

Chemical Characterization, Antimicrobial, Antiproliferative and Antioxidant Activities of the Essential Oil of *Monarda citriodora* Growing in Kashmir

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ABSTRACT

Objectives: The main aim of this research was to evaluate phytochemicals, antimicrobial, antiproliferative and antioxidant Activities of the essential Oil of *Monarda citriodora* growing in Kashmir (India). **Materials and Methods:** Essential oil obtained by hydrodistillation from the flowers of *Monarda citriodora* was analysed by a combination of capillary gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID) techniques. The essential oil was evaluated for its antimicrobial and antioxidant potential. Antimicrobial activity was evaluated against two fungal and seven bacterial strains. The antiproliferative efficacy was evaluated by Sulphorhodamine-B assay. **Results:** A total of 13 components were identified constituting 99.4% of the total oil composition. Thymol (79.7%), P-cymene (7.7%), Tricyclene (4.5%) and carvacrol (3.0%) were found to be the major components. The oil composition was totally dominated by oxygenated monoterpenes constituting 83.2%. Additionally, potent and broad-spectrum of antimicrobial activities were shown by the essential oil. *In vitro* cytotoxic studies revealed concentration dependent growth inhibition of all target cancer cell lines in the 10-100 µg/ml dilution range, with IC₅₀ values of 24.2µg/ml (THP-1), 55.5µg/ml (A-549), 31µg/ml (HEP-2) and 37.5µg/ml (Caco-2). Antioxidant studies of the essential oil

evaluated by two complementary assays viz DPPH and hydroxyl radical assays confirmed that the oil exhibited potent and dose-dependent inhibition of the DPPH and hydroxyl radicals with IC₅₀ values of 19.3µg/ml and 39.2µg/ml respectively. **Conclusion:** We concluded that the essential of *Monarda citriodora* growing in Kashmir is rich in chemical composition and possess significant antimicrobial, antioxidant and antiproliferative activities.

Key words: *Monarda citriodora*, GC-MS, Antibacterial activity, antiproliferative activity, Essential oil

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INTRODUCTION

In addition to being ineffective in many cases due to emergence of resistant microbial strains, Present antibiotic therapies are frequently accompanied by a large number of toxic side effects,¹ These resistant strains such as methicillin-resistant *S. aureus* (MRSA), Vancomycin-resistant Enterococci (VRE) and multi drug-resistant strains of *K. pneumonia* and *P. aeruginosa* give birth to nosocomial infections which are characterized by high morbidity and mortality rates and hence pose a serious problem worldwide.²⁻⁴ Novel and sustainable antimicrobials are crucial in solving this problem. Plant extracts and essential oils, which represent natural complex multi-component systems composed mainly of terpenes in addition to some other non-terpene components, have always had choice of use for different purposes.^{5,6} Plant essential oils have been screened for their antimicrobial, carminative, antioxidant, spasmolytic, antiviral, hepato-protective, and anti-cancer properties.⁷⁻¹¹ A great concern in studying antimicrobial properties of essential oils is emergence of antibiotic resistance. These detailed investigations culminated into results depicting significant antibacterial activity of many plant essential oils against a range of resistant microbial strains.¹²⁻¹⁴ This special property of essential oils, being effectively active in resistant strains is governed by their diverse chemical profile which as a tool empowers essential oils to be multi target oriented in their action against various infections.

In addition to being anti-microbial and antioxidant in nature, some essential oils along with their constituents have been reported to exert

both *in-vitro* and *in-vivo* anti-tumour activity against human breast adenocarcinoma cell lines, colon neuroblastoma, lung cancer cell lines, oral carcinoma (KB) murine leukemia (P388) and melanoma cells.¹⁵⁻¹⁹ Further, the chemotherapeutic values of some essential oil components against cancer cells have been under scrutiny in Phase I clinical trials which authenticate their anti-tumour activity.²⁰

Monarda citriodora var. *citriodora*, which is native to North America and naturalized in Europe, is an annual herbaceous plant. Common names of this plant include purple horsemint, lemon mint, and lemon bee balm.²¹ Plants of *Monarda* species have been used as garden plants, flavoring and food additives and for medicinal purposes. Oils isolated from plants of the same species grown in diverse geographical areas may be very different. Reminiscent odor of lemons is evolved on crushing its leaves.

Reviewing the potential ethnomedicinal promises of this plant, we have undertaken a research programme to investigate not only volatile chemical composition of essential oil but also screen it for various biological activities like those of antimicrobial, antioxidant and cytotoxicity which as per literature survey constitutes the first documentary report on this plant.

MATERIALS AND METHODS

Chemicals

DPPH (2,2-diphenyl-1-picryl hydrazyl), dry ferric chloride, dimethyl sulfoxide, L- ascorbic acid and methanol, were procured from Sigma (St. Louis, MO). All the chemicals/solvents used were analytical grade.

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Plant material and Essential oil extraction

The aerial part of *Monarda citriodora* was collected from Gulmarg (Kashmir) during July-2019. Five kilograms of the fresh plant material in six installments was subjected to hydrodistillation using a Clevenger type apparatus for 3 hr according to the procedure described in the European Pharmacopoeia. To eliminate the moisture essential oil was treated with anhydrous sodium sulphate and then stored at 4°C. Calculation of the yield was performed on fresh weight basis and was found to be 0.50 % (V/W).

Gas Chromatography/Flame Ionization Detector and Gas Chromatography Mass Spectrometry analysis

GC/FID was carried out on Perkin Elmer auto system XL Gas Chromatograph 8500 series equipped with flame ionization detector (FID) and head space analyzer using a fused silica capillary RTX-5 column (30m x 0.32 mm, film thickness 0.25µm) coated with dimethyl polysiloxane. Oven temperature was programmed from 60°C to 280°C and varied at a rate of 3°C/min. Injector temperature was maintained at 230°C and detector temperature 250°C. Injection volume of 1µl was used and nitrogen was used as a carrier gas with flow rate of 1.0 mL/min. GC-MS analysis was carried on a Varian Gas Chromatograph series 3800 fitted with a VF-5 ms fused silica capillary column (60m x 0.25mm, film thickness 0.25µm) coupled with a 4000 series mass detector under the following conditions: injection volume 0.5 µl with split ratio 1:60, helium as carrier gas at 1.0 mL/min constant flow rate, injector temperature 230°C oven temperature 60°C/min. Mass spectra: electron impact (EI+) mode, 70 eV and ion source temperature 250°C. The a.m.u range of 50-500 a.m.u was set to record the mass spectra.

Identification of various constituents was done on the basis of Retention Index (RI), co-injection with standards (Sigma Aldrich and standard isolates), MS Library search (NIST 05 and WILEY), by comparing with the MS literature data.^{22,23} The individual components relative percentages were measured without using correction factors based on GC peak area (FID response).

Microbial Strains, Antimicrobial Activity Assay and Minimum Inhibitory Concentrations

The antimicrobial activity of essential oil of *Monarda citriodora* was tested against seven aerobic, Gram positive and Gram negative bacterial strains and two fungal strains which account for the major group of pathogens. The bacterial and fungal strains against which activity was assessed included *Bacillus subtilis* MTCC-441, *Staphylococcus epidermidis* MTCC-435, *Pseudomonas aeruginosa* MTCC-1688, *Staphylococcus aureus* MTCC 96, *Salmonella typhi* MTCC-531, *Shigella dysenteriae* MTCC-369, *Klebsiella pneumonia* MTCC-19, *Penicillium chrysogenum* MTCC 947 and *Aspergillus niger* MTCC 1344 and were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. All the bacterial strains were sub-cultured on Muller-Hinton agar (Himedia) slants every fifteen days to maintain the bacterial viability. Test conditions were in full agreement with CLSI Protocol M7A6 requirements.

Antibacterial activity of essential oil of *Monarda citriodora* was determined by agar well diffusion method. Bacterial strains were grown overnight at 37°C in Mueller-Hinton Broth (Himedia). 100µl of standardized inoculum (0.5 Mac-Farland) of each strain was inoculated on molten Muller-Hinton agar, homogenized and poured into sterile 90 mm petri dishes and were allowed to solidify under laminar air flow. *Standard cork borer* of 5mm diameter was used to make wells, into which 50µl essential oil dissolved in DMSO was added. Vancomycin and Ampicillin (10µg/disc) were used as positive control and DMSO alone as negative control.

A laboratory film was used to cover each petri dish to evade evaporation followed by incubation at 37 ± 1°C for 24 hr. Finally, a standard scale was used to mark the inhibition zone to the nearest size.²⁴ Each experiment was replicated in triplicates and data was revealed as mean ± SD.

Minimum inhibitory concentrations were obtained using broth dilution method.²⁵ Desired concentrations (0.125-625 µg/ml) were generated by diluting each sample in sequence with Muller Hinton broth. Bacterial strains were adjusted to 0.5 McFarland standard turbidity. Each sample was stirred and inoculated with 50 µl of tested bacterial suspension containing 2 × 10⁵ CFU/ml and incubated at 37± 1°C for 24 hr. The lowest concentration at which no evident growth was seen post incubation in test bacteria was considered as MIC value.

Antioxidant activity by Free radical-scavenging and Hydroxyl radical scavenging methods

Antioxidant properties of *Monarda citriodora* essential oil were evaluated as both free radical scavenging capacity (RSC) and protective effect on the lipid peroxidation (LP). Free radical scavenging activity of the oil was assessed by evaluating the scavenging potency (Table 1) on 1-diphenyl-2-picryl hydrazyl radical (DPPH).¹² A 0.5 mM solution of DPPH and a stock solution of sample (1mg/ml) in methanol were prepared. Different concentrations of essential oil (10-100 µg/ml) were added to 1 ml (0.5 mM DPPH) and final volume was made to 3ml with methanol. The mixture was shaken vigorously and kept standing at room temperature for 10 min. Then the absorbance of the mixture was measured at 517 nm on spectrophotometer. The decrease in the absorbance indicates an increase in DPPH-radical scavenging activity. The percentage inhibition was calculated by the following equation.

$$\text{DPPH radical scavenging (\%)} = \left[\frac{A_c - A_s}{A_c} \right] \times 100$$

Where: A_c is the absorbance of control, A_s is absorbance of sample.

Table 1: Chemical profile of the essential oil from the flowers of *Monarda citriodora*.

| S. No | Compound | % Peak Area | Methods of identification |
|-------|----------------------------|-------------|---------------------------|
| 1 | α-Thujene | 0.2 | MS, RI |
| 2 | α-Pinene | 1.0 | MS, RI |
| 3 | β-Pinene | 0.9 | MS, RI |
| 4 | α-Phellandrene | 0.1 | MS, RI |
| 5 | α-Terpinene | 2.5 | MS, RI |
| 6 | P-Cymene | 6.7 | MS, RI, |
| 7 | Limonene | 0.2 | MS, RI |
| 8 | Tricyclene | 4.5 | MS, RI, |
| 9 | 4-Terpeneol | 0.1 | MS, RI |
| 10 | Methyl carvacrol | 0.4 | MS, RI |
| 11 | Thymol | 76.7 | MS, RI, Std |
| 12 | Carvacrol | 6.0 | MS, RI, Std |
| 13 | β-Caryophyllene | 0.1 | MS, RI |
| | Total (%) | 99.4 | |
| | Monoterpene hydrocarbons | 16.1 | |
| | Oxygenated monoterpenes | 83.2 | |
| | Sesquiterpene hydrocarbons | 0.1 | |
| | Oxygenated sesquiterpenes | nil | |

The experiment was done in triplicate. IC_{50} value was calculated and indicated the concentration to scavenge 50% of DPPH free radicals.

The scavenging activity was performed by calculating the competing the essential oil and deoxyribose for hydroxyl radicals forming in Fenton reaction. Thiobarbituric acid reactive substances (TBARS) are formed when hydroxyl radicals get degraded by deoxyribose which can be spectroscopically measured at 532 nm.¹² The reaction mixture containing 25 mM deoxyribose, 10 mM ferric chloride, 100 mM ascorbic acid, 2.8 mM H_2O_2 in 10 mM KH_2PO_4 (pH 7.4) and various concentrations of essential oil of *Monarda citriodora*. The reaction mixture was incubated at 37°C for 1h. Then 1 ml each of 3% trichloroacetic acid and 1.6% thiobarbituric acid were supplied to the mixture followed by heating at 100°C for 20 min. The TBARS was measured spectrophotometrically at 532 nm. The results were expressed as percentage inhibition of deoxyribose oxidation and are determined by the following mathematical equation.

$$\% \text{ Inhibition} = [(A - B)/A] \times 100$$

Where: malonaldehyde generated in Fenton reaction is represented by "A" and malonaldehyde generated in presence of essential oil is represented by "B".

Anticancer activity

Human cell lines and culture

Optimum density of seeded cell suspension was plated to each compartment of 96-well plates (Iwaki) and exposed to various essential oil concentrations ranging from 10-100µg/ml. Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2 . In the cultured RPMI-1640 medium, standards which included 5-Fluoro uracil and Mitomycin-C (Sigma-Aldrich, Madrid, Spain) were used. The cells were incubated with sample for 48 hr and fixed in cold ice TCA at 4°C for 1.5 h. Plates were washed with distilled water followed by drying in open air in absence of light. Sulpharhodamine-B (SRB) solution (0.4%) was added to each well of 96-well plates and allowed to stain at room temperature for 30 min. The unbound SRB solution was removed by washing the plates quickly with 1% (v/v) acetic acid. The bound SRB dye was solubilised by adding 100 µl of 10 mM unbuffered Tris base (PH = 10.5) to each well and shaken for 5 min on shaker platform. The plates were read in a 96-well plate reader at 540 nm.

Cytotoxicity assay

Sulpharhodamine-B assay was performed against four different human cancer cell lines which included Caco-2 (colon), HEP-2 (liver), A-549 (lung), and THP-1 (leukemia). An increase in growth of inhibition during 48 h incubation at all concentrations of 10, 20, 40, 60, 80 and 100 µg/ml of essential oil was seen. DMSO was set up separately as negative control to check cell death occurred by DMSO alone, which acted as solvent for dissolving samples homogeneously. Results were expressed as concentration of drug suppressing 50% cell growth (IC_{50}) and a significant cellular cytotoxic effect due to different essential oil concentrations was seen on different human cancer cell lines of varying tissue origin

Statistical analysis

Each separate experiment was executed in triplicate replicas, and mean values were calculated. The data were recorded as means \pm standard deviations. Analysis of variance for individual parameters was performed on the basis of mean values to find out the significance at $p < 0.05$.

RESULTS

Chemical composition of the essential oil

The various chemical constituents identified in the essential oil of *Monarda citriodora* by making use of GC-MS technique are listed in Table 1, in order of their elution from an RTX-5 column. The GC-MS total ion chromatogram (TIC) of the essential oil is shown in Figure 1. A total of 20 constituents were identified from the essential oil comprising 91.9 % of overall oil composition. The oil composition is dominated by the presence of monoterpene hydrocarbons and oxygenated sesquiterpene hydrocarbons accounting for 59.25 % and 20.81 % of the total oil composition respectively. Thymol, p-cymene, and carvacrol were identified as the dominant compounds.

Antibacterial activity

The antimicrobial efficacies of *Monarda citriodora* essential oil were evaluated against a set of seven bacterial strains including both Gram +ve and Gram -ve strains and two fungal strains. Potency of essential oil against microbes was assessed qualitatively and quantitatively by the presence or absence of zone diameters (ZD_s), inhibition zones, and respective MIC values. The results demonstrated in Table 2 represent that this essential oil displayed a variable degree of antimicrobial activity. *Staphylococcus epidermidis* MTCC-435, *P. aeruginosa* and *S. typhi* were the most sensitive bacterial strains to the essential oil. *B. subtilis* and *K. pneumonia* were moderately sensitive while as *S. dysenteriae* and *S. aureus* were least sensitive towards essential oil of *Monarda citriodora*. In case of antifungal activity essential oil was appreciably potent towards both the fungal strains. Antimicrobial efficacies of essential oil were further compared with that of standard antibiotics by disc diffusion method.

Antioxidant activity

DPPH free radical scavenging activity

Figure 2 shows the scavenging effect of essential oil of *Monarda citriodora* on the DPPH radical. DPPH is a stable radical that loses its purple colour when it undergoes reduction by accepting an electron from an antioxidant molecule. Ascorbic acid was considered as reference compound. A comparable and dose-reliant scavenging effectively was shown by the oil to that of reference standard (ascorbic acid) at higher concentrations. The IC_{50} value of the essential oil was found to be 19.3µg/ml.

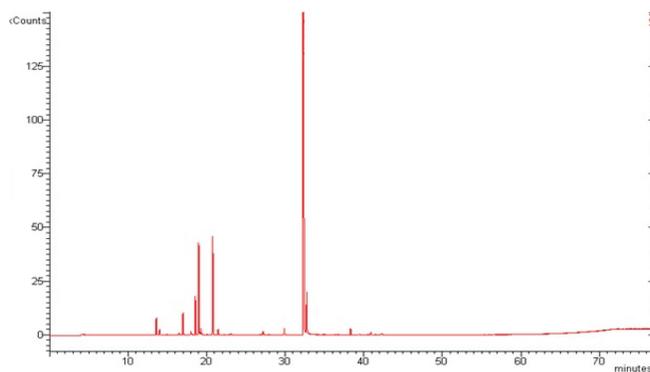
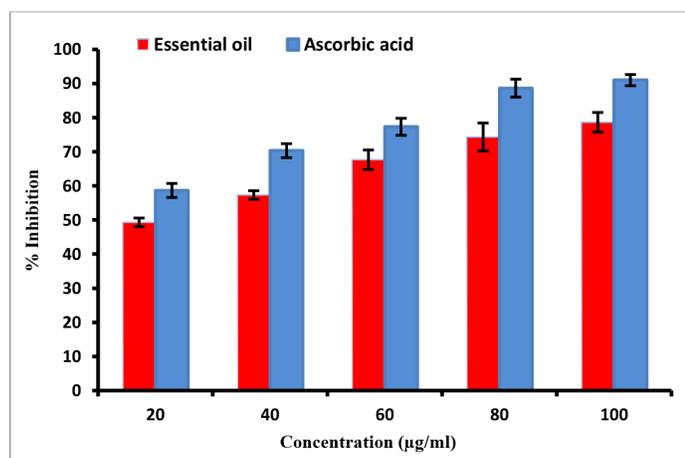


Figure 1: GC-MS Total Ion Chromatogram (TIC) of the flower essential oil of *Monarda citriodora*.

Table 2: Antimicrobial activity of the essential oil of *Monarda citriodora* (MIC and MBC values).

| S.NO | Micro-organism | Essential oil($\mu\text{g/ml}$) | | Ampicillin | | Vancomycin | | Fluconazole | |
|------|-----------------------|-----------------------------------|-----|------------|-----|------------|------|-------------|-----|
| | | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| 1 | <i>B. subtilis</i> | 64 | >64 | 0.5 | >1 | 0.5 | 1 | NT | NT |
| 2 | <i>S. epidermidis</i> | 16 | >16 | 2 | 4 | 1 | 2 | NT | NT |
| 3 | <i>P. aeruginosa</i> | 16 | 32 | 2 | 4 | 2 | 4 | NT | NT |
| 4 | <i>S. aureus</i> | 8 | 16 | 0.5 | 1 | 2 | 4 | NT | NT |
| 5 | <i>S. typhi</i> | 16 | >32 | 4 | >4 | 0.5 | >0.5 | NT | NT |
| 6 | <i>S. dysenteriae</i> | 16 | >32 | 4 | 8 | 1 | 2 | NT | NT |
| 7 | <i>K. pneumonia</i> | 32 | >64 | 1 | >2 | 0.5 | 1 | NT | NT |
| 8 | <i>P. chrysogenum</i> | 32 | 64 | NT | NT | NT | NT | 0.5 | 1 |
| 9 | niger | 32 | >64 | NT | NT | NT | NT | 0.5 | >1 |

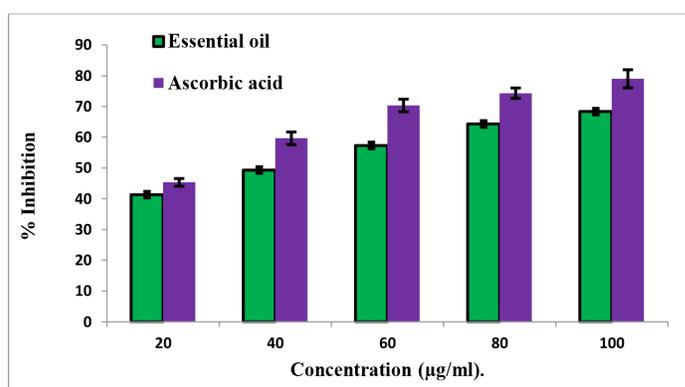
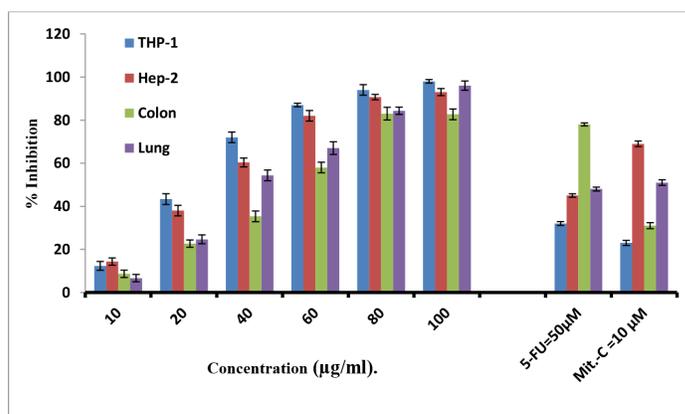
**Figure 2:** Antioxidant activity of the essential oil of *Monarda citriodora* as evaluated by DPPH radical scavenging assay. All the values are mean \pm SEM (n =3).

Hydroxyl radical scavenging activity

In the present study (Figure 3), hydroxyl radical scavenging potential of essential oil was estimated by generating hydroxyl radicals using ascorbic acid-iron- H_2O_2 (Fenton reaction) and their subsequent scavenging in presence of essential oil samples. The antioxidant efficacy of essential oil was found to be comparable to the reference standard (ascorbic acid). This ability of the essential oil to quench hydroxyl radicals could be coordinated to the prevention of lipid peroxidation, carcinogenesis and mutagenesis. The IC_{50} value was found to be $39.2 \mu\text{g/ml}$. subsequent scavenging in presence of essential oil samples. The antioxidant efficacy of oil was found to be comparable against reference standard (ascorbic acid). This ability of the essential oil to quench hydroxyl radicals could be coordinated to the prevention of lipid peroxidation, carcinogenesis and mutagenesis. The IC_{50} values of the essential oil was found to be $39.2 \mu\text{g/ml}$. oil to quench hydroxyl radicals can be related to the prevention of lipid peroxidation, carcinogenesis and mutagenesis. The IC_{50} value was determined to be $39.2 \mu\text{g/ml}$. subsequent scavenging in presence of essential oil samples. The antioxidant activity of the essential oil was found to be comparable to the reference standard (ascorbic acid). The IC_{50} values of the essential oil was found to be $39.2 \mu\text{g/ml}$.

Cytotoxic activity

The cytotoxic potential of the *Monarda citriodora* essential oil was studied using cultured Caco-2, Hep-2, A-549, and THP-1 cancer cell lines by

**Figure 3:** Antioxidant activity of the essential oil of *Monarda citriodora* as evaluated by hydroxyl radical scavenging assay. All the values are mean \pm SEM (n =3).**Figure 4:** Cytotoxic activity of essential oil against human cancer cell lines. All the values are mean \pm SEM (n =3).

sulphorhodamine-B assay. The cell lines were subjected to increasing dose of the essential oil ranging from 10-100 $\mu\text{g/ml}$ for 48 hr which reduced the viability of these cell lines. Figure 4 shows that the oil was significantly active against all the four tested cell lines. The percentage of the dead cells in case of THP-1 (leukemia), Hep-2 (liver), Caco-2 (colon) and A-549 (lung) was found to be 99%, 93%, 85% and 97% respectively for the essential oil concentration of 100 $\mu\text{g/ml}$. The essential oil exhibited a dose-dependent inhibitory effect on all cell lines tested in the 10-100 $\mu\text{g/ml}$

dilution range (Figure 2). The IC₅₀ values of the essential oil were found to be 24.2µg/ml (THP-1), 55.5µg/ml (A-549), 31µg/ml (HEP-2) and 37.5µg/ml (Caco-2).

DISCUSSION

Medicinal and aromatic plants are important and valuable gifts of nature. They are botanical raw materials and also named as herbal drugs. Medicinal and aromatic plants are principally incorporated in aromatic, therapeutic and culinary purposes as modules of medicinal products, healthy foods, cosmetics and natural products.²⁶ They also act as starting material in value-added processed natural ingredients including dry and liquid extracts, essential oils and oleoresins.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) production is continuously balanced by natural antioxidant defence system, but with aging this defence system becomes weak and as such some other alternative has to be there which should be strong enough to take care of the in significant natural antioxidant potential.²⁷ Consumption diet rich in antioxidant content is always a vital strategy in regulating the oxidation of desired vulnerable substrates present in cells and hence results in prevention of a number of disorders relevant to this change. Flavonoids, phenolic acids, tannins and essential oils have been well documented to possess antioxidant activity and thus represent an important choice to be used in case of resulting circumstances. Hydroxyl radical is known for its extremely reactive nature in biological systems with capacity of causing fragmentation of genetic material hence contributes to carcinogenesis, mutagenesis and cytotoxicity. In order to avoid a major toll to DNA damage there should be some systems either in built or transported from outside at the time of urgency which are capable of quenching these super reactive species. From literature survey, mono and sesqui terpenes which form the bulk of essential oils are gifted with such properties. This ability of the essential oil to quench hydroxyl radicals can be related to the prevention of lipid peroxidation, carcinogenesis and mutagenesis.²⁸ The essential oil obtained from under investigation plant showed high variety of chemical composition responsible for its phenomenal antioxidant potential.

Different nature of interactions between receptors and essential oil constituents, permeability and diffusion of various hydrophobic agents across the cell membrane etc. may influence the extent of the microbial susceptibility. Herein, we found that *Monarda citriodora* essential oil possesses significant antimicrobial activity which may be attributed to its chemical constituents identified.

Moreover, it has been well documented that essential oils obtained from medicinal plants show efficient antiproliferative activities against a broad spectrum of human cancer cell lines.²⁹ Medicinal plants and their derived phytochemicals have been a backbone for current day pharmacy and several chemotherapeutic agents from plant source have been already approved and some are in clinical trials.³⁰ In the present research, we investigated the antiproliferative activity of the *Monarda citriodora* essential oil against Caco-2 (colon), HEP-2 (liver), A-549 (lung), and THP-1 (leukemia) cells. The results showed efficient inhibitory effects for the *Monarda citriodora* essential oil against these selected target cell lines.

CONCLUSION

The current study revealed potential antimicrobial, antiproliferative and antioxidant activities of *Monarda citriodora* essential oil can be of immense importance to devise further experiments for standardizing the activity profile of this essential oil so that it can find potential applications in pharmaceutical industries for curbing microbe born infectious diseases.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest to indicate.

ABBREVIATIONS

ROS: Reactive Oxygen Species; **RNS:** Reactive Nitrogen Species; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **GC-FID:** Gas Chromatography Flame Ionization Detection; **GC-MS:** Gas Chromatography-Mass Spectrometry.

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