

RESEARCH ARTICLE

Chemical profiling of endangered *Citrus macroptera* leaf extracts and evaluation of its cytotoxic activity

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Abstract

The main component of drug production and formulation, with minimal to no adverse effects, is plant-based medicine. *Citrus macroptera* is an endangered species of North-Eastern India and has a diverse array of pharmaceutical applications owing to the presence of several bioactive compounds. The study aimed to evaluate the presence of bioactive metabolites, antioxidants, and cytotoxic mechanisms of the *in vitro* and *ex vitro* leaf extracts of *C. macroptera*. Gas chromatography–mass spectrometry (GC-MS) profiling was performed using the ethanolic leaf extracts. Methanolic and aqueous extracts were profiled using High-performance thin-layer chromatography (HPTLC). Moreover, the cytotoxicity of the methanolic leaf extracts was assessed using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test against SH-SY5Y, human neuroblastoma cell lines. GC-MS analysis revealed the existence of numerous pharmacologically significant components such as esters, hydrocarbons, fatty alcohols, and ester compounds. The presence of flavonoids and stigmasterol was exhibited by the HPTLC profile. The *in-situ* HPTLC analysis revealed white fluorescence after derivatization under white light that explicitly confirms the antioxidant potential of the extracts and can serve as a lead source of natural antioxidants. The cytotoxic efficacy of the extracts was observed in a concentration-dependent manner. However, the *in vitro* leaf extract displayed better suppressive effects against the SH-SY5Y cells with a 24-h IC₅₀ of 167.71 µg/mL compared to the *ex vitro* leaf extract. Further, a comprehensive study is necessary to identify the cytotoxic compounds and their mode of action. The outcome of our investigation provides empirical evidence that phytoconstituents present in the leaf extracts of *C. macroptera* can be used as an effective pharmaceutical agent to combat several diseases.

Keywords

Antioxidants; cytotoxicity; flavonoids; GC-MS; HPTLC; phytoconstituents

Abbreviations:

- Citrus macroptera*- *C. macroptera*
- *Citrus macroptera* methanolic leaf extract -CML (MeOH)
- *Citrus macroptera* ethanolic leaf extract-CML (EtOH)
- Citrus macroptera* aqueous leaf extract- CML (Aq)
- *Citrus macroptera in vitro* methanolic leaf extract-CML (IN MeOH)

- Citrus macroptera* *in vitro* ethanolic leaf extract- CML (IN EtOH)
- DPPH-1,1-diphenyl-2-picrylhydrazyl
- HPTLC- High-performance Thin Layer Chromatography
- GC-MS- Gas Chromatographic and Mass spectrometry
- MTT-3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
- Fetal Bovine Serum (FBS)
- Minimum Essential Media with High Glucose (MEM-HG)
- 1X Dulbecco's Phosphate Buffered Saline (DPBS)
- Dimethyl sulfoxide (DMSO),
- Polyethylene Glycol (PEG)
- Dulbecco's Modified Eagle's Medium, high glucose (DMEM-HG)

Introduction

Medicinal plant use has been practiced in traditional healthcare systems since prehistoric times and the majority of the world's population still relies on it. *Citrus* species have a wide range of applications in the food, beverage, pharmaceutical, and nutraceutical industries due to the presence of abundant secondary metabolites (1, 2). Citrus natural products are famous for their aroma, which is attributed to flavonoids and limonoids found in the rind (3). *C. macroptera* is a native and endangered species of India's northeastern region, found in the Khasi Hills of Meghalaya and the districts of Manipur, Tripura, and Assam. *C. macroptera* fruit, juices, pulp, and peel have been shown to have a wide range of ethnomedicinal activities. The Khasi and Garo tribes employ *C. macroptera* peel in culinary applications, particularly as a flavoring element in non-vegetarian foods. "Wak Chambal Phura" is a Garo delicacy made from this fruit. The pulp is used to make pickles and the whole fruit is strung with strings around the hips and swung to represent protection of the fields from pests (4). *C. macroptera* is a pharmacologically important medicinal plant that has been used as a folk medicine to treat a variety of diseases since ancient times. Phytochemicals found in its fruits include limonene, β -pinene, β -caryophyllene, geraniol, edulinine, isoplatydesmine, and ribalinine among others (5, 6).

Identification of chemical structures and their pharmacological relevance has always been critical for screening novel chemical compounds. *Citrus* L. is one of the most studied fruit species in terms of bioactive content (7). Because of the complicated nature of the phytoconstituents, there is a lack of conventional quality control, which raises concerns for their safety and efficacy (8, 9). HPTLC is a sensitive method for analyzing complicated combinations of chemical compounds. It can be used for a quick study of the antioxidant activity of extracts and the HPTLC-DPPH test is used for qualitative analysis, which aids in overcoming the problem of interference from color and other compounds contained in natural products (10, 11). GC-MS is another cutting-edge

chromatographic technology widely used in identification of the unknown compounds in a complex mixture. The metabolites can be determined by comparing them to the reference library (12).

Oxidative stress has been linked to several neurological disorders such as Parkinson's and Alzheimer's. It is triggered by various factors such as the secretion of cytokines, and the accumulation of abnormal proteins and calcium ions across the neurons that induce nerve cell death due to the activation of several biochemical pathways and reactive oxygen species (13, 14). Therefore, from this perspective, bioactive compounds such as alkaloids, flavonoids, and terpenoids can play a pivotal role in attenuating such disease development (15, 16). A previous study using plant extracts has been utilized against SH-SY5Y cells to evince the neuroprotective potential (17). Despite having abundant metabolites in various plant parts of *C. macroptera* there is no report to date, depicting the metabolites and bioactivity of *in vitro* and *ex vitro* leaf extracts. The objective of the current study is to showcase the fingerprint profile of the phytoconstituents through GC-MS and HPTLC analysis and to evaluate the cytotoxic activities of *C. macroptera* against the human neuroblastoma, SH-SY5Y cell line. Thus, this study can further be validated with an *in vivo* animal model to corroborate the current findings.

Materials and Methods

Chemicals and Reagents

The HPTLC plates pre-coated with silica gel 60 F254 were from Merck, Darmstadt, Germany. Stigmasterol, ascorbic acid, and DPPH were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL), Mumbai, India. Cell line: SH-SY5Y-human neuroblastoma cell line was procured from the National Centre for Cell Science (NCCS), Pune, Maharashtra, India; Cell culture media- MEM-HG supplemented with 10% FBS; DPBS, MTT reagent, 0.25% trypsin EDTA solution was procured from MP Biomedicals, Germany; DMSO, cell culture grade was from Merck Germany.

Plant Material and Sample Preparation

The plants of *C. macroptera* were procured from the ICAR Research Complex of the NEH region, Lamphelpat, Imphal, Manipur, India, sub-tropical zone hill zone (NEH-3), 24°50' N latitude, 93°55' E longitude during the December (2017) under the DBT project sanction no. (BT/PR16132/NER/95/160/2015) with the collector code: RCM_CM_01 and established in the organic field of Amity University Noida, Uttar Pradesh, Noida, India. Leaves (*ex vitro*) from the aerial part were collected from the field-established plants. *In vitro*, leaves were collected from the micro propagated plants of *C. macroptera*. Both the leaf samples were dried and ground before use and the extract was prepared by the cold extraction method (maceration) with some modifications (18). The dried powder samples were soaked in the respective solvents, viz., ethanol (95%), methanol (95%), and aqueous in the ratio of 1:10, for 72 h

at room temperature with occasional shaking every 6-7 h. The extract was filtered with 11 μ m pore-size filter paper. The filtrate was concentrated, dried, and stored at 4 °C for further use.

GC-MS Analysis

Gas chromatographic-mass spectrometry analysis was carried out to identify the bioactive or organic compounds present in the CML (IN EtOH) and CML (EtOH) using Autoexcel Turbo Mart, (Perkins Elmer) equipped with column hp-5Ms capillary. The column length was 30m and the injector temperature was 260 °C at a 10 °C/min rate up to 280 °C. The flow rate was 1 ml/min with helium gas used as a carrier gas. The Electron spray Ionization (ESI) force was 220 °C with a scan range of 20-610 AMU/sec. The significant bioactive compounds were assessed based on the MS spectrum pattern for both extracts and were compared against the standard mass spectra from the database of the National Institute of Standards and Technology (NIST) library using TurboMass Software.

HPTLC Profiling Analysis

HPTLC fingerprinting was performed to assess the presence of flavonoids and the antioxidant activity of the different leaf extracts of *C. macroptera*. 10 mg of each sample was weighed and added to 1 ml of methanol (10 mg/ml), which was then sonicated for 10 min at 40 Hz. The supernatant was collected after centrifugation. HPTLC separations were run on stationary phase TLC aluminum plates with silica gel 60 F254 200 X 100 mm. For the qualitative analysis of flavonoids, the extracts: CML (MeOH); CML (IN MeOH); CML (Aq) were spotted on the plate having a band length of 8.0 mm using a CAMAG ATS-4 sampler on a preactivated 60 F254 HPTLC plate and Ethyl Acetate: Water: Formic Acid: Acetic Acid (100: 26: 11: 11 v/v/v) as mobile phases for chromatogram development. Similarly, for HPTLC-DPPH activity, the mobile phase remains the same as that used in flavonoid detection. The standards (ascorbic acid and stigmasterol) and *C. macroptera* leaf extracts were spotted on HPTLC plates. Another TLC plate was used for studying the presence of stigmasterol in all the samples. The process is analogous to the flavonoid sample application with mobile phase Toluene: Ethyl Acetate (9.3: 0.7 v/v/v). Before setting up the mobile phase, all the sample-loaded plates were dried. The plates were saturated with the solvent system by placing them in the twin TLC trough developing chamber for 20 min at room temperature with a distance coverage of 70 mm. The retardation factor (Rf) values and color were detected.

Derivatization and Data Acquisition

HPTLC plates were dried (50-60°C) for complete evaporation of the mobile phase or the solvent from the plate after the development (19). The plates were dipped in DPPH reagent for a fraction of a second using the Chromatogram Immersion System III (CAMAG). For stigmasterol analysis, the plates were dipped in Anisaldehyde Sulphuric Acid Reagent and for flavonoid detection, the plate was dipped in the Natural Product reagent followed by PEG reagent. The spots were visible at

254 nm (deuterium lamp, absorption node filter K400) and 366 nm (mercury lamp, fluorescence filter K400) in the case of the DPPH-HPTLC assay, whereas in the identification of compounds (flavonoids and stigmasterol), a mercury lamp at 366 nm was used. The images of the plates were captured with a TLC Visualizer Documentation System (CAMAG, Muttenz, Switzerland) equipped with Vision CATS software operated with LABSERVER, version 3.1.21109. The scanner operating parameters were (Mode: adsorption/ reflection; slit dimension; 6 X 0.45 mm; scanning speed: 20 mm/s). The plates were kept in a photo-documentation chamber and images were captured at visible light, 254 nm (short wavelength) and 366 nm (long wavelength).

Determination of Cytotoxic Activity

SH-SY5Y (human neuroblastoma) cell lines were determined for cytotoxic activity. Under the controlled conditions, the cells were grown in MEM-HG supplemented with 10% FBS and maintained in a humidified atmosphere with 5% CO₂ at 37 °C and regularly passaged to maintain the confluence of approximately 70%. The cytotoxic potential of CML (MeOH) and CML (IN MeOH) was examined by the ability of the cells to cleave the tetrazolium salt MTT by the enzyme succinate dehydrogenase following the procedure as described in the previous study (20). The SH-SY5Y cells were cultured in T-25 flasks, trypsinized, and aspirated using DMEM-HG to obtain the cell density of 2 x 10⁴ cells/well in 200 mL of the cell suspension which was seeded in each well of the 96 well micro titre plate. The plate was incubated at 37 °C and 5% CO₂ atmosphere for 24 h. Subsequent 24 h incubation, different concentrations (25-200) μ g/mL of test drugs/extracts and standards were added to the respective wells. The plates were incubated again under similar conditions. After the removal of the plate, the drug/ extract was aspirated from the wells. 200 μ l of medium containing 10% MTT reagent was added to each well to get a final concentration of 0.5 mg/mL and incubated in the dark at 37 °C and 5% CO₂ atmosphere for 3 h. The solubilization solution, DMSO was used to dissolve the purple formazan crystals by gentle shaking in a gyratory shaker. The negative control was used as the media itself or the media with cells without drug/extract and the positive control was media with cell and stigmasterol. Plates were assessed using a microplate reader by measuring the absorbance at 570 nm and 630 nm as references. The % growth inhibition was calculated after subtracting the background and the blank and the concentration of the test drug needed to inhibit cell growth by 50% (IC₅₀) was generated from the dose-response curve for the SH-SY5Y cell line.

Statistical analysis

All the experiments had three copies of each run. The findings were presented as mean standard deviation. The observations were interpreted using linear regression study to establish the correlation between the cell viability and concentration of the extract to determine the (IC₅₀) values and the statistical validation was significant at P<0.05.

Results

Analysis by GC-MS

CML (IN EtOH) and CML (EtOH) were chemically profiled through GC-MS analysis and it was discovered that out of 100 volatile chemicals, CML (IN EtOH) had the highest number of compounds. Esters, hydrocarbons, and fatty alcohols make up the majority of the chemical classes in the CML (IN EtOH), while the CML (EtOH) is composed primarily of the following chemical classes: hydrocarbons, fatty alcohol, terpenoids, ether, fatty acid and ester (Fig. 1). It has been reported that esters have several commercial and pharmaceutical applications (24). Moreover, there were no similar compounds found in both extracts. The mass spectra of the identified compounds of CML (IN EtOH) and CML (EtOH) are presented (Fig. 2 and 3). Several other bioactive constituents found in the extracts exerting pharmacological activities are depicted in (Table 1). CML (IN EtOH) had more active components compared to CML (EtOH).

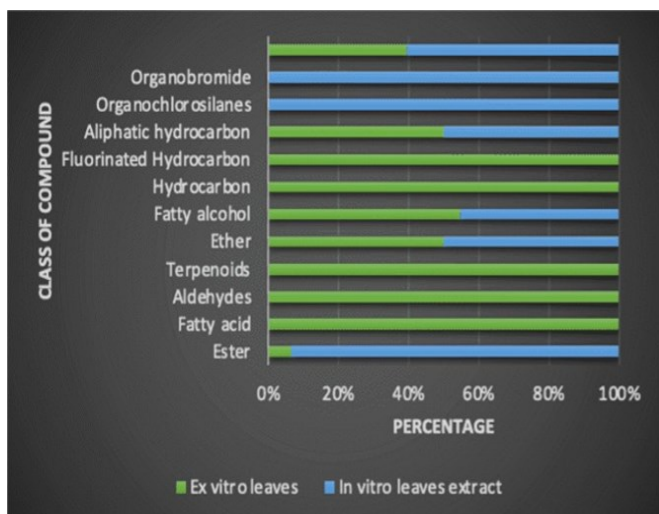


Fig. 1. Representation of the various classes of volatile compounds obtained after GC-MS analysis of CML (IN EtOH) and CML (EtOH) of *C. macroptera*

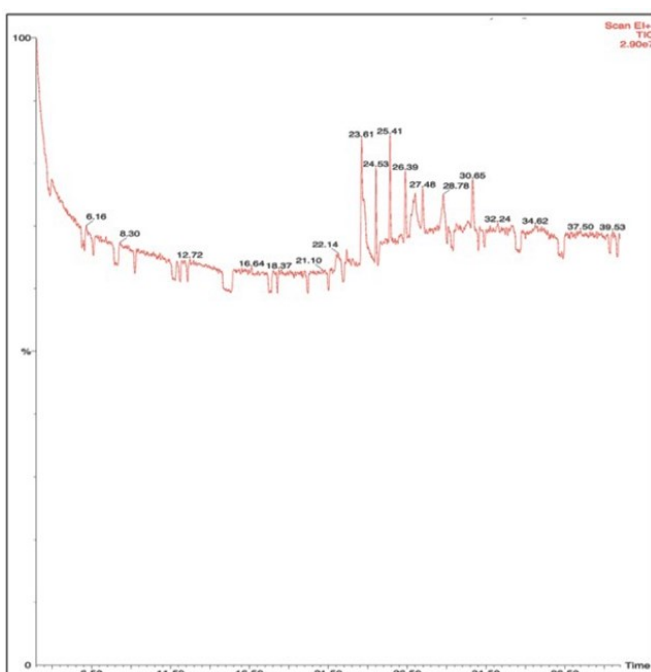


Fig. 2. Chromatogram of the phytoconstituents detected in CML (IN EtOH) using GC-MS

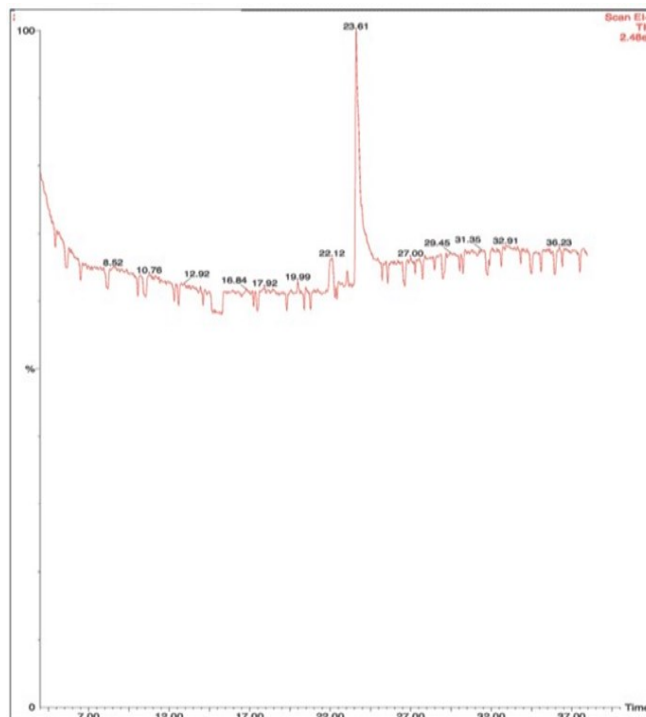


Fig. 3. Chromatogram of the phytoconstituents detected in CML (EtOH) using GC-MS

Estimation of Stigmasterol in the Extracts

There is no report of the quantification of stigmasterol in the leaf (*in vitro* and *ex-vitro*) extracts of *C. macroptera* by HPTLC. The solvent system combination, toluene: ethyl acetate (9.3: 0.7 v/v), gave the best separation. The peak that showed an R_f value at 0.47 was identified as stigmasterol. By comparing the retention factors of the extracts with those of stigmasterol, it was found that all the samples contained stigmasterol in varying concentrations. The fingerprint profile depicts the levels of stigmasterol in CML (MeOH) and CML (EtOH) were excess whereas in CML (Aq) and CML (IN MeOH) were found in traces (Fig. 4).

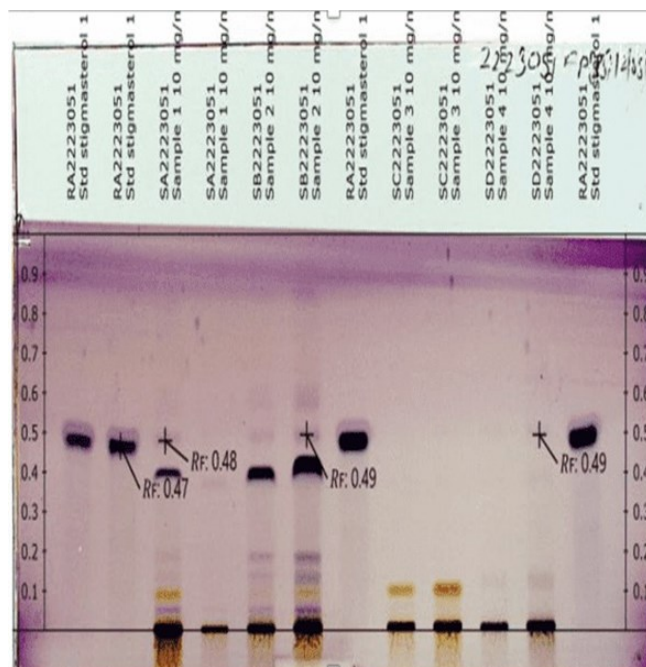


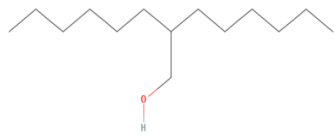
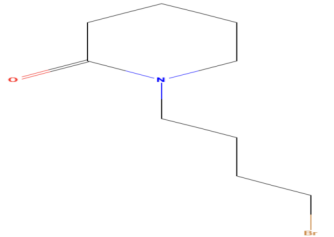
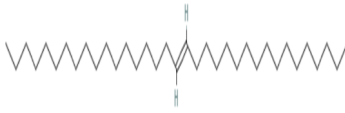
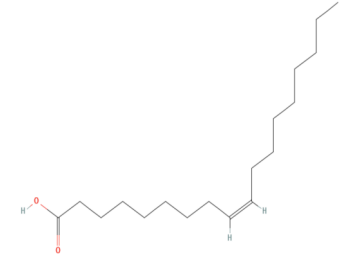
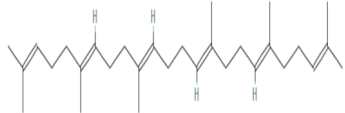
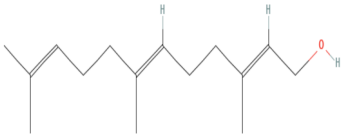
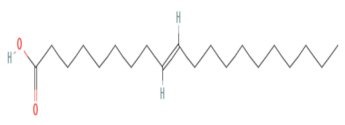


Fig. 4. HPTLC chromatogram at white light representing the qualitative assessment of stigmasterol in the extracts {Sample 1-CML (MeOH); Sample 2-CML (EtOH); Sample 3-CML (Aq); Sample 4- CML (IN MeOH)}

Table 1. The pharmacological activity, molecular weight, and chemical structure of some of the bioactive components of CML (IN EtOH) obtained from GC-MS profiling

Chemical compound	RT	Molecular weight	Molecular formula	Pharmacological activity	Chemical structure
Silane, Trichlorodocosyl-	23.61	442	C ₂₂ H ₄₅ Cl ₃ Si	Metabolite observed in Cancer metabolism (34)	
1-hexacosanol	23.61	382	C ₂₆ H ₅₄ O	Natural agents for mosquito repellents (35)	
2-hexyl-1-Octanol	23.61	214	C ₁₄ H ₃₀ O	Antioxidant and Anti-inflammatory properties (36)	
2-piperidinone, N-[4-Bromo-N-Butyl]-	23.61	233	C ₉ H ₁₆ ONBr	Antimicrobial (25, 37)	
17-Pentatriacontene	28.77	490	C ₃₅ H ₇₀	Antimicrobial (38)	
Oleic acid	23.59	282	C ₁₈ H ₃₄ O ₂	Anticancer, Autoimmune and inflammatory diseases (39, 40)	
Squalene	30.64	410	C ₃₀ H ₅₀	Anti-tumor, oxygen scavenging activities, antimicrobial (41, 42)	
Trans-farnesol	30.64	222	C ₁₅ H ₂₆ O	Antimicrobial natural compound, Antioxidant (27-29)	
9-Eicosenoic acid, (z)-	23.59	310	C ₂₀ H ₃₈ O ₂	Belongs to a class of omega-3 fatty acids possessing potential health-promoting attributes (43)	

Assessment of Flavonoids in the Extracts

The HPTLC chemical profiling revealed the presence of metabolites such as flavonoids and stigmasterol in various solvent leaf extracts of *C. macroptera*. The presence of flavonoids in *C. macroptera* has been previously reported from the fruit, peel, and juice but this is the first report depicting the presence of flavonoids in the *in vitro* and *ex vitro* leaves extracts of *C. macroptera*.

The appearance of the greenish-blue fluorescent bands at 366 nm after derivatization confirms the presence of flavonoids in all extracts, CML(MeOH), CML (IN MeOH), and CML(Aq) (Fig. 5).

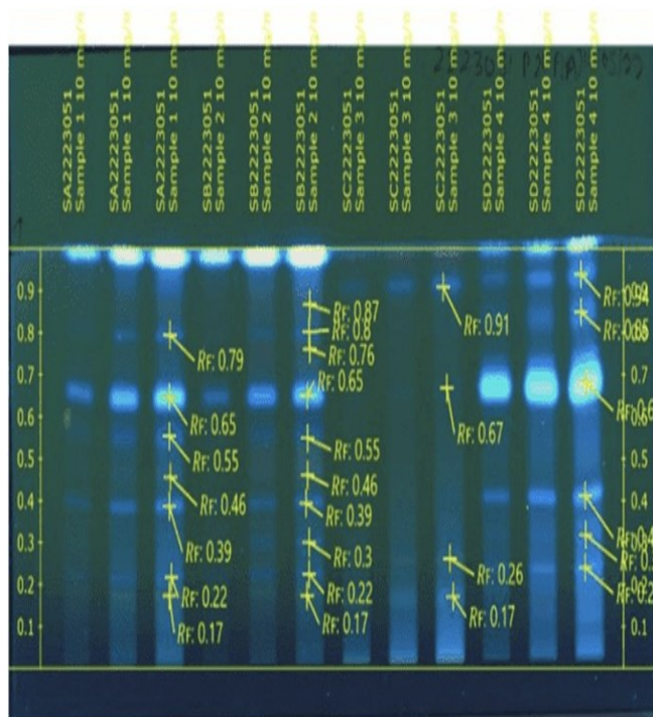


Fig. 5. HPTLC chromatogram after derivatization by NP reagent followed by PEG at 366nm represents the presence of flavonoids (Track 1-3: CML (MeOH); Track 4-6: CML (EtOH); Track 7-9: CML (Aq); Track 10-12: CML (IN MeOH))

In situ HPTLC-DPPH assay

HPTLC-DPPH assay was performed to examine the *in-situ* antioxidant activity of the extracts by dipping them in DPPH solution. The appearance of white fluorescence in all the tracks after derivatization under white light explicitly confirms the antioxidant activity exhibited by the extracts (Fig. 6). The chromatographic plate also shows varying band intensities in different tracks. The standards exhibited stronger band intensities compared to the test extracts. The number of bands in the CML (Aq) was the least resolved, whereas, in all other extracts, better band resolution was observed. Therefore, our study is the first report employing this rapid (high throughput) screening for the evaluation of metabolites exhibiting antioxidant potential from the leaf extract of *C. macroptera*.

Table 2. The percentage viability of the SH-SY5Y cells from the *C. macroptera* leaf extracts was determined by MTT assay after 24 h exposure.

Sample	Concentration(µg/ml)					IC ₅₀ (µg/ml)
	25	50	100	150	200	
Stigmasterol	74.19%	73.50%	50.69%	42.80%	26.67%	117.68
CML (IN MeOH)	99.06%	92.02%	87.56%	46.83%	39.79%	167.71
CML(MeOH)	96.09%	98.97%	96.48%	72.30%	47.08%	216.39

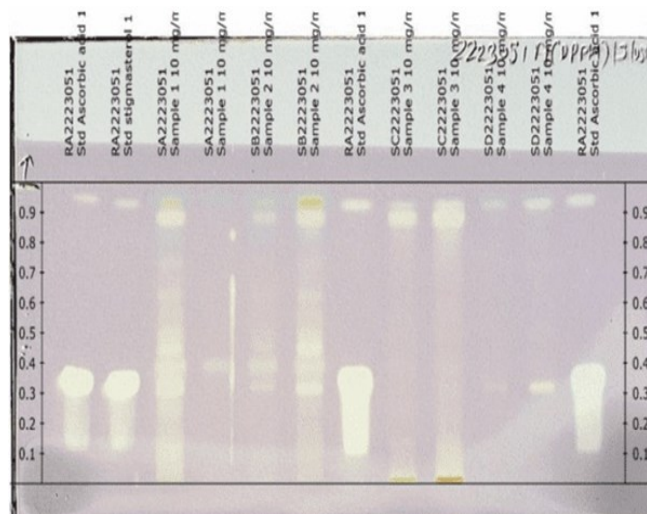


Fig. 6. HPTLC chromatogram profile after post chromatographic derivatization with DPPH reagent under white light (Sample 1-CML(MeOH); Sample 2-CML(EtOH); Sample 3-CML(Aq); Sample 4- CML (IN MeOH))

Cytotoxicity Assessment

The cytotoxic effect of CML (MeOH) and CML (IN MeOH) against the human neuroblastoma, SH-SY5Y cell lines was expressed as the percentage of relative viability when compared to the untreated cells (Table 2). The result reveals the morphological alterations of the cells treated with extracts (Fig. 7) and (Fig. 8). Stigmasterol exhibited the highest toxicity. As it was evident the % of cell viability decreases with increased dosage of the extracts. However, the CML (IN MeOH) had better efficacy in contrast to CML (MeOH), i.e., at the concentration of 200 µg/ml, a high degree of cytotoxicity to the SH-SY5Y cells from CML (IN MeOH) and CML (MeOH) with cell viability % of 39.79% and 47.08% respectively was obtained.

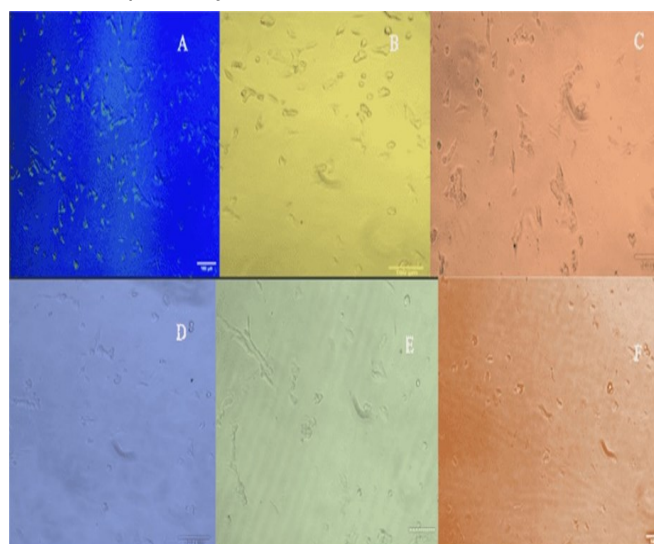


Fig. 7. Representative images of the dose-dependent effect of CML (IN MeOH) on the morphology of SH-SY5Y cells (magnification X100): A) Untreated B) 25µg/ml

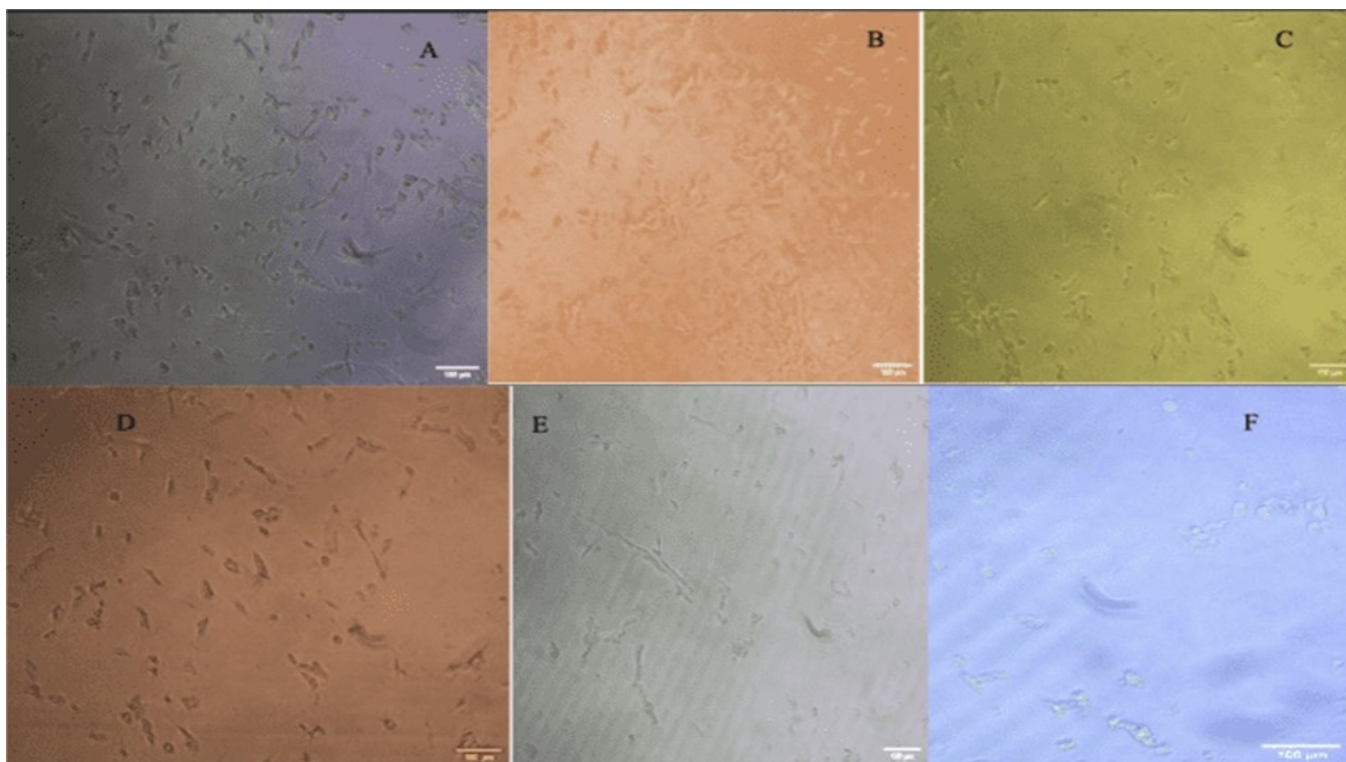


Fig. 8. Representative images of the dose-dependent effect of CML(MeOH) on the morphology of SH-SY5Y cells (magnification X100): A) Untreated B) 25µg/mL C) 50 µg/mL D) 100 µg/mL E) 150 µg/mL F) 200 µg/mL

Discussion

The bioactive compounds and polyphenols can be recovered by an appropriate solvent system (21). The abundance of natural bioactive compounds, exhibiting remarkable therapeutic attributes present in medicinal and aromatic plants has been known from time immemorial (22, 23). It has been reported that esters have several commercial and pharmaceutical applications (24). 2-Piperidinone, N-[4-Bromo-N-Butyl]-, 17-Pentatriacontene, Squalene found in the *in vitro* leaves extract have the potential to exhibit antimicrobial activity (25, 26). Farnesol, another compound found in the leaf extract can exhibit anti-inflammatory properties in dealing with asthma, gliosis, and edema (27-29). There is a possibility of bioactive component enhancement during *in vitro* regeneration of *C. macroptera* (30, 31).

HPTLC is a simple and precise method of identification for the marker compound *viz* flavonoids stigmasterol, terpenoids, lupeol, etc. (32). Previous studies have been reported using the HPTLC method to evaluate the neuroprotective and antidiabetic activity of the essential oils of the Lamiaceae family (33). It is vital to characterize the antioxidant activity using several approaches because different antioxidant assays produce a range of results (34). In this study, different *C. macroptera* leaf extracts were investigated using DPPH radical scavenging activity and showed varying band resolutions exhibiting antioxidant potential. When the DPPH solution is exposed to the tested materials, it donates a hydrogen atom, resulting in the reduction of the compound to diphenylpicrylhydrazine (35).

The cytotoxicity of the leaf extracts (*in vitro* and *ex vitro*) of *C. macroptera* has not been studied so far. The previous studies conducted utilizing the fruit extracts

using Brine shrimp lethality bioassay LC₅₀ value of 30.90 µg/ml was recorded (36). In our study, the CML (IN MeOH) was more cytotoxic against the human neuroblastoma cells (SH-SY5Y). The antiproliferative activity conducted with different cell lines, A549, MCF-7 and HepG2 and on normal C2C12 mouse myoblast cell line using the *C. macroptera* peel extract with IC₅₀ values for 24 h were 284.289 ± 84.978 µg/ml, 1313.982 ± 202.293 µg/ml, 1760.486 ± 95.202 µg/ml and 1506.954 ± 72.383 µg/ml respectively (6). The current study shows the opportunity for *C. macroptera* leaf extracts to be developed as an anti-cancer candidate against the SH-SY5Y cell line, the IC₅₀ values for 24 h in CML (IN MeOH) and CML (MeOH) were recorded to be 167.71µg/ml and 216.39µg/ml respectively. Therefore, *C. macroptera* leaf extracts can be further explored to identify the neuroprotective potential and can plausibly be utilized in the therapeutic industries.

Conclusion

The GC-MS and HPTLC metabolite profiling of the leaf extracts of *C. macroptera* has revealed a variety of phytoconstituents having diverse pharmacological importance. The study also validates the antioxidant potential of the extracts by DPPH-HPTLC analysis. Cell death was evidenced by the cytotoxic effects of the leaf extracts on SH-SY5Y cell lines in a dose-dependent manner and CML (IN MeOH) showed better efficacy than CML (MeOH). Therefore, the current findings provided preliminary data of the *in vitro* and *ex vitro* leaf extracts of *C. macroptera* which can further be utilized for demystification of lead compounds exhibiting anti-cancerous and neuroprotective potential.

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Authors' contributions

RS contributed to the experimentation, data analysis, interpretation, and manuscript preparation. SKS aided in the collection of samples. SS and DPK were involved in the conception, design, and overall reviewing and editing of the manuscript. We ensure and hereby declare that all authors have read and approved the manuscript.

Compliance with ethical standards

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