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Original Research Paper

Bioactive Potential of *Rosmarinus officinalis* Essential Oil: Phytochemical Profiling and In Vitro Antioxidant and Anti-Inflammatory Activities

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Abstract: *Rosmarinus officinalis* L. (syn. *Salvia rosmarinus*), commonly known as rosemary, is a medicinally important aromatic plant rich in bioactive volatile constituents with potential antioxidant and anti-inflammatory properties. The present study evaluated the phytochemical composition, antioxidant activity, cytotoxicity, and in vitro anti-inflammatory potential of *R. officinalis* essential oil. Fresh leaves were collected, authenticated, shade-dried, and subjected to hydrodistillation using a Clevenger-type apparatus. The obtained essential oil was characterized by GC–MS, while antioxidant activity was evaluated using the DPPH radical-scavenging assay. Cytotoxicity was assessed in RAW 264.7 macrophage cells using the MTT assay, and anti-inflammatory activity was evaluated through inhibition of protein denaturation. GC–MS analysis identified several volatile constituents, including α-pinene, limonene, 1,8-cineole, linalool, camphor, borneol, α-terpineol, eugenol, β-caryophyllene, α-humulene, germacrene D, and caryophyllene oxide. Eugenol (43.52%) and β-caryophyllene (19.51%) were the predominant constituents, suggesting their possible contribution to the biological activity of the essential oil. In the DPPH assay, the essential oil exhibited moderate antioxidant activity with an IC_{50} of 136.5 ± 0.05 µg/mL compared with 2.502 ± 0.02 µg/mL for ascorbic acid. The MTT assay indicated moderate cytotoxicity toward RAW 264.7 cells, with an IC_{50} of 101.5 ± 0.11 µg/mL. In the protein denaturation assay, the essential oil demonstrated moderate inhibitory activity with an IC_{50} of 195.3 ± 0.07 µg/mL compared with 97.26 ± 0.01 µg/mL for diclofenac.

Keywords: Antioxidant, Anti-inflammatory, Cytotoxicity, Macrophage, Essential oil, MTT assay.

Introduction- *Rosmarinus officinalis* L. (syn. *Salvia rosmarinus*), commonly known as rosemary, is an aromatic medicinal plant belonging to the family Lamiaceae and has been traditionally used as a culinary herb and herbal medicine. The plant is characterized by a rich phytochemical profile comprising phenolic diterpenes, phenolic acids, flavonoids, triterpenes, and volatile constituents present in its essential oil. Among these bioactive compounds, 1,8-cineole, α-pinene, camphor, borneol, verbenone, and camphene are frequently reported as important constituents of rosemary essential oil. The chemical composition of the oil can vary considerably depending on geographical origin, plant maturity, extraction technique, and environmental conditions [1,3,5]. Inflammation is a complex biological response involving the coordinated activation of inflammatory cells and mediators such as cytokines, prostaglandins, nitric oxide (NO), and reactive oxygen species (ROS). Excessive or persistent activation of inflammatory pathways is associated with several chronic disorders. Consequently, identification of plant-derived compounds capable of modulating inflammatory mediators has attracted considerable interest in natural-product research. Essential oils are particularly promising because their low-molecular-weight volatile constituents can interact with multiple cellular and molecular targets. The

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anti-inflammatory activity of *R. officinalis* essential oil has been associated with its ability to modulate oxidative stress and inflammatory signaling pathways. Its major monoterpenes and oxygenated monoterpenes may contribute to inhibition of inflammatory mediators, reduction of ROS generation, and modulation of pathways involving NF-κB, cyclooxygenase (COX), lipoxygenase (LOX), inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines. In addition, phenolic constituents such as rosmarinic acid, carnosic acid, and carnosol, although their abundance in essential oil differs from that of nonvolatile extracts, provide an important phytochemical basis for the broader anti-inflammatory potential of rosemary [1,2]. Therefore, systematic evaluation of *R. officinalis* essential oil, combined with phytochemical characterization and correlation of individual constituents with anti-inflammatory activity, may provide valuable insight into its therapeutic potential. The present study can consequently focus on establishing a relationship between the essential oil's chemical composition and its ability to inhibit inflammatory responses, thereby supporting its potential application as a natural source of anti-inflammatory agents [5].

% RSA= (ABS control-Abs Sample)/Abs control) x100

Methodology

Plant Material Collection and Identification- Fresh, healthy leaves of *Rosmarinus officinalis* L. were collected from the medicinal plant garden of Maharana Pratap College of Pharmacy, Kanpur, India. The plant material was authenticated by the Department of Pharmacognosy, Maharana Pratap College of Pharmacy, and a voucher specimen was prepared and deposited in the departmental herbarium for future reference. The collected leaves were carefully inspected and freed from adhering soil and other extraneous materials. They were subsequently washed with distilled water and shade-dried at room temperature until a constant weight was obtained.

Extraction of *Rosmarinus officinalis* Essential Oil- The essential oil was extracted from the dried leaves of *R. officinalis* by hydro distillation using a Clevenger-type apparatus. Briefly, 500 g of dried and coarsely powdered leaves were transferred into a round-bottom flask containing 3 L of distilled water. The mixture was subjected to hydro distillation for approximately 3–4 h, allowing the volatile constituents to be co-distilled with water and subsequently condensed in the Clevenger apparatus. The separated essential oil was carefully collected from the graduated receiver, and any residual moisture was removed using anhydrous sodium sulphate. The obtained essential oil was transferred to an amber-coloured, airtight glass vial and stored at 4 °C until further phytochemical and biological investigations [6]. The percentage yield of essential oil was calculated using the following formula:

Essential oil yield (%) = (Weight of essential oil obtained / Weight of dried plant material used) × 100

IN VITRO ANTIOXIDANT ACTIVITY

DPPH Scavenging Assay-To evaluate antioxidant activity, 10 µL of different concentrations of test samples or standard (Ascorbic Acid, SRL Cat. No. 23006) were mixed with 200 µL of 0.1 mM DPPH solution (SRL Chem, Cat. No. SR-29128) in a 96-well plate. Reactions were prepared in quadruplicate; duplicate blanks (sample + methanol without DPPH), red blanks (without DPPH), and untreated wells served as controls. Plates were incubated in the dark for 30 min, and absorbance was read at 517 nm using a microplate reader (iMark, Bio-Rad). A combination of test solution with 20 µL deionized waters served as control. Scavenging activity (%) was calculated relative to control [7]. IC_{50} values were derived using GraphPad Prism 6 by plotting inhibition (%) vs. sample concentration.

Calculations:

GC-MS Analysis-GC-MS analysis of *R. officinalis* essential oil was performed using a PerkinElmer Clarus 600 system equipped with an Rtx-5MS capillary column. Helium (99.99%) was used as the carrier gas at a constant flow rate of 1.0 mL/min. Injection volume: 1 µL, split ratio: 10:1, ionization energy: 70 eV, scan range: 40–650 m/z, scan time: 0.2 sec. Injector temperature: 260°C. Oven temp held at 50°C for 3 min, then ramped to 300°C at 10°C/min. Compounds were identified by comparing retention times and mass spectra with those in the NIST and Wiley-8 libraries [8].

CELL VIABILITY ASSAY

MTT Assay- The cytotoxicity of the provided samples on RAW 264.7 (Passage no. 27, Procured from NCCS Pune, India and RRID- CVCL_0493) cell line was determined by MTT Assay. The cells (10000 cells/well) were cultured in 96 well plate for 24 h in DMEM medium (Dulbecco's Modified Eagle Medium-AT149-1L-HIMEDIA) supplemented with 10% FBS (Fetal Bovine Serum - HIMEDIA-RM 10432) and 1% antibiotic solution (Penicillin-Streptomycin-Sigma-Aldrich P0781) at 37°C with 5% CO_2. Next day cells were treated from different concentrations of the samples (concentration as per mentioned in excel sheet). Stock solution of samples was prepared in DMSO and further diluted to get different concentrations in incomplete Cell culture Medium (Without FBS). After incubation for 24 hours, MTT Solution (5 mg/ml) was added to cell culture and further incubated (Air- jacketed CO2 incubator-Heal force-HF90) for 2 h. Cells without

treatment were considered as Control and cells without MTT were considered as Blank. At the end of the experiment, culture supernatant was removed, and cell layer matrix was dissolved in 100 µl Dimethyl Sulfoxide (DMSO–SRL- Cat no.- 67685) and read in an Elisa plate reader (iMark, Biorad, USA) at 540 nm. *IC50* was calculated by using software Graph Pad Prism -6. Images were captured under inverted microscope (Olympus ek2) using Camera (AmScope digital camera 10 MP Aptima CMOS). 50% inhibitory concentration (*IC50*) was presented as Mean ± SEM [9].

Cell line Culture and Treatment -Anti-Inflammatory Effect- Raw 264.7- Raw 264.7 cell line was procured from NCCS Pune. The cells were harvested from flask at 90% confluency and seeded in 96 well plates at appropriate density in DMEM medium (Dulbecco's Modified Eagle Medium-AT149-1L) supplemented with 10% FBS (Fetal Bovine Serum - HIMEDIA-RM 10432) and 1% antibiotic solution at 37°C with 5% CO_2. Next day, the medium was removed and fresh culture medium was added to each well of the plate and then 5- 50 µl (10% of total medium of the cell culture) of prepared treatment dilutions was added to the defined wells and incubated (Air-Jacketed CO_2 Incubator- Heal Force -HF90) the treated plate for 24 hr. Then the cell culture becomes ready for further associated experimental works. Images were captured under inverted microscope (Olympus ek2) using Camera (AmScope digital camera 10 MP Aptima CMOS) [10].

Anti-inflammatory Assay

Protein Denaturation Inhibition Assay- 5 µL of the sample/ standard at different concentrations were mixed with 45 µL of 5% bovine serum albumin (BSA) prepared in PBS and incubated at 37°C for 30 minutes. After incubation, 150 µL of sodium phosphate buffer (pH 6.3) was added to each tube, and the samples were further incubated at 70°C for 30 minutes. Following the incubation, the turbidity of the reaction mixture was measured spectrophotometrically at 660 nm. For the control, 5 µL of PBS was used instead of the sample, whereas the product control was prepared without bovine serum albumin [11-12].

The percentage inhibition of protein denaturation was calculated as follows:

% Inhibition = (A-Control - A Test /A Control) ×100

Statistical analysis-The statistical analysis was performed in IBM SPSS version 18 (SPSS version 18.0; IBM Corporation, Armonk, NY, USA). The entire assay was performed in triplicate and the values were expressed as mean ± standard deviation (SD). Descriptive statistics were used for continuous variables and expressed in mean and standard deviation. A test of normality was applied.

Result and Discussion

Table-1 DPPH Scavenging Assay of *R. officinalis* essential oil

Sample code	IC_{50} value (µg/ml)
Ascorbic Acid	2.502±0.02
R. officinalis essential oil	136.5±0.05

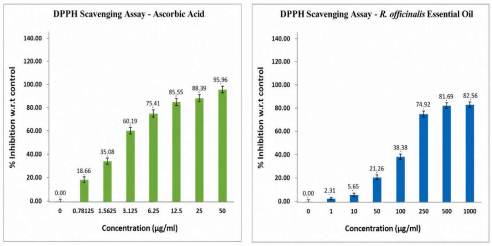


Figure-1 Graph represents the DPPH Scavenging Assay of *R. officinalis* essential oil

Based on the results obtained from the experimental work, Antioxidant activity (DPPH Assay) was estimated in sample, and 50% inhibitory concentration (IC_{50}) was mentioned in table 1. Sample of *R. officinalis* was found to be moderately active. 136.5 µg of *R. officinalis* was found equivalent to 2.502 µg of standard Ascorbic acid. The standard compound, being a purified compound and highly active substance, was assessed using a lower concentration range (0 to 50 µg/ml). In contrast, the sample's potency remains unbound; therefore, a broader concentration range (0 to 1000 µg/ml) was employed for the sample analysis.

Identification of components-Identification was done using the calculated fragments, molecular mass, and molecular structure. The NIST database and the Willy8-library, which has more than 62,000 pattern entries, were used to interpret the GC-MS spectra. Together with their molecular weights and chemical structures, the components of the test materials were determined. The percentage amounts discovered in the sample were contrasted with component spectra from the Willy8 collection and the NIST library. This is done in order to determine whether this plant species has any chemicals or a combination of them that could support its traditional and commercial therapeutic purposes. It also helps determine the most effective methods for removing harmful compounds. The test materials' constituent parts were determined (Table 2, and Figure 2).

Table 2- Compounds of *R. officinalis* essential oil

Peak	R.Time	Area	Area %	Name	Common Name
	7.264	8774889	0.13	Bicyclo[3.1.1] Hept-2-Ene, 2,6,6-Trimethyl-	A-Pinene
	11.05	7655928	0.11	Limonene	Limonene
	11.15	21012463	0.31	Eucalyptol	1,8-Cineole
	14.32	23778505	0.35	Linalool	Linalool

	16.248	12552874	0.19	Camphor	Camphor
	17.572	47253198	0.70	Bicyclo[2.2.1] Heptan-2-Ol, 1,7,7-Trimethyl-, (1s-Endo)-	Borneol (Endo-Borneol)
	18.621	2783311	0.04	(+)-Alpha-Terpineol (P-Menth-1-En-8-Ol)	A-Terpineol
	27.123	2946108909	43.52	Phenol, 2-Methoxy-3-(2-Propenyl)-	Eugenol
	29.017	1320554051	19.51	Caryophyllene <(E)->	B-Caryophylle
	30.110	102395620	1.51	Humulene <Alpha->	A-Humulene
	31.152	141603900	2.09	1,6-Cyclodecadiene, 1-Methyl-5-Methylene-8-(1-Methylethyl)-, [S-(E, E)]-	Germacrene D
	35.025	110272506	1.63	Caryophyllene Oxide	Caryophyllene Oxide

GC–MS analysis revealed several phytoconstituents with reported anti-inflammatory properties, including α-pinene, limonene, 1,8-cineole, linalool, borneol, α-terpineol, eugenol, β-caryophyllene, α-humulene, germacrene D, and caryophyllene oxide. Among these, eugenol (43.52%) and β-caryophyllene (19.51%) were the predominant constituents and may substantially contribute to the anti-inflammatory potential of *Rosmarinus officinalis* essential oil. Eugenol has been reported to suppress COX-2 and NF-κB-mediated inflammatory responses, whereas β-caryophyllene exhibits CB2 receptor-mediated anti-inflammatory effects. The presence of these bioactive constituents therefore provides a plausible phytochemical basis for the observed anti-inflammatory activity of the essential oil.

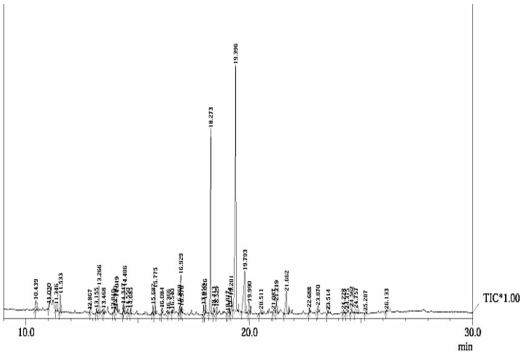


Figure-2 *R. officinalis* essential oil chromatogram

Cell Viability Results- Based on the results obtained from the MTT assay, it was observed that when the RAW 264.7 cell line was exposed to different concentrations of the samples, cytotoxic activity was estimated for sample and 50% inhibitory concentration as mentioned in table 3. *R. officinalis* essential oil was found to be moderately cytotoxic. *IC50* is the concentration of an inhibitor/sample/ formulation at which the viable cells are reduced by half.

Table 3: Represents the MTT values of *R. officinalis* essential oil

Sample Code	***IC*$_{50}$ value**
Doxorubicin	Doxorubicin
R. officinalis essential oil	101.5±0.11

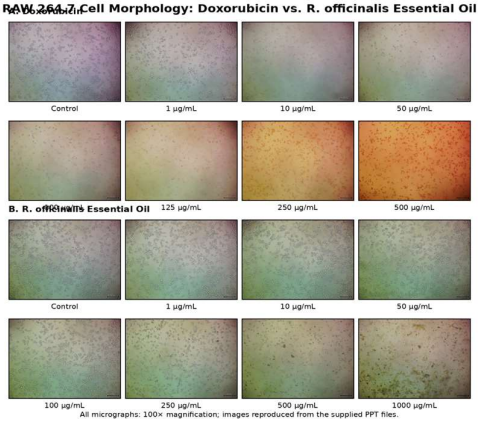


Figure-3 Microscopic results of RAW 264.7 cells. A- showing doxorubicin vs. *R. officinalis* and B- Showing *R. officinalis* essential oil

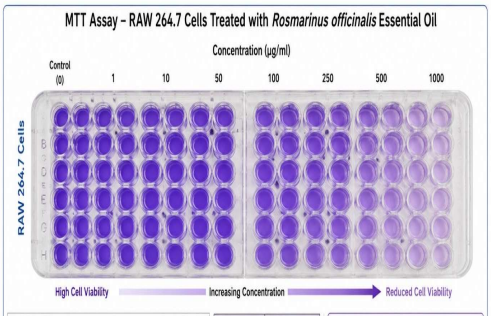


Figure-4 MTT Assay RAW 264.7 cells treated with *R. officinalis* essential oil

Protein Denaturation Inhibition Assay- Based on the results obtained from the experimental work, Protein Denaturation Inhibition Assay was estimated for sample and 50% inhibitory concentration (*IC50*) were mentioned in table 4. Sample- *Rosmarinus officinalis* essential oil was found to be moderately active as compared to the standard Diclofenac. 195.3µg of samples–*Rosmarinus officinalis* essential oil was found equivalent to 97.26µg of the standard Diclofenac.

Table 4: Represents the Protein Denaturation of *R. officinalis* essential oil

Sample Code	IC_{50} value
Diclofenac	97.26±0.01
R. officinalis essential oil	195.3±0.07

Discussions- The present study demonstrates that *Rosmarinus officinalis* essential oil possesses moderate antioxidant and anti-inflammatory potential, which may be related to its diverse volatile phytochemical composition. GC–MS analysis identified several biologically relevant constituents, including α-pinene, limonene, 1,8-cineole, linalool, camphor, borneol, α-terpineol, eugenol, β-caryophyllene, α-humulene, germacrene D, and caryophyllene oxide. Notably, eugenol (43.52%) and β-caryophyllene (19.51%) were the predominant compounds, suggesting that these constituents may contribute substantially to the observed biological effects. The DPPH assay showed moderate radical-scavenging activity of the essential oil, with an IC_{50} of 136.5 ± 0.05 µg/mL, whereas ascorbic acid exhibited considerably stronger activity (2.502 ± 0.02 µg/mL). This difference indicates that the essential oil has antioxidant capacity but is less potent than the purified reference antioxidant. The activity may arise from the combined effects of oxygenated monoterpenes and phenolic volatile constituents. The MTT assay using RAW 264.7 macrophages demonstrated an IC_{50} of 101.5 ± 0.11 µg/mL. Microscopic observations further indicated relatively preserved cellular morphology at low-to-moderate concentrations, while greater morphological alterations occurred at higher concentrations, suggesting concentration-dependent effects on cell viability. Importantly, the protein denaturation assay demonstrated moderate anti-inflammatory activity, with an IC_{50} of 195.3 ± 0.07 µg/mL compared with 97.26 ± 0.01 µg/mL for diclofenac. Overall, these findings support the potential of *R. officinalis* essential oil as a natural source of antioxidant and anti-inflammatory compounds, although further mechanistic and in vivo studies are required to validate its therapeutic relevance.

Conclusion- The present study concludes that *Rosmarinus officinalis* essential oil possesses promising in vitro antioxidant and anti-inflammatory properties, supported by its diverse phytochemical composition. GC–MS analysis identified several bioactive constituents, with eugenol (43.52%) and β-caryophyllene (19.51%) being the predominant compounds and potentially contributing to the observed biological activities. The essential oil demonstrated moderate DPPH radical-scavenging activity with an IC_{50} of 136.5 ± 0.05 µg/mL and moderate inhibition of protein denaturation with an IC_{50} of 195.3 ± 0.07 µg/mL, although its activity was lower than that of the respective standards. The MTT assay indicated moderate effects on RAW 264.7 cell viability, with an IC_{50} of 101.5 ± 0.11 µg/mL. Overall, these findings support *R. officinalis* essential oil as a potential natural source of antioxidant and anti-inflammatory agents. Further mechanistic, dose-response, and in vivo investigations are required to establish its therapeutic efficacy and safety.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Ethics Statement

Ethical approval was not required for this study.

Data Availability Statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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