 and incubated at 30 ºC with continuous agitation (150 rpm). Illumination was provided at a light intensity of 90 μE/m^2/s with a photoperiod of 10 h light and 14 h dark, maintained for duration of 10 days. Following cultivation, biomass was harvested and rinsed twice with distilled water and centrifuged again to eliminate residual impurities. The purified biomass was dried at 40 ºC until a constant weight was reached and subsequently used for methanolic extraction.

Ultrasonic-assisted methanolic extract: Dried biomass (50 g) was suspended in 100 mL of methanol in a 250 mL beaker, resulting in a slurry volume of approximately 120 mL. Sonication was performed using a micro-tip horn transducer (6.5 mm diameter) at 0.35 W/mL for 20 min [12]. The temperature of the mixture was continuously monitored with a sensor and maintained at 30 ºC by immersing beaker in an ice bath to prevent overheating. After treatment, the extract was filtered through Whatman No. 42 filter paper. The UAME was concentrated under reduced pressure using a rotary evaporator at 45 ºC, vacuum-dried, weighed and subsequently stored at 4 ºC until further analysis.

Phytochemical screening: Phytochemicals including alkaloids, carbohydrates, glycosides, proteins, free amino acids, flavonoids, phenolics, tannins, terpenoids, carotenoids and phytosterols were screened using standard qualitative assays [13].

Estimation of TPC, TFC and total carotenoids: The concentrations of TPC, TFC and total carotenoids (TC) in the UAME derived from the biomass of strains KMS2 and MCH4 were estimated spectrophotometrically using the methods described by Singleton & Rossi [14], Chang *et al.* [15] and Zavrel *et al.* [16], respectively. Gallic acid and quercetin were used as a standard for TPC and TFC estimation, respectively.

Evaluation of antioxidant activity: *In vitro* antioxidant activity of UAME of *C. humicola* MCH4 was evaluated *via* DPPH assay [17] and ABTS assay [18]. Different concentrations of the UAME ranging from 50 to 300 μg/mL were mixed with an equal volume of DPPH solution and incubated in the dark at 37 ºC for 25 min. The absorbance of each reaction mixture was measured at 517 nm, while the DPPH solution without UAME served as the control. Likewise, 5 μL of the UAME at concentrations ranging from 50 to 300 μg/mL was mixed with 4 mL of $ABTS^{+\bullet}$ solution and kept in the dark at room temperature for 2 h. The absorbance of the reaction mixture was then recorded at 734 nm. The ascorbic acid was used as the standard reference in both assays. The percentage of radical scavenging activity was determined using the following equation:

$$\text{Radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of sample

Evaluation of anti-inflammatory activity: *In vitro* anti-inflammatory activity of UAME of *C. humicola* MCH4 was evaluated by inhibition of albumin denaturation [19], proteinase activity [19] and lipoxygenase (LOX) activity [20]. UAME of *C. humicola* MCH4 were prepared at concentrations of 50,

100, 200, 300, 400 and 500 µg/mL in deionised water. For positive controls, ibuprofen (IPF) and prednisolone (PNL) were employed. Deionised water served as the negative control. The percentage of inhibition of each assay was determined using the following equation:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Determination of IC_{50}: The IC_{50} (half-maximal inhibitor concentration) values of UAME for DPPH and ABTS radicals, BSA denaturation, proteinase activity and lipoxygenase activity was determined by plotting the percentage inhibition against varying UAME concentrations and analyzing the trend using linear regression. Notably, a lower IC_{50} value indicates higher antioxidant and anti-inflammatory potential.

FTIR characterisation: The major functional groups of UAME of *C. humicola* MCH4 were identified by FTIR spectroscopy. Approximately 1.5 mg of UAME was finely pulverised with 100 mg of KBr and compressed into a transparent pellet under vacuum. The spectra were then recorded at the wavenumber range of 4000-500 cm^{-1} with a resolution of 4 cm^{-1} using an FTIR spectrometer. Replicate spectra were obtained to ensure reproducibility and the spectral data were compared with a reference to identify the functional groups.

Statistical analysis: All experiments were performed in triplicate and the results are presented as mean ± standard deviation. Statistical differences among treatments were evaluated using one-way ANOVA at a significance level of $p < 0.05$, followed by appropriate post hoc comparisons. Regression equations and corresponding R^2 values were also determined. All statistical analyses were performed using MINITAB software (version 12).

RESULTS AND DISCUSSION

Growth performance of wild and mutant *C. humicola* in UPMWW: Cultivation of the wild strain *C. humicola* KMS2 and the double mutant strain *C. humicola* MCH4 in 75% UPMWW resulted in pronounced differences in biomass accumulation. After 10 days of cultivation, the mutant strain produced 3.98 g/L, whereas the wild strain produced only 1.01 g/L, representing a 3.94-fold enhancement in biomass yield. This clearly demonstrates the effectiveness of mutagenesis in improving algal growth in nutrient-rich wastewater media. Biomass productivity is a crucial parameter for industrial scalability. The biomass productivity of mutant strain achieved 0.323 g/L/d, which was 4.25 times higher than the wild strain (0.076 g/L/d). This productivity exceeds values reported for several wastewater-grown microalgae (0.08 g/L/d), confirming the effectiveness of strain improvement strategies [21]. One-way ANOVA revealed that strain type had a highly significant effect ($p < 0.001$) on biomass yield and biomass productivity. Post-hoc Tukey analysis confirmed that the mutant strain differed significantly from the wild strain in all measured growth conditions. The very high F-values (biomass yield: F = 152.6; biomass productivity: F = 187.1) indicate strong experimental reproducibility and confirm the reliability of the observed improvements.

Phytochemical profiling: The ultrasonic-assisted methanolic extraction of *C. humicola* MCH4 was highly effective, yielding ≈ 24.72 g of extract from 50 g of dried biomass of wild and mutant stain and enabling the detection of a wide range of phytochemicals, including free amino acids, proteins, carbohydrates, flavonoids, glycosides, alkaloids, tannins, phenolics, terpenoids, phytosterols and carotenoids (Table-1). This result suggests that the UPMWW-grown double mutant *C. humicola* MCH4 serves as a superior source of diverse bioactive compounds compared to the wild strain, as glycosides, tannins and phytosterols were absent in the wild strain. The findings are consistent with earlier reports on phytochemical screening of microalgae [22,23]. Ultrasonic-assisted extraction is enhanced the solvent penetration and mass transfer compared to conventional extraction methods, thereby increasing yield and enabling the recovery of a broader spectrum of metabolites [23]. The chemical composition of microalgal biomass varies considerably among species and differs markedly from terrestrial plants. Therefore, the observed extraction yield likely reflects the recovery of a broad spectrum of methanol-soluble cellular components rather than exclusively bioactive secondary metabolites. The biomass used in this study was thoroughly dried prior to extraction to minimize the influence of residual moisture. Consequently, the high yield can be attributed to the efficient solubilisation of intracellular metabolites and the comparatively low amount of insoluble residual biomass after extraction.

TABLE-1
ISOLATED PHYTOCHEMICAL CONSTITUENTS OF UAME OF WILD AND MUTANT *C. humicola*

Phytochemical	UAME (1%, w/v)	
	Double mutant *C. humicola*	Wild *C. humicola*
Carbohydrates	+++	+++
Proteins	+++	+++
Free amino acids	++	+
Alkaloids	++	+
Glycosides	+	–
Flavonoids	+++	+
Phenolics	+++	+
Tannins	+	–
Terpenoids	++	+
Phytosterols	+	–
Carotenoids	+++	++

(+++) = Protuberant; (++) = Medium; (+) = very less present; (–) = absent

TPC, TFC and TC of UAME derived from *C. humicola* MCH4: The quantitative estimation of phenolic, flavonoid and TC constituents revealed a pronounced enhancement in the double mutant strain. The UAME of *C. humicola* MCH4 exhibited a TPC of 59.47 ± 0.09 mg GAE/g DW and a TFC of 62.47 ± 0.12 mg QE/g DW, whereas the wild strain produced significantly lower levels, with a TPC of 11.24 ± 0.11 mg GAE/g DW and a TFC of 13.91 ± 0.08 mg QE/g DW. This corresponds to a 5.29-fold increase in TPC and a 4.49-fold increase in TFC in the mutant strain, demonstrating that mutagenesis markedly enhanced the biosynthesis of secondary metabolites. One-way ANOVA indicated that strain type had a highly

significant effect on both TPC ($F(1,4) = 128{,}462.3$, $p < 0.001$) and TFC ($F(1,4) = 196{,}874.6$, $p < 0.001$) and Tukey's HSD post-hoc test confirmed that the mutant strain differed significantly from the wild strain for both parameters ($p < 0.001$). The TFC value ($p < 0.05$) obtained here is slightly higher than the TPC, indicating that flavonoids constitute a substantial proportion of the total phenolic pool in *C. humicola* MCH4. Similarly, 4.11 mg/g TC was obtained from the UAME of *C. humicola* MCH4, whereas the wild strain yielded 1.73 ± 0.05 mg/g. The TC content of the mutant strain was 2.4-fold higher than that of the wild strain ($p < 0.05$), indicating a statistically significant increase in TC production in the mutant compared to the wild type. This aligns with previous studies on microalgae, where high levels of flavonoids were reported to correlate with antioxidant and antimicrobial activities [24,25]. The TPC and TFC values found in this study are comparable to or higher than those reported for several other freshwater and marine microalgae, highlighting the efficiency of ultrasonic assisted methanolic extraction in recovering bioactive secondary metabolites [25]. Moreover, the strong phenolic and flavonoid content of *C. humicola* MCH4 underscores its possible as a sustainable source of natural antioxidants and anti-inflammatory agents. The enhanced accumulation of phenolic, flavonoid and carotenoids compounds can be attributed to mutation-induced upregulation of phenylpropanoid biosynthetic pathways and improved stress adaptation under UPMWW cultivation. The significantly higher biomass productivity of the mutant strain during cultivation likely contributed to the increased metabolite yield, suggesting that genetic improvement enhanced both growth and secondary metabolite production, thereby highlighting the strong potential of *C. humicola* MCH4 as a valuable source of antioxidant bioactive compounds. Hence, the UAME of the double mutant *C. humicola* MCH4 was selected for further characterisation.

***In vitro* antioxidant activity of UAME derived from *C. humicola* MCH4:** The UAME of *C. humicola* MCH4 exhibited strong FR scavenging activity in a dose dependent manner, similar to the standard antioxidant ascorbic acid. As shown in Fig. 1a, at 50 µg/mL, the UAME inhibited of 51.42% DPPH radicals, while ascorbic acid showed 52.08% inhibition, both surpassing 50% threshold ($p < 0.05$). With increasing concentrations, activity further increased, reaching 85.42% for the extract and 86.46% for ascorbic acid at 300 µg/mL. Similarly, ABTS radical scavenging assay showed that the UAME exhibited strong antioxidant activity, with values comparable to those of ascorbic acid across all concentrations tested (Fig. 1b). At 50 µg/mL, the UAME inhibited 50.67% of ABTS radicals, nearly equal to ascorbic acid (51.53%). With increasing concentrations, scavenging activity improved steadily, reaching 87.58% for the extract and 90.54% for ascorbic acid at 300 µg/mL ($p < 0.05$). This finding suggests that the phenolic and flavonoid compounds present in *C. humicola* MCH4 play a crucial role in its radical scavenging activity. Alike strong antioxidant activities have been reported for other microalgae, where high phenolic and carotenoid content directly contributes to DPPH and ABTS scavenging efficiency [26,27].

The IC_{50} values obtained from both DPPH (50 µg/mL; $y = 6.2423x + 51.512$, $R^2 = 0.91$) and ABTS (50 µg/mL; $y = 6.7x + 56.097$, $R^2 = 0.9158$) assays indicate that the UAME of *C. humicola* MCH4 exhibits significant antioxidant activity. The standard antioxidant also showed strong dose response linearity ($y = 6.3603x + 52.017$, $R^2 = 0.91$). The high correlation coefficients confirm good linear relationships between concentration and radical scavenging activity. These findings suggest that *C. humicola* MCH4 contains phenolic compounds and flavonoids that contribute to its antioxidant potential. For plant-derived extracts, IC_{50} values below 100 µg/mL are typically regarded as evidence of strong antioxidant capacity, largely due to the high levels of phenolic and flavonoid compounds present in medicinal and edible plants and microalgae [28-30]. These benchmarks are widely employed for comparative evaluation and highlight that both plant and algal sources serve as valuable reservoirs of natural antioxidants, with microalgae in particular representing a sustainable alternative owing to their rapid growth rates and diverse secondary metabolite profiles.

Inhibition of albumin denaturation by UAME: The UAME derived from *C. humicola* MCH4 showed a dose-dependent inhibition of BSA denaturation. At 50 µg/mL, the UAME exhibited 49.66% inhibition ($p < 0.05$), which increased to 83.78% at 500 µg/mL (Fig. 2). Standard drugs IPF and PNL showed slightly higher inhibitory effects across concentrations, reaching 85.49% and 84.33% inhibition, respectively, at 500 µg/mL. The IC_{50} value of UAME was estimated as 59.4 µg/mL ($y = 7.0486x + 39.953$, $R^2 = 0.9761$), which was slightly higher than that of IPF (52 µg/mL; $y = 7.2811x + 44.029$, $R^2 =$

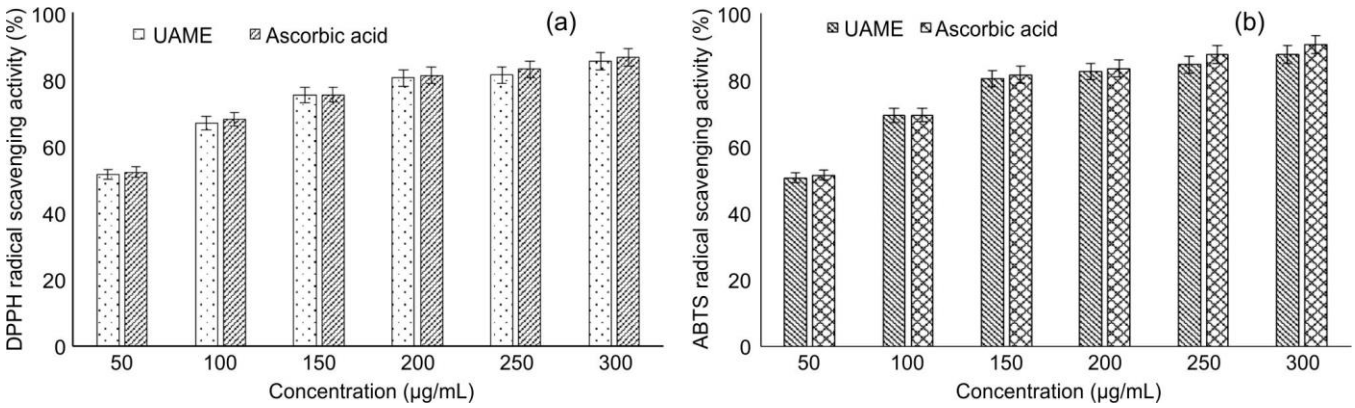

Fig. 1. Antioxidant activity of UAME of *C. humicola* MCH4 by (a) DPPH assay and (b) ABTS assay

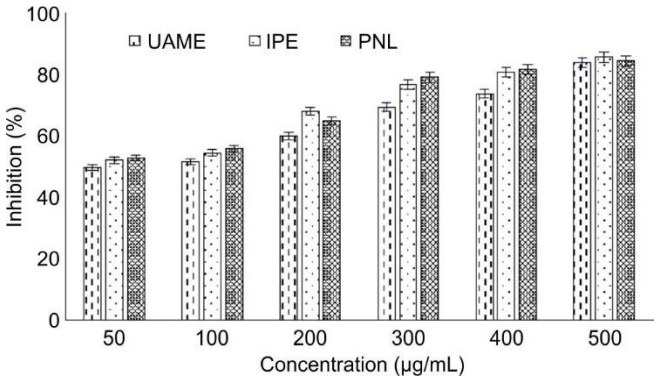

Fig. 2. Effect of UAME of *C. humicola* MCH4 on inhibition of BSA denaturation

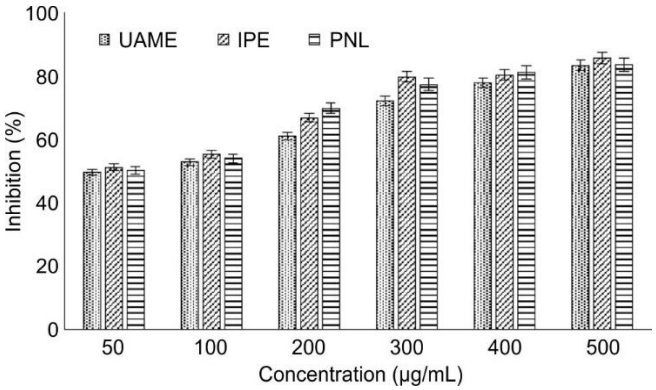

Fig. 3. Effect of UAME of *C. humicola* MCH4 on inhibition of proteinase activity

0.9599) and PNL (50 µg/mL; $y = 7.1366x + 44.752$, $R^2 = 0.9394$). The high R^2 values indicate good linearity of the dose–response relationships. However, UAME is a complex mixture of bioactive constituents, whereas the reference drugs are purified and well-characterised pharmaceutical compounds.

Moreover, the results were obtained from an *in vitro* enzyme inhibition assay; therefore, these findings indicate promising preliminary anti-inflammatory potential of the extract. The BSA denaturation assay is a widely accepted *in vitro* model to screen anti-inflammatory potential, as protein denaturation is closely linked with inflammatory processes [31]. In this study, the strong activity observed in the UAME may be ascribed to the occurrence of phenolic compounds, flavonoids and bioactive lipids that are known to interact with protein structures and act as antioxidants, thereby preventing denaturation. Similar findings have been reported in microalgal extracts where secondary metabolites effectively inhibited protein denaturation and reduced inflammation [32,33].

Inhibition of proteinase activity of UAME: The crude UAME of *C. humicola* MCH4 showed marked inhibition of proteinase activity in a concentration-dependent manner. At 50 µg/mL, the crude UAME inhibited 49.55% of proteolytic activity, exceeding 50% inhibition at 100 µg/mL and reaching a maximum of 83.33% at 500 µg/mL (Fig. 3). Standard anti-inflammatory drugs IPF and PNL also displayed strong inhibitory effects, reaching 85.65% and 83.64% inhibition respectively at 500 µg/mL ($p < 0.05$). The crude UAME exhibited concentration-dependent proteinase inhibitory activity with an IC_{50} value of 57.0 µg/mL, supported by a strong linear regression ($y = 7.286x + 40.627$, $R^2 = 0.981$). In comparison, IPF and PNL showed IC_{50} values of 51 µg/mL ($y = 7.4337x + 43.862$, $R^2 = 0.9432$) and 50 µg/mL ($y = 7.3226x + 43.749$, $R^2 = 0.928$), respectively, with high correlation coefficients indicating good linearity of the dose–response relationships. However, UAME is a complex mixture of bioactive constituents, whereas the reference drugs are purified and well-characterised pharmaceutical compounds.

Moreover, the results were obtained from an *in vitro* enzyme inhibition assay; therefore, these findings indicate promising preliminary anti-inflammatory potential of the extract. Proteolytic enzymes such as serine proteinases, released by neutrophils at sites of tissue injury, are associated with inflammatory damage and disease progression. Their inhibition is therefore a crucial mechanism for reducing inflammation and tissue degradation [34]. The comparable inhibitory potential of *C. humicola* MCH4 extract and standard drugs suggests that the bioactive metabolites present in the UAME, likely phenolics and flavonoids, contribute to protease inhibition. Previous reports on microalgal extracts have demonstrated similar activities, attributing protease inhibition to secondary metabolites capable of binding enzyme active sites and stabilizing protein structures [35,36].

Inhibition of lipoxygenase activity of UAME: The UAME of *C. humicola* MCH4 exhibited a strong and dose-dependent inhibition of LOX activity. At 50 µg/mL, the extract achieved 49.54% inhibition, exceding the 50% threshold at 100 µg/mL and reaching a maximum of 84.21% at 500 µg/mL (Fig. 4). The standard drugs IPF and PNL also showed comparable inhibitory effects, with 85.34% and 84.54% inhibition ($p < 0.05$), respectively, at the highest tested concentration. The crude UAME showed concentration-dependent LOX inhibitory activity with an IC_{50} value of 55.2 µg/mL, supported by a strong linear regression ($y = 7.1306x + 41.318$, $R^2 = 0.9953$). The reference drugs IPF and PNL exhibited IC_{50} values of 54.1 µg/mL ($y = 7.5274x + 40.951$, $R^2 = 0.9832$) and 50 µg/mL ($y = 7.1834x + 43.121$, $R^2 = 0.9661$), respectively, with high correlation coefficients indicating good dose–response linearity. However, UAME is a complex mixture of bioactive constituents, whereas the reference drugs are purified and well-characterised pharmaceutical compounds.

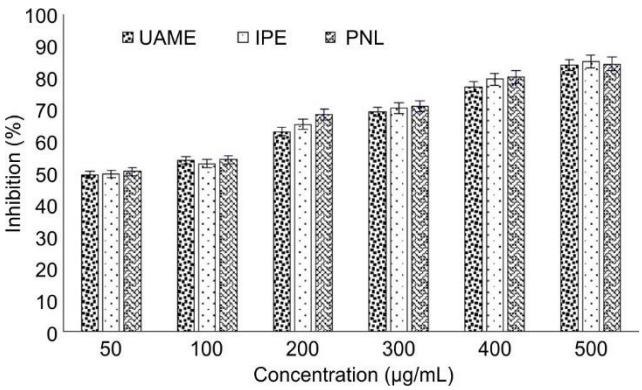

Fig. 4. Effect of UAME of *C. humicola* MCH4 on inhibition of lipoxygenase activity

Moreover, as the results were obtained from an *in vitro* enzyme inhibition assay, these findings indicate promising

preliminary anti-inflammatory potential of the extract due to its LOX inhibitory activity. LOX are key enzymes in arachidonic acid metabolism, catalyzing the synthesis of leukotrienes and hydroperoxides, which are the potent mediators of inflammation. Excessive LOX activity has been implicated in asthma, arthritis and other inflammatory disorders [37]. Inhibitors of LOX are therefore regarded as important therapeutic candidates for controlling inflammatory disorders. The strong inhibitory effect observed in *C. humicola* MCH4 extract suggests that the bioactive constituents, particularly phenolics, flavonoids and lipid-derived metabolites, may interfere with LOX catalytic activity or scavenge reactive intermediates generated during lipid peroxidation. Similar inhibitory trends have been reported in recent studies on microalgal and plant extracts, which attribute LOX inhibition to polyphenolic compounds with redox and enzyme-binding properties [35,36].

The crude UAME of *C. humicola* MCH4 demonstrated strong, concentration-dependent anti-inflammatory potential in all three *in vitro* assays performed. The extract effectively inhibited albumin denaturation ($IC_{50} \approx 59.4$ µg/mL), proteinase activity ($IC_{50} \approx 57.0$ µg/mL) and lipoxygenase activity ($IC_{50} \approx 55.2$ µg/mL). These values are only marginally higher than those of the standard anti-inflammatory drugs IPF (≈ 51-54 µg/mL) and PNL (≈ 50 µg/mL), highlighting the potency of algal extract. At higher concentrations (400-500 µg/mL), the inhibitory effects of the extract were nearly comparable to those of the standards across all assays.

The slight differences in potency are expected, as the UAME is a complex mixture of bioactive compounds rather than a single purified molecule. Nevertheless, its ability to achieve inhibition levels similar to established drugs underscores its therapeutic promise. The activity is likely due to phenolics, flavonoids and lipid-derived metabolites that stabilize proteins, inhibit proteolytic enzymes and interfere with LOX activity, thereby reducing inflammatory mediator production. Similar outcomes have been reported in recent studies emphasizing the anti-inflammatory properties of microalgal metabolites and their potential as alternatives to synthetic drugs [35,36].

FTIR characterisation: As shown in Fig. 5, the FTIR spectrum of the UAME of *C. humicola* MCH4 shows a set of well-defined bands that together indicate a complex mixture of hydroxylated, carbonyl-bearing, aliphatic and oxygenated compounds. A broad band at 3416.2 cm^{-1} is diagnostic of O–H stretching vibration and may also include N–H stretching contributions; this feature is typical of free or hydrogen-bonded hydroxyl groups found in phenolics, alcohols or residual water and of amine functionality in proteins or amino-containing metabolites. The peak at 2926.4 and 2855.7 cm^{-1} corresponds to aliphatic C–H stretching vibration, supporting the presence of fatty acids, lipids or other long-chain hydrocarbons within the extract. Two distinct absorptions in the carbonyl region, 1731.2 cm^{-1} and 1711.1 cm^{-1}, indicate multiple C=O environments; the higher-frequency band (≈1731 cm^{-1}) is most consistent with ester or esterified lipid carbonyls, whereas the slightly lower band (≈1711 cm^{-1}) can reflect ketone or conjugated carbonyl species. These carbonyl signals, together with the strong aliphatic C–H bands, point to significant lipidic/esterified material in the methanolic fraction.

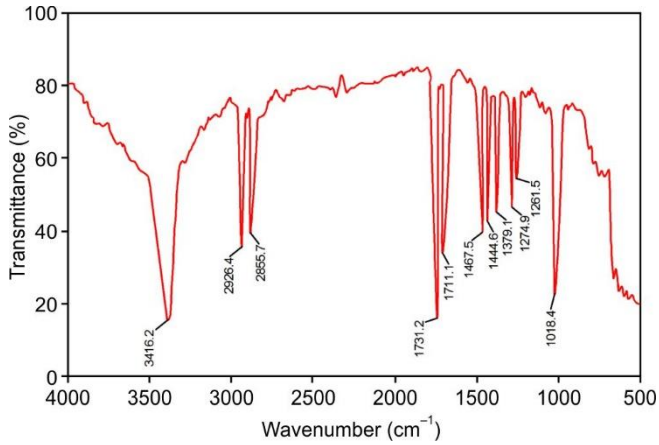

Fig. 5. FTIR spectrum of the UAME of *C. humicola* MCH4

Bands at 1467.5 cm^{-1} and 1444.6 cm^{-1} are corresponding to C–H bending vibration of methylene and methyl groups and further confirm an aliphatic/lipid component. The absorption centered at 1379.1 cm^{-1} is constant with O–H bending vibration in phenolic compounds or with methyl deformation modes and thus suggests the presence of phenolics or methylated aromatics. Two adjacent bands at 1274.9 cm^{-1} and 1261.5 cm^{-1} fall in the C–O stretching region and likely reflect aryl–O (ether/ester) and aliphatic C–O linkages, these are commonly observed for aromatic esters, glycosidic bonds and etherified polyphenols. Finally, the band at 1018.4 cm^{-1} is representative of C–O/C–O–C stretching vibration and is typical of carbohydrates, polysaccharide fragments or glycosylated metabolites. The predominance of hydroxyl, carbonyl and aliphatic functional groups highlights the biochemical richness of the extract, which likely underpins its antioxidant and bioactive potential.

Conclusion

This investigation demonstrated that UAME obtained from *C. humicola* MCH4 cultivated in UPMWW represents an efficient and sustainable approach for recovering diverse bioactive compounds compared with the wild strain *C. humicola* KMS2. The extraction yielded 24.72 g of UAME from 50 g of dry biomass, indicating effective solvent penetration and metabolite recovery. Qualitative phytochemical screening further confirmed the presence of a wide range of primary and secondary metabolites, highlighting the enhanced metabolic potential of *C. humicola* MCH4 relative to the wild strain. Quantitative assays revealed notably high TPC (59.47 ± 0.09 mg GAE/g DW), TFC (62.47 ± 0.12 mg QE/g DW) and TC (4.11 mg/g) suggesting that *C. humicola* MCH4 is an abundant source of antioxidant and therapeutic metabolites. The strong FR scavenging activity observed in both DPPH and ABTS assays ($IC_{50} \approx 50$ µg/mL), comparable to ascorbic acid, confirmed the potent antioxidant potential of the UAME. Furthermore, the UAME exhibited significant anti-inflammatory activity across all *in vitro* assays, effectively inhibiting BSA denaturation, proteinase activity and LOX activity with IC_{50} values (55–59 µg/mL) similar to those of standard drugs IPF and PNL. FTIR spectral analysis further validated the biochemical complexity of the extract, revealing functional groups corresponding to carbonyl, hydroxyl, aliphatic and

ether linkages associated with phenolics, lipids and carbohydrates. These findings collectively confirmed the chemical diversity and pharmacological relevance of *C. humicola* MCH4 metabolites.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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