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RESEARCH ARTICLE

In vitro assessment of *Bionectria ochroleuca* metabolites: A promising approach for controlling root-knot nematode, *Meloidogyne incognita*

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Abstract

Meloidogyne incognita, a highly destructive root-knot nematode, causes substantial crop losses worldwide by infesting plant roots, which disrupts nutrient and water uptake. This pest is notoriously challenging to manage due to its broad host range and growing resistance to many chemical nematicides, emphasizing the urgent need for sustainable, eco-friendly alternatives. In this context, the current study investigated the nematicidal potential of secondary metabolites derived from the entomopathogenic fungus, *Bionectria ochroleuca* against *M. incognita*. The *in vitro* assays demonstrated a dose- and time-dependent inhibition of egg hatching and juvenile survival. At a crude metabolite concentration of 100%, egg hatching was reduced to 5.64% and juvenile mortality increased to 95.4% after 72 h. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified key metabolites, including palmitic acid, butanedioic acid, lactic acid and oleic acid, which appear to inhibit nematode growth through mechanisms that impair cell membrane integrity, disrupt energy metabolism and interfere with essential metabolic pathways. Further, metabolite enrichment analysis revealed their involvement in the biosynthetic pathways, such as unsaturated fatty acids, galactose metabolism and phenylalanine metabolism. Molecular docking studies supported these findings by showing high binding affinities of these metabolites to virulent nematode proteins, including Cytochrome c oxidase subunit 1 and NAD(H) oxidase, suggesting interference with essential biological processes within the nematode. Overall, these findings position the metabolites of *B. ochroleuca* as promising candidates for managing nematode infestations, offering a potent alternative to chemical nematicides and thereby contributing to sustainable agricultural practices.

Keywords

Bionectria ochroleuca; docking studies; GC-MS; *Meloidogyne incognita*; nematicidal activity; secondary metabolites

Introduction

Plant-parasitic nematodes (PPNs) pose a significant threat to global agriculture, accounting for over 30% of crop yield losses worldwide (1). Among them, the root-knot nematode, *Meloidogyne incognita*, is one of the most destructive species. It attacks a wide range of crops, leading to severe root galling, reduced plant vigor, and

heightened susceptibility to other biotic and abiotic stresses (2, 3). The damage inflicted by *M. incognita* often results in poor yields and significant economic losses for farmers (4). Traditionally, integrated nematode management strategies have relied on crop rotation systems to control PPNs (1). However, due to the polyphagous nature of *M. incognita*, these methods alone are insufficient, often necessitating the frequent application of nematicidal products to suppress nematode populations. Recently, many synthetic chemical nematicides have been phased out due to their adverse effects on human health and the environment which created a pressing need for sustainable, bio-based alternatives. In this context, soil-borne microorganisms, particularly fungi, have emerged as promising biological control agents against nematodes.

Entomopathogenic fungi (EPF) have garnered considerable attention for their dual role in both plant and soil ecosystems. In terms of biocontrol, EPF directly infects nematodes, insects and soil-borne pathogens by penetrating their cuticle through enzymes like chitinases, proteases, and lipases, which break down the structural components of the pest's outer protective layers. Following entry, the fungi proliferate inside the pest, ultimately leading to its death. Additionally, EPF produces various secondary metabolites with nematicidal, insecticidal and antimicrobial properties, further reinforcing their efficacy against a broad spectrum of pests (5). As endophytes, EPF colonize plant tissues non-pathogenically, enhancing plant defenses by triggering systemic resistance. This resistance often involves the induction of key plant signaling pathways, such as jasmonic acid (JA) and salicylic acid (SA) pathways, which prime the plant's innate immunity to withstand pathogen attacks. EPF also improves nutrient uptake, facilitating the absorption of essential minerals like nitrogen and phosphorus through interactions within the rhizosphere or with the plant itself. Additionally, EPF can synthesize phytohormones like indole-3-acetic acid (IAA), promoting root and shoot growth, and thus enhancing overall plant vigor. This multifaceted functionality highlights EPF as potent agents for integrated pest management and sustainable agriculture (6-8).

One promising species of EPF is *Bionectria ochroleuca*, a fungus isolated from soils and plant tissues worldwide (9, 10). This fungus is known for its versatility, displaying both sexual and asexual reproductive phases and thriving in various ecological niches, including roles as a plant pathogen, endophyte, or free-living organism (8, 11). *B. ochroleuca* has been found in association with several crops, showcasing its potential as a bio-based tool for integrated pest management (12). In addition to its ecological versatility, *B. ochroleuca* produces a range of bioactive secondary metabolites with antimicrobial and cytotoxic properties. These include polyketide glycosides, peptides, sesquiterpenes and macrolides, many of which exhibit strong antibacterial and antifungal activity (13). Notable metabolites from the genus *Bionectria*, include dioxopiperazine, gliocladicillin A-C, bionectin D-E, clonostachin B, bionectrins A-C, tetramic acid derivatives, virgineone, virgineone aglycone, pullularins E and F, ochrolines, usnic acid A-C, TMC-151F and bionectriol A-D, have demonstrated potent biological activities. These findings highlight *Bionectria* as a valuable source of secondary metabolites with diverse applications (14-17). With its wide distribution and proven bioactivity, *B. ochroleuca* shows significant potential as a biological control agent against PPNs. However, several limitations must be considered: variability in metabolite production due to different growth conditions, challenges in the environmental persistence of its bioactive compounds, potential host range limitations against various nematode species, compatibility issues with existing agricultural practices, uncertain non-target effects on beneficial soil organisms, regulatory hurdles for approval and commercialization, a lack of comprehensive field studies validating its effectiveness and concerns about the economic viability of its production and application. Although previous studies have demonstrated its antagonistic effects against plant pathogens such as *Rhizoctonia solani* and *Pythium aphanidermatum* (18), its effectiveness against nematodes, particularly *M. incognita*, remains largely unexplored.

The current study aims to investigate the nematicidal potential of *B. ochroleuca* through *in vitro* testing, followed by metabolite profiling using gas chromatography-mass spectrometry (GC-MS). Furthermore, the identified bioactive compounds will be subjected to *in-silico* molecular docking to assess their interaction with virulent protein targets of *M. incognita*. This research aims to explore the potential use of *B. ochroleuca* as a sustainable biocontrol agent, offering an eco-friendly solution to combat nematode-induced crop damage.

Materials and Methods

Biocontrol strain and growth conditions

The strain of *B. ochroleuca* (MT605140), sourced from the Department of Nematology at Tamil Nadu Agricultural University in Coimbatore, India, was isolated from mites. To confirm the identity of this strain, both morphological and molecular characterizations were conducted. DNA was extracted using the Cetyltrimethylammonium bromide (CTAB) method and PCR was performed with ITS1 and ITS4 primers (ITS1 - Forward: 5'-TCCGTAGGTGAACCTGCGG-3'; ITS4 - Reverse: 5'-TCCTCCGCTTATTGATATGC-3'). The PCR conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Fig. S1-S3 provide visual details of this characterization. The PCR product was sequenced to confirm the genetic identity of the strain. The resulting sequence data was analyzed using BLAST (Basic Local Alignment Search Tool) in the NCBI database, revealing a high similarity to known *B. ochroleuca* sequences. Fig. S1-S3 illustrate the morphological and molecular characterization results. To ensure strain purity, it was cultured on potato dextrose agar (PDA) under aseptic conditions and incubated at 24 ± 1°C for 7 days. Regular sub-culturing was performed to maintain viability and genetic consistency throughout the experiment.

Maintenance of root knot nematode, M. incognita

Three-week-old tomato plants (*Solanum lycopersicum* cv. PKM 1) were used to propagate a *virulent population of M. incognita* under greenhouse conditions. After a 45-days of incubation period following inoculation, egg masses were collected from the galled roots of the infected plants. These egg masses were placed in Petri dishes containing fresh tap water and incubated

at 27°C for five days to stimulate hatching. The resulting egg masses and hatched juveniles were then used to assess the efficacy of *B. ochroleuca* metabolites in inhibiting egg hatching and causing juvenile mortality at different concentrations.

Extraction of crude metabolites

Sterilized 100 mL of potato dextrose broth (PDB) was prepared in 250 mL conical flasks, and two 5 mm discs from an actively growing colony of *B. ochroleuca* were inoculated into each flask. The flasks were incubated for 20 days at 25±1°C. After incubation, the mycelia were removed by filtration through filter paper. Metabolites were extracted from the culture filtrate using ethyl acetate solvent extraction in a 1:1 (v/v) ratio. This process was repeated three times using a separatory funnel. The organic phase was transferred to a clean conical flask through a glass funnel containing a layer of anhydrous sodium sulfate on a glass funnel to remove moisture. The collected organic phase was then concentrated using a rotary evaporator. The resulting crude extract, a brown-colored substance, was stored in glass vials for future use (19).

In vitro bioassay against M. incognita

Mature egg masses of *M. incognita* were collected from infected tomato roots, ensuring eggs were at the same embryonic stage. In petri plates, 500 eggs were exposed to 1 mL of crude metabolite from *B. ochroleuca* at concentrations of 25%, 50% and 100%. A control treatment, using sterilized distilled water, was also included. Each treatment was replicated four times. After 72 h of incubation, the number of hatched juveniles was recorded to calculate the hatching percentage. The percentage of egg hatch inhibition was determined by counting the non-hatched eggs and data were corrected for mortality using Abbott's formula: Egg inhibition % = [(m-n)/ (100-n)] × 100; where 'm' and 'n' represent the percentages of non-hatched eggs in the treatment and control, respectively (20).

To assess nematode mortality, 100 second-stage juveniles of *M. incognita* were exposed to 1 mL of *B. ochroleuca* crude metabolites at concentrations of 25%, 50% and 100% in petri plates. Each treatment, along with a control using sterilized distilled water, was replicated four times. After 72 h of incubation, nematode mortality was evaluated by transferring the inactive juveniles to sterile distilled water overnight to confirm death. Mortality was confirmed by the straight posture of the dead juveniles and their lack of response when touched with a nematode-picking needle. The percentage of mortality was calculated and corrected using Abbott's formula to eliminate any mortality observed in control: Juvenile mortality % = [(m-n)/ (100 -n)] × 100; where, 'm' and 'n' stands for mortality percentage in treatment and control (20).

To maintain standardized environmental conditions during culturing and bioassays, all experimental Petri dishes containing *M. incognita* eggs or juveniles were incubated in a temperature-controlled incubator at 28±2 °C with 80% relative humidity. This setup was used to ensure consistent conditions across all treatments. A 12-h light/dark cycle was maintained throughout the experiment to replicate natural light conditions and minimize light-induced stress on the samples.

Metabolite profiling and pathway enrichment analysis

Metabolites were analyzed using Gas Chromatography and Mass Spectrometry (GC-MS), with identification based on comparison to NIST 14 standards. Compounds showing a mass spectral similarity of over 90% were identified as potential active compounds. Metabolite Set Enrichment Analysis (MSEA) was performed using MetaboAnalyst (https://www.metaboanalyst.ca/). The GC-MS data for the detected metabolites were preprocessed and formatted according to MetaboAnalyst's requirements. The data were uploaded to the platform, normalized, log-transformed, and scaled. Each metabolite was annotated with its corresponding KEGG IDs. To identify active biological pathways, we conducted an over-representation analysis (ORA). This analysis determined which metabolites or pathways were present more frequently in our dataset than expected by chance, allowing us to highlight the biological processes most relevant to our findings.

Computational docking studies of B. ochroleuca biomolecules with M. incognita

The identified biomolecules were subjected to molecular docking studies to assess their potential inhibitory effects against key proteins of *M. incognita*. The target proteins included Cytochrome cytochrome c oxidase subunit 1 (UniProt: A0A193H5K2), NAD(P)H oxidase (UniProt: Q4JJA9), Putative aspartyl protease (UniProt: F0UYY7), Venom allergen-like protein, Mi-vap-2 (UniProt: A7X975), Prefoldin-2 (UniProt: A0A0U1ZVZ2), MiPDI1 (GenBank: MT370326), MiEFF18 (GenBank: ASX95047.1), Mi8D05 (GenBank: AEV57568.1), Minc00344 and MiPM (WormBase ParaSite, accession number: PRJEB8714). Protein sequences were retrieved from UniProt (https://www.uniprot.org/) using a threshold of >60% query coverage and percent identity. Those sequences meeting these criteria were modeled using homology modeling, while those with <30% query coverage or lacking suitable templates underwent ab initio modeling. The proteins viz., Cytochrome c oxidase subunit 1, Prefoldin-2, Putative aspartyl protease have experimentally and computationally predicted AlphaFold structure. The 3D AlphaFold structures in PDB format were downloaded from the UniProt database. Protein Disulfide Isomerase 1 was modeled using a template protein structure (PDB ID- 4EKZ_A) due to its significant query coverage and percent identity. Minc00344, Mi8D05, MiEFF18 and MiPM, NAD(P)H oxidase and Venom allergen-like protein (Mi-vap-2) lacked homologs and had lower percent identity (PI), I-TASSER was employed and the best models were selected based on high c-scores. Ligands were sourced in a two-dimensional structure (SDF format) from PubChem. Docking studies were executed using the BIOVIA Discovery Studio Visualizer (DS4.5), focusing on interactions between *B. ochroleuca* compounds and the target proteins (21, 22). The CDock module was used to evaluate maximum binding affinity, indicated by negative "C docker energy values (kcal/mole)". The docked conformations were visualized using Biovia Discovery Studio Client version 2022 (23, 24).

Statistical analysis

The experimental data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) software version 26.0 (IBM Corp., Armonk, NY, USA). For the *in vitro* bioassays, the data were subjected to analysis of variance (ANOVA) to determine the significance of treatment effects. Means were compared using Duncan's Multiple Range Test (DMRT) at a significance level of $p \leq 0.05$ (25, 26).

Results

The investigation on the nematicidal effects of secondary metabolites derived from *B. ochroleuca* against *M. incognita* yielded promising results and significantly impacting on nematode egg hatching and juvenile mortality. The results strongly indicate that the metabolites exhibit dose and time-dependent efficacy, making them potential candidates for biocontrol strategies. At a relatively low concentration of 25% concentration, the egg hatching was substantially reduced to 17.6% and increased juvenile mortality to approximately 71.25% after 72 h. As the concentration increased to 100%, the impact on nematodes became even more pronounced, with egg hatching reduced to 5.64% and percent J2 mortality reached 95.4%. These results are statistically significant ($p \leq 0.05$), confirming that the secondary metabolites from *B. ochroleuca* are highly effective in impairing nematode development and survival. Further, the dead and disintegrated juveniles showed the presence of vacuoles inside the body (Fig. 1 & Fig. 2).

GC-MS analysis revealed a range of bioactive compounds belonging to diverse chemical classes like alcohol, alpha-keto acids, fatty acids, alkenes, hydroxybutyrates. Notable metabolites identified were butanedioic acid, lactic acid, oleic acid, myristic acid, palmitic acid, tyrosol, 4-hydroxybenzoic acid, phthalic acid (Table 1) (Fig. 3). These metabolites are known for their broad-spectrum bioactive properties, including antimicrobial, antifungal and nematicidal effects. These metabolites likely disrupt key biological processes in nematodes, contributing to their mortality. Enrichment analysis of the identified metabolites revealed their participation in several critical metabolic pathways e.g., biosynthesis of unsaturated fatty acids, galactose metabolism, linoleic acid metabolism, phenylalanine metabolism and butanoate metabolism Moreover, KEGG pathway mapping further connected these metabolites to broader biosynthetic processes, including microbial metabolism in diverse environments and biosynthesis of plant secondary metabolites (Fig. 4). This suggests that the metabolites from *B. ochroleuca* not only target nematodes but also potentially interact with plant metabolic pathways, enhancing the overall plant defense system.

Molecular docking studies provided deeper insights into how these metabolites interact with key virulence proteins in *M. incognita*. Several metabolites showed strong binding affinities with critical nematode proteins, which were essential for nematode survival and pathogenicity. For instance, palmitic acid exhibited strong binding energies of -39.1804 kcal/mol with Cytochrome c oxidase subunit 1, forming hydrogen bonds with residues ASP 83, GLY 28, and ALA 85. It also showed a binding energy of -44.5856 kcal/mol with MiPM, forming hydrogen bonds with VAL 24 residues. Other significant interactions included a binding affinity of -36.4877 kcal/mol with NAD(H) oxidase (H-bond: ARG 211) and -36.0897 kcal/mol with Putative aspartyl

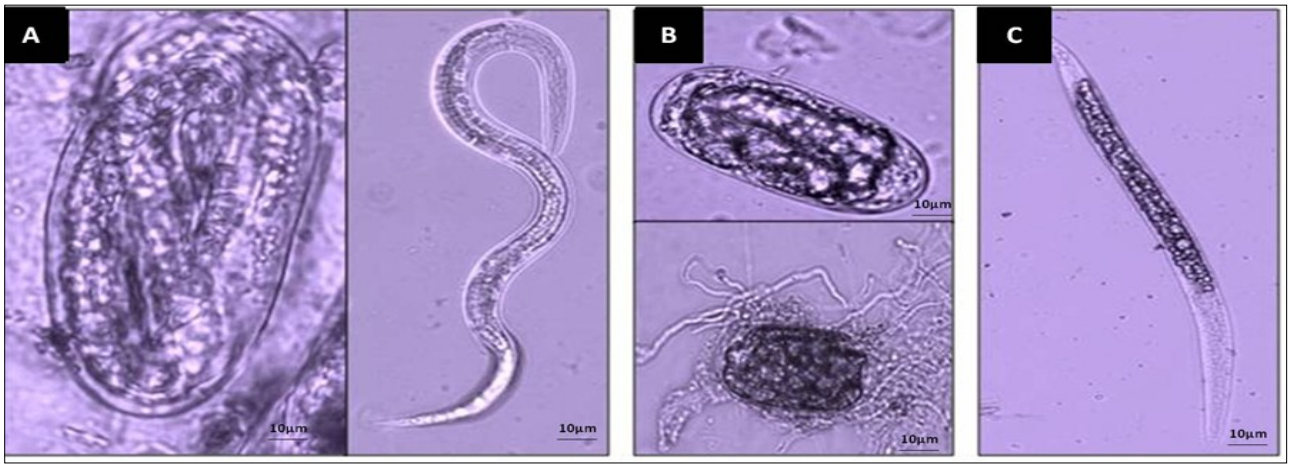

Fig. 1. Effect of crude metabolite of *B. ochroleuca* on *M. incongita* egg hatching and juvenile mortality.

(A) Healthy egg (Left) and infective juvenile J2 (right) (B, C). Disintegrated egg and infective juvenile J2

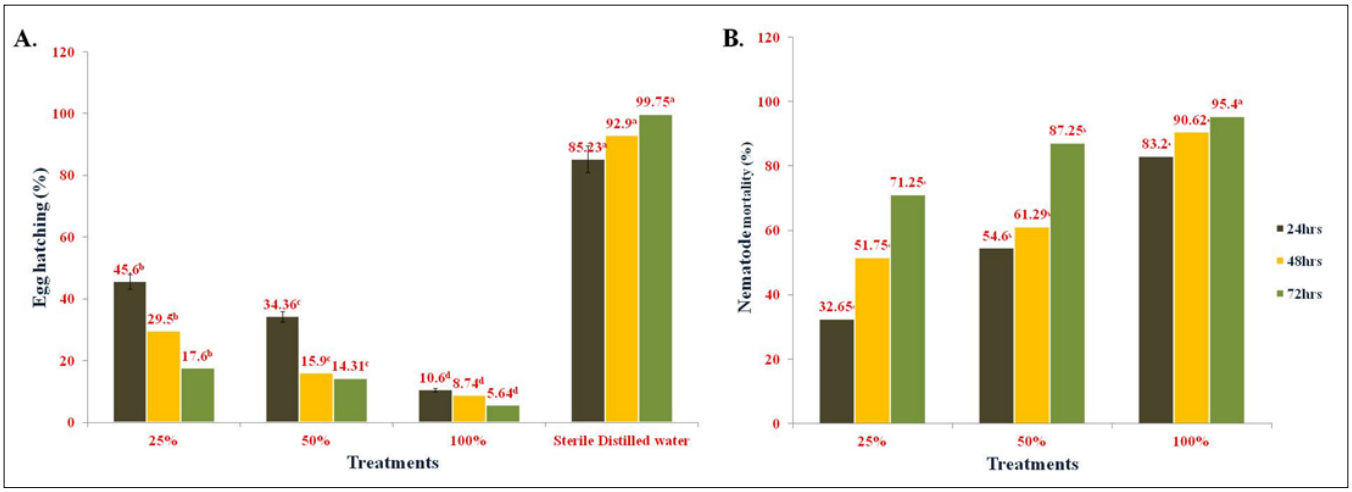

Fig. 2. Effect of *B. ochroleuca* crude metabolite on egg hatching and J2 mortality of *M. incognita* (A, B). Graph depicting the egg hatching and J2 mortality at different concentrations of crude metabolite upto 72h of incubation time. Means followed by same letters are not significantly different according to DMRT test at $p \leq 0.05$. Error bars represent standard error.

Table 1. Metabolites extracted from ethyl acetate fraction of *C. rosea* (TNAU CrN02)

Retention Time	Area%	Compound
6.401	2.52	2,3-Butanediol, 2TMS derivative
6.616	3.1	2,3-Butanediol, 2TMS derivative
6.777	0.55	Pyruvic acid-meto-TMS
7.045	6.11	Lactic Acid,2TMS derivative
8.184	0.2	2-Keto-isovaleric acid-meto-TMS
9.537	3.82	3-Hydroxyisobutyric acid-2TMS
11.531	0.26	4-Hydroxybutanoic acid, 2TMSderivative
12.548	0.74	Glycerol, 3TMS derivative
12.81	0.43	2-Methyl-4-oxo-3,4-dihydro-2H-pyran-3-ylisobutyrate
13.125	0.2	Phenylacetic acid-TMS
13.606	15.68	Butanedioic acid,2TMS derivative
13.699	15.44	Butanedioic acid,2TMS derivative
14.163	0.74	Uracil, 2TMS derivative
14.301	0.79	Acetoin,TMS derivative
14.484	1.28	2-Butenedioic acid,(E)-,2TMS derivative
14.596	4.64	2-Butenedioicacid,(E)-,2TMS derivative
17.52	0.29	3-Hydroxy-3-methylpentanoic acid,2TMS derivative
17.91	0.36	Malic acid-3TMS
18.025	1.96	Malic acid-3TMS
18.219	0.73	2,2'-(But-2-yne-1,4-diylbis(oxy))bis(ethan-1-ol), 2TMS
18.317	0.78	Pyroglutamic acid,TMS derivative
18.443	1.62	Diethylene glycol, 2TMS derivative
18.618	1.79	5-Oxoproline-2TMS
18.802	9.2	L-5-Oxoproline,2TMS
19.798	0.64	2-Hydroxybenzeneacetic acid,2TMS derivative
19.964	3.85	Tyrosol, 2TMS derivative
20.087	0.97	2-Ketoglutaricacid-meto-2TMS
20.526	0.33	E-14-Hexadecenal
20.687	0.75	Pentanedioic acid,3-methyl-3-((trimethylsilyl)oxy)-,bis(trimethylsilyl)ester
21.271	2.6	4-Hydroxybenzoic acid-2TMS
21.498	0.59	4-Hydroxyphenylacetic acid-2TMS
23.85	0.84	Tris(trimethylsiloxy)ethylene
23.976	0.64	Aconitic acid-3TMS
24.949	0.99	n-Heptadecanol-1
25.428	1.76	Citric acid-4TMS
26.054	1.05	Myristic acid-TMS
26.501	0.22	D-Fructose,1,3,4,5,6-pentakis-O-(trimethylsilyl)-,O-methyloxime
26.999	2.23	Glucose-meto-5TMS(1)
27.357	0.41	d-Mannose,2,3,4,5,6-pentakis-O-(trimethylsilyl)-,o-methyloxyme,(1Z)-
27.634	0.7	D-Glucitol,6TMS
28.959	0.57	1-Tetracosene
29.917	2.53	Palmitic acid-TMS
32.652	0.35	1-Hexacosene
32.886	0.35	Linoleic acid-TMS
33.028	1.2	Oleic Acid,(Z)-,TMS derivative
33.601	0.96	Stearic acid-TMS
40.984	0.93	1-Monopalmitin, 2TMS derivative
44.231	0.51	Glycerol mono stearate, 2TMS derivative
49.493	0.24	Glycerol-3TMS
30.304	0.56	Decanoic acid

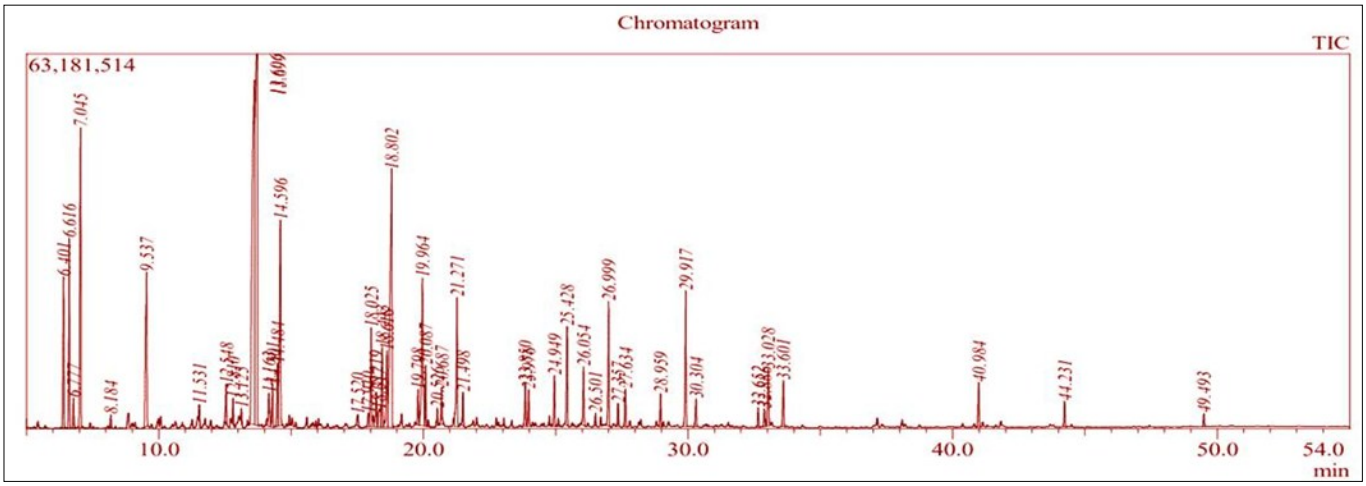

Fig. 3. Gas chromatography-mass spectrometry (GC-MS) chromatogram of the *B. ochroleuca.* The peak numbers in this Fig.correspond to those used in Table 1.

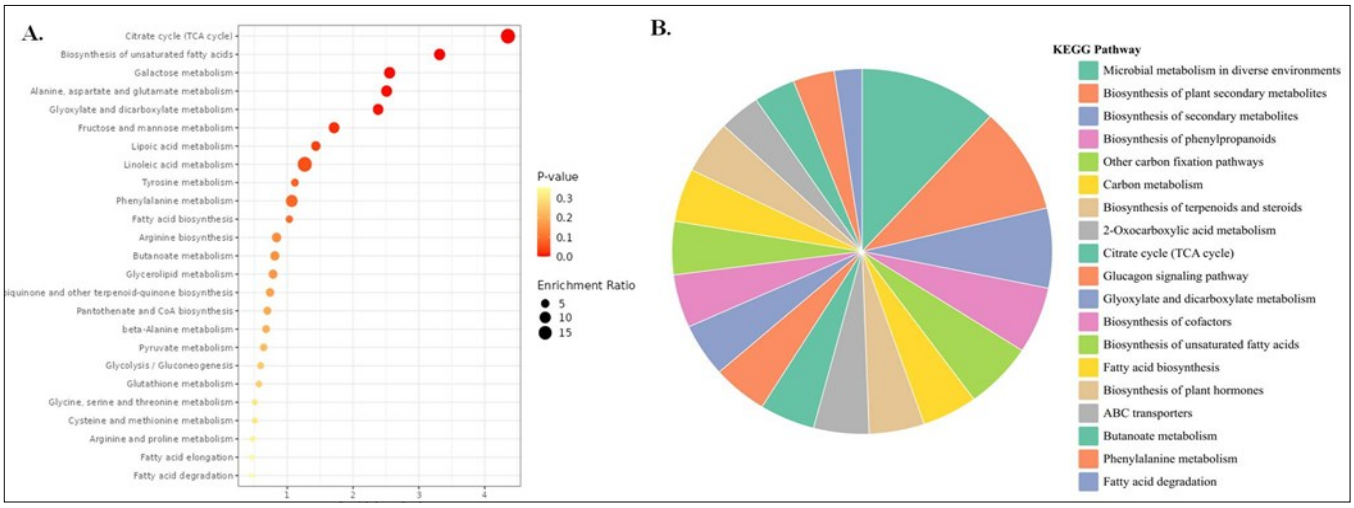

Fig. 4. Metabolite set enrichment analysis and KEGG Pathway mapping of *B. ochroleuca* metabolites

(A) Metabolite set enrichment analysis showing significant metabolic pathways.

(B) KEGG pathway mapping of identified metabolites from *B. ochroleuca*.

protease (H-bond: ARG 52). Succinic acid showed a binding energy of -35.9047 kcal/mol with Putative aspartyl protease (H-bonds: SER 31, THR 301, TYR 46, ARG 47) and -26.9114 kcal/mol with Cytochrome c oxidase subunit 1 (H-bonds: THR 301, ARG 47). Diethylene glycol had a binding energy of -23.9406 kcal/mol with Putative aspartyl protease (H-bonds: GLU 367, THR 301, ARG 47) and -19.1239 kcal/mol with Cytochrome c oxidase subunit 1 (H-bond: HIS 44). In contrast, Carbofuran 3G, used as a positive control, demonstrated significantly lower binding energies, such as -11.3085 kcal/mol with Cytochrome c oxidase subunit 1 (H-bond: ARG 30) and -13.7872 kcal/mol with Putative aspartyl protease (H-bond: ARG 30). Notably, hydrogen bonds were formed between the compounds and the binding site residues. Additionally, various other interactions occurred, including hydrophobic contacts, van der Waals forces, and different types of stacking and bridging interactions. These findings indicate the potential of these compounds to effectively target the protein sites (Table 2) (Fig. 5 & Fig. 6). These molecular interactions suggest that the metabolites from *B. ochroleuca* effectively target vital proteins in nematodes, impairing their functionality and contributing to the overall nematicidal activity. These results collectively indicate the potential of *B. ochroleuca* metabolites in the development of sustainable, natural nematode control strategies.

Discussion

The findings from our research align closely with existing literature on the nematicidal effects of various metabolites and biological agents against *M. incognita*, highlighting the potential of biocontrol products as effective alternatives to synthetic nematicides. For instance, *Myrothecium verrucaria* metabolites identified via GC-MS were shown to significantly reduce *M. incognita* egg hatching by 94.23% and increase juvenile mortality by 96.70%, validating the potent bioactivity of fungal metabolites in nematode control (27). The extracts from *Phyllanthus amarus* (28) and actinomycete strains such as *Micromonospora* sp. (29, 30) have reported substantial mortality rates against various nematodes, reinforcing the effectiveness of higher metabolite concentrations. Moreover, endophytic bacteria from the genera *Pseudomonas*, *Arthrobacter*, *Bacillus* and *Serratia* isolated from diverse plant species, have demonstrated high mortality rates against *Meloidogyne* spp., with *B. thuringiensis* AK08 showing the highest efficacy. The production of secondary metabolites, such as flavonoids, phenols, and terpenoids, further underscores their potential as biocontrol agents. Among these, cholest-5-en-3β-ol-3-carbonochloridate was identified as a major compound, present at a concentration of 25.35% (31). Furthermore, the effectiveness of secondary metabolites, such as 2,6-pyridinedicarboxylic acid from *Simplicillium chinense* and oxalic acid from *Aspergillus tubingensis*, highlights the potential of naturally derived products in nematode management (32, 33). These compounds act by disrupting nematode cell membranes, inhibiting enzyme activities crucial for survival, and supporting plant resilience through enhanced root growth and stress tolerance, as seen with *Simarouba glauca* and various fungal bioagents (34, 35). Together, these studies underscore the viability of metabolite-rich extracts and fermentation products as eco-friendly and effective biocontrol agents against nematode infestations. This aligns with our findings and reinforcing the value of diverse natural sources for sustainable agriculture.

The identification of key bioactive metabolites through our GC-MS analysis, such as butanedioic acid, lactic acid, oleic acid, myristic acid, palmitic acid, tyrosol and 4-hydroxybenzoic acid, highlights the nematicidal potential of *B. ochroleuca*. These metabolites exhibit strong bioactive properties, such as antimicrobial, antifungal and nematicidal effects, suggesting they contribute to fight nematodes by disrupting their essential biological functions, ultimately leading to nematode mortality. Fatty acids like palmitic and oleic acid are particularly effective at destabilizing cell membranes and interfering with nematode metabolism, resulting in cell death (36). The amphiphilic nature of these fatty acids, characterized by hydrophilic carboxyl groups and hydrophobic carbon chains, enhances their bioactivity against nematodes. Previous research has demonstrated that oleic acid's effectiveness in controlling nematodes is significantly amplified by the presence of a double bond at the α, β-position of the carbonyl group, which boosts its potency against *M. incognita* (37). Likewise, lauric acid, myristic acid, and palmitic acid have shown to exert concentration-dependent effects on nematode populations, functioning as either lethal agents or repellents (38, 39).

Table 2. C Docker energy values of small molecules of TNAU CrN 02 (Palmitic acid, Succinic acid, Diethylene glycol, Decanoic acid) and Carbofuran with virulent target and H-bond formed

C docker energy (kcal/mol) of small molecules and carbofuran on different targets						H-Bonds				
Targets	**Palmitic acid**	**Succinic acid**	**Diethylene glycol**	**Decanoic acid**	**Carbofuran**	**Palmitic acid**	**Succinic acid**	**Diethylene glycol**	**Decanoic acid**	**Carbofuran**
Cytochrome c oxidase subunit 1	-39.1804	-26.9114	-19.1239	-33.2168	-11.3085	ASP 83; GLY 28; ALA 85	THR 301 ; ARG 47	HIS 44	ASP 122; HIS 44	ARG 30
NAD(P)H oxidase	-36.4877	-24.4977	-20.3842	-41.3034	-6.76117	ARG 211	ARG 211	ARG 211; THR 86	ARG 211	GLN 87
Putative aspartyl protease	-36.0897	-35.9047	-23.9406	-10.6708	-13.7872	ARG 52	SER 31; THR 301; TYR 46; ARG 47	GLU 367 ; ARG 47 ; THR 301	ARG 47	ARG 30
Mi-vap-2	-29.4199	-25.6162	-15.5022	-29.3024	-5.91573	THR 212	TYR 59 ; ASN 152 ; ASP 206	ASP 206 ; ASN 152	ASN 85	LYS 208
Prefoldin-2 protein	-33.1514	-21.4768	-13.7913	-32.567	-4.05822	LYS 94 ; ILE 87	HIS 52 ; ARG 70 ; GLN 71	ASN 56	ASN	HIS 52 ; ARG 70
MiEFF18	-31.7011	-22.9109	-16.2256	-33.6746	-8.36952	LYS 259 ; GLY 260	LYS 298 ; LYS 272	LYS 298 ; GLY 273	GLN ; 291	LYS 272 ; LYS 298
MiPM	-44.5856	-27.4848	-18.0611	-31.1033	-10.1205	VAL 24	GLU 20	GLN 47	PHE 21	GLN 47
MiPDI11	-24.1557	-26.9565	-17.7249	-21.6109	-1.938032	VAL 265	LEU 338 ; LYS 272; ILE 271	LYS 272 ; GLY 269 ; PHE 267	LYS 272	LYS 272
Minc03344	-38.7911	-27.2735	-15.6281	-27.3155	-10.5589	TYR 70	THR 73 ; ASN 71	ASN 56	ASN 56	ASN 71
Mi-Mi8D05	-35.1013	-22.4457	-14.3781	-18.6252	-9.49714	GLN 4; GLU 68	LYS 176	GLU 174	ARG 114	TYR 56

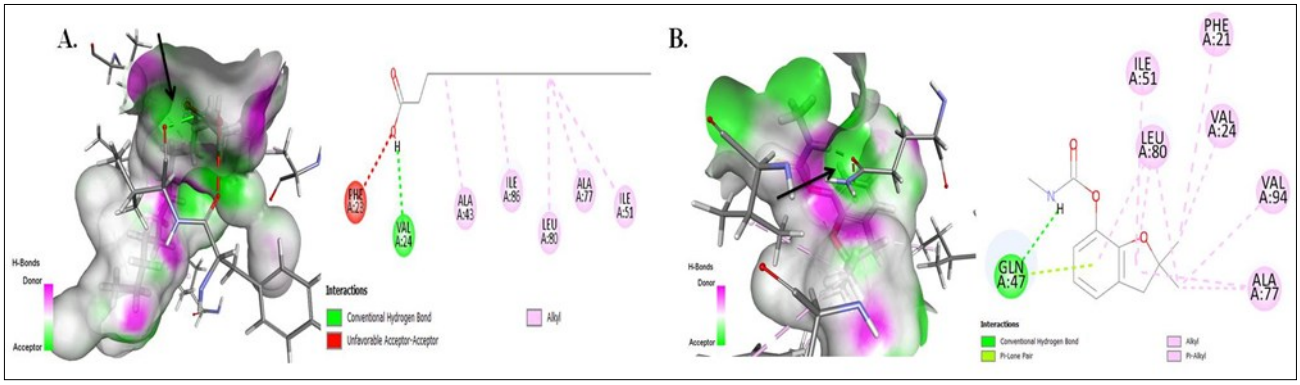

Fig. 5. Molecular docking interaction of Palmitic acid (-44.58 kcal/mol) (A) and Carbofuran (-10.12 kcal/mol) (B) with MiPM (Mi Pausse muraille): arrow represents H-bond interactions.

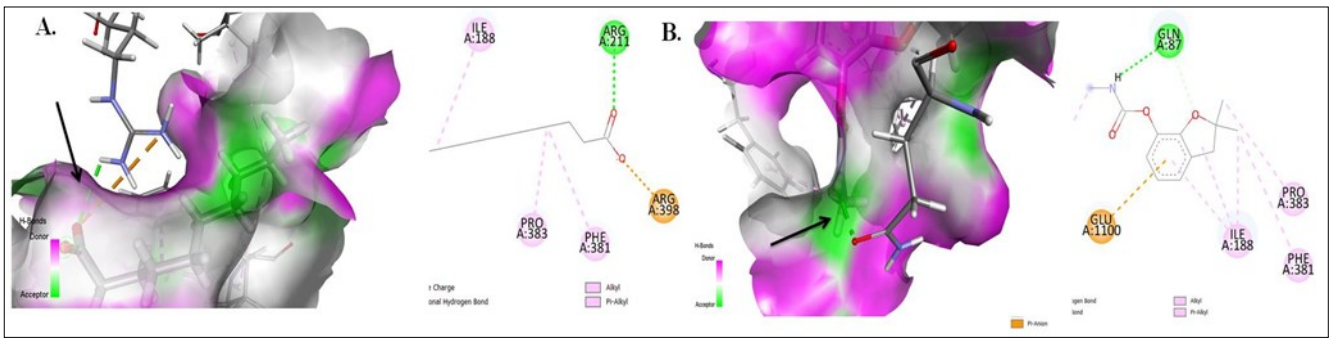

Fig. 6. Molecular docking interaction of Decanoic acid (-41.30 kcal/mol) (A) and Carbofuran (-6.76 kcal/mol) (B) with NAD(P)H oxidase: arrow represents H-bond interactions.

The presence of these fatty acids in *B. ochroleuca* corresponds well with their reported ability to suppress nematode survival through diverse mechanisms, such as membrane disruption and metabolic interference. Lactic acid, another prominent metabolite identified in our analysis, has been reported to induce high mortality rates in *M. incognita* juveniles, likely through interference with their energy metabolism and movement (40-43). Similarly, butanedioic acid and 4-hydroxybenzoic acid, both identified in our study, have been noted for their pesticidal and antimicrobial activities. Butanedioic acid likely exerts its nematicidal effects by interfering with key metabolic pathways, while 4-hydroxybenzoic acid has been shown to inhibit nematode growth through its antimicrobial properties (44, 45). Further, compounds such as tetradecanoic acid and undecanoic acid demonstrate significant effectiveness in suppressing nematode activity (46, 47). These findings underscore the promise of fatty acids as key components in the development of novel nematicidal biomolecules, paving the way for sustainable pest management strategies in agriculture.

The influence of alcohol functional groups on nematicidal activity is indeed well-documented, with their position playing a critical role in determining the strength of inhibition (48). The four-diethylene glycol dialkanoates e.g., dipropionate, dibutanoate, dipentanoate and di-(2-ethylhexanoate)-exhibited strong nematicidal effects against *Rotylenchulus reniformis* juveniles, with EC50 values ranging from 21.53 to 24.14 ppm, underscoring their efficacy (49). Enrichment analysis of the identified metabolites revealed their participation in several critical metabolic pathways and broader biosynthetic processes, suggesting that the metabolites from *B. ochroleuca* target nematodes and may also interact with plant metabolic pathways, enhancing the overall plant defense system.

The molecular docking approach models the interaction between small molecules and proteins at the atomic level, enabling the characterization of how these molecules behave within the target protein's binding site and providing insight into key biochemical processes. In our study, we found significant interactions between key metabolites and virulence proteins in *M. incognita*, revealing the potential mechanisms through which these compounds exert their nematicidal activity. Notably, palmitic acid demonstrated strong binding affinities with several critical nematode proteins, including Cytochrome c oxidase subunit 1 and MiPM. The binding energies of -39.1804 kcal/mol for Cytochrome c oxidase subunit 1 and -44.5856 kcal/mol for MiPM highlight palmitic acid's potential to interfere with essential metabolic and pathogenic processes within the nematode. These interactions may disrupt the nematodes's respiratory function and structural integrity, of the nematode, leading to impaired survival (50). Furthermore, our analysis identified that other metabolites, such as succinic acid, also exhibited significant binding affinities with virulence proteins, suggesting a broader array of compounds contributing to the overall nematicidal effects observed. Interestingly, diethylene glycol, another metabolite identified in this study, demonstrated moderate binding energies, including -23.9406 kcal/mol with Putative aspartyl protease and -19.1239 kcal/mol with Cytochrome c oxidase subunit 1. Though weaker than those of palmitic and succinic acid, these interactions suggest that diethylene glycol may still contribute to nematode inhibition, possibly through secondary or synergistic mechanisms. Recent study demonstrated the compound, l-proline-catalyzed pyrano[3,2-c]pyridone derivatives exhibited significant nematicidal activity against *M. incognita*, comparable to the commercial nematicide carbofuran, with promising *in vitro* results and supporting *in silico* docking analyses indicating their potential as effective nematicidal agents (24). In contrast, the positive control, Carbofuran 3G exhibits lower binding affinities with both Cytochrome c oxidase subunit 1 (-11.3085 kcal/mol) and Putative aspartyl protease (-13.7872 kcal/mol), indicating that the natural metabolites may disrupt nematode biology more effectively. Additionally, these natural compounds are generally biodegradable and less toxic to non-target organisms, contributing to a lower environmental impact compared to Carbofuran, which is associated with risks of soil and water contamination and poses significant hazards to human health and beneficial species. While conventional nematicides may offer lower initial costs and ease of application, their long-term economic viability is diminished by potential resistance development, environmental cleanup costs and health risks. Although initial costs for extracting and purifying natural metabolites may be higher, their potential for lower application rates and reduced environmental remediation expenses could enhance their cost efficiency over time. Overall, the promising effectiveness, environmental safety and potential cost efficiency of natural metabolites position them as viable alternatives for sustainable agricultural practices. Future research should focus on optimizing their extraction and application methods to ensure their successful integration into existing pest management systems.

While our molecular docking study demonstrates significant interactions between metabolites from *B. ochroleuca* and virulence proteins of *M. incognita*, several limitations must be acknowledged. Although molecular docking has predictive power, it is inherently theoretical and does not account for the complexities of biological environments. Therefore, experimental validation through *in vitro* and *in vivo* assays is essential to confirm the nematicidal effects of these compounds at various concentrations (24). *In vitro* assays could involve exposing nematodes, such as *M. incognita*, to various concentrations of key metabolites like palmitic acid and succinic acid, followed by monitoring mortality rates over specific time intervals. Behavioral studies can assess the effects of these metabolites on nematode movement and feeding behavior. Additionally, *in vivo* trials should be performed in greenhouse or field settings, applying the metabolites to plants infested with nematodes, measuring plant growth, yield and nematode populations over time to evaluate the efficacy of these natural compounds compared to standard chemical nematicides. Mechanistic studies could include enzyme activity assays to explore how the metabolites influence metabolic processes in nematodes and molecular techniques, such as RT-qPCR, to analyze the expression of nematode genes involved in virulence and stress responses. Moreover, the effectiveness of metabolites in agricultural environments is significantly influenced by factors such as bioavailability, stability and metabolite concentration. To thoroughly evaluate these factors, various methods can be utilized, including field trials to observe real-world conditions, laboratory simulations to mimic environmental factors, chromatographic techniques for detailed analysis, assessments of soil microbial activity, root uptake assays to study bioavailability, controlled stability testing and analytical modeling to predict persistence and impact. Additionally, the focus on a limited number of metabolites and proteins highlights the need for further exploration of other

potential targets and compounds to achieve a comprehensive understanding of nematicidal mechanisms. Comprehensive biosafety assessments, evaluations of environmental impact, and long-term efficacy studies of these metabolites are critical for large-scale agricultural applications. Field trials should be conducted in diverse agricultural settings, such as greenhouse conditions, organic farms and conventional cropping systems, across various regions to evaluate their effectiveness under different environmental conditions and soil types. Also, research should focus on developing practical formulations that enhance the application of these metabolites, such as liquid concentrates, granules, or encapsulated products, ensuring ease of use for farmers. Future research should address these considerations to fully realize the benefits of *B. ochroleuca* metabolites to enhance crop health and reduce reliance on synthetic chemicals, promoting viable solutions for nematode control.

Overall, our findings, supported by the existing literature, emphasize the significant nematicidal potential of *B. ochroleuca* metabolites, particularly through mechanisms like membrane disruption, metabolic interference and antimicrobial activity. These compounds collectively provide a robust strategy for sustainable pest management.

Conclusion

The exploration of *B. ochroleuca* metabolites as a nematicidal agent presents a promising frontier in sustainable agriculture. These metabolites have demonstrated significant potential by effectively reducing nematode egg hatching and increasing juvenile mortality in a dose- and time-dependent manner. Key bioactive compounds such as butanedioic acid, lactic acid, oleic acid, and palmitic acid disrupt critical biological processes in nematodes, while enrichment analysis suggests their involvement in essential metabolic pathways that may also enhance plant defenses. Molecular docking studies revealed strong interactions between palmitic acid, butanedioic acid and diethylene glycol with virulent nematode proteins, indicating mechanisms that impair nematode functionality. This combination of strong nematicidal efficacy, a diverse array of bioactive compounds and the potential to enhance plant health, positions *B. ochroleuca* as a unique alternative to other biological control agents. To shift from lab-based research to practical field application, future experiments should focus on conducting field trials to evaluate the effectiveness of *B. ochroleuca* metabolites under varied agricultural conditions. Essential steps include optimizing formulation techniques, assessing the stability and bioavailability of metabolites in field conditions, and investigating potential interactions with other agricultural inputs. The economic and environmental implications of utilizing *B. ochroleuca* metabolites on a large scale could be substantial, as they may decrease reliance on synthetic pesticides, thus reducing production costs and mitigating negative environmental impacts associated with chemical usage. This innovative approach not only addresses nematode infestations but also responds to the increasing demand for eco-friendly pest management solutions. Ultimately, a multifaceted approach that combines rigorous laboratory research with practical field applications will unlock the full potential of *B. ochroleuca* as a vital player in the future of sustainable agriculture.

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Authors' contributions

NS, JI and GJ conceptualized the research. PV, JI, AS and KE helped in GC MS sample preparation and analysis. VS performed *in vitro* study and molecular docking experiments and PV illustrated the docking files. VS, PV, NS and GJ drafted the manuscript. The authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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