Applied Biological Research 26(1): 136-141; (2024)
DOI: 10.48165/abr.2024.26.01.14

SHORT COMMUNICATION

IN VITRO STUDIES ON THE ANTI-INFLAMMATORY EFFECTS OF ESSENTIAL OIL OF *Rosmarinus officinalis* L. IN MACROPHAGE CELLS

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(Received 21 November, 2023; accepted 30 January, 2024)

ABSTRACT

The use of plant-based drugs against various human disorders have attained global interest. Multicomponent essential oils are used to cure inflammation-related disorders. The present study was aimed to assess the anti-inflammatory potential of essential oil from *Rosmarinus officinalis* (EO-RO) using *in vitro* model of LPS-activated RAW macrophage cell lines. EO-RO was hydro-distilled using Clevenger apparatus and evaluated first for protein denaturation and proteinase inhibitory activities, followed by assays for cyclooxygenase (COX) and lipoxygenase (LOX) activities in RAW 264.7 macrophage cell lines. The data was statistically analysed by one-way analysis of variance. Dose-dependent inhibition was observed in protein denaturation with 50 µg mL^{-1} EO-RO exhibiting a maximum inhibition of 67.77%. Similarly, proteinase inhibitory assay depicted 72.47% inhibition. The COX and LOX activities showed increased inhibitions in dose-dependent manner i.e. 25 to 100 µg mL^{-1} EO-RO displayed 58.72 and 75.33%; 28.58-40.86% inhibition, respectively.

Keywords: Anti-inflammation**,** essential oil, macrophage cells, protein denaturation, *Rosmarinus officinalis*

INTRODUCTION

Inflammation is a physiological reaction of cellular injury, infection or autoimmune activation. Over-expression of pro-inflammatory mediators may lead to multiple disorders including atherosclerosis. Essential oils of many *Lamiaceaea* species have been used in ethnic medicine for curing the inflammation-related disorders (Furman *et al.*, 2019; Kamelnia *et al.*, 2023). The macrophages are important inflammatory cells implicated in the initiation of inflammatory responses, which play critical role in pathogenesis of numerous inflammatory disease processes by secreting a number of pro-inflammatory mediators and pro-inflammatory cytokines. Lipopoly-saccharides (LPS), a compound present om cellular wall of Gram-negative bacteria, play important role in inducing inflammatory response and lead to various inflammatory disorders.

Essential oil (EO) are secondary metabolites comprising of volatile complex compounds with a strong odour. Many aromatic plants synthesize EO and possess 20-60 components at various levels (Leon-Mendez *et al.*, 2019). EO consists of monoterpenes, sesquiterpenes, and phenylpropane, which have several functional groups, including alkanes, alcohols, aldehydes, ketones, esters, and acids which have various pharmacological properties (Sadgrove *et al.*, 2022). EO's from *Pinus densiflora, P. koraiensis, Cinnamomum subavenium* and *Chamaecyparis obtusa* exhibit anti-

inflammatory biological activities (Lee *et al.,* 2017; Hao *et al.,* 2019; Yang *et al.,* 2021). Generally, the EOs block the JNK, NF-κB, and ERK pathways in macrophages as demonstrated in *Citrus medica* (Kim *et al.,* 2013). Although various members from Rutaceae and Verbenaceae were evaluated for their inflammation-related disorders, but research work on anti-inflammatory activity of EOs form *Rosmarinus officinalis* is scanty in comparison to other *Lamiaceae* species. Hence, this study was aimed to evaluate the anti-inflammatory activity of EOs from *R. officinalis* on LPS-induced RAW 264.7 macrophage cells under *in vitro* conditions.

MATERIALS AND METHODS

Plant material and extraction of essential oil

Rosmarinus officinalis leaves (1.0 kg) were subjected to hydro-distillation (1:1 ratio, w/v) at 105±2°C using a Clevenger-type apparatus until no more essential oil was obtained (Javanmardi *et al*., 2002). The essential oils were collected, dried under anhydrous sodium sulphate, and stored in sealed vials in dark at 4°C until use.

Protein denaturation assay

The *in vitro* protein denaturation assay was used to estimate the potential of essential oil from *R. officinalis* as reported by Mirke *et al*. (2020). Bovine serum albumin (BSA) was chosen as a protein model. To realize the experiments, 0.10 mL samples (@ 6.25, 12.5, 25.0, and 50.0 µg mL^{-1} in DMSO) were added to 2.40 mL of 3.5% BSA water solution. In positive control test, diclofenac sodium (250 µg mL^{-1}) was used instead of samples, while product control groups were prepared without BSA. The pH was adjusted at 6.3 with 1 N HCl, then incubated at 37°C for 20 min and heated at 57°C for 3 min. Phosphate buffered saline (pH 6.3, 2.5 mL) was added to each sample once they have cooled. The turbidity was measured by UV-visible spectrophotometer (SL119, Systronics) at 416 nm and the percent inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = 100 - \left\{\frac{\text{OD of test solution} - \text{OD of product control}}{\text{OD of test control}}\right\} \text{x } 100$$

Proteinase inhibitory activity

The test was performed as per the method of Sakat *et al*. (2010). The reaction mixture (2 mL) was containing 0.06 mg trypsin, 1 mL 20 mM Tris HCl buffer (pH 7.4) and 1 mL EO-RO of different concentrations (6.25, 12.5, 25.0, and 50.0 µg mL^{-1}). The mixture was incubated at 37ºC for 5 min and then 1 mL of 0.8% (w/v) casein was added. The mixture was incubated for additional 20 min and then 2 mL of 70% perchloric acid was added to halt the reaction. Cloudy suspension was centrifuged and the absorbance of supernatant read at 200 nm against buffer as blank. The percentage inhibition of proteinase inhibitory activity was calculated.

$$\text{Percentage inhibition} = 100 - \left\{\frac{\text{OD of test solution} - \text{OD of product control}}{\text{OD of test control}}\right\} \text{x } 100$$

Varying concentrations of EO extract used were 6.25, 12.5, 25.0, and 50.0 µg mL^{-1}. This range was chosen because concentrations below 6.25 µg mL^{-1} yielded insignificant inhibition and at concentrations above 50 µg mL^{-1} the value became too high.

Cell culture

The RAW 264.7 macrophage cell line (procured from National Centre for Cell Sciences, Pune, India) was used to evaluate the COX and LOX inhibition assay. The cells were cultured in plastic culture flasks in Dulbecco's modified Eagle medium (DMEM) containing L-glutamine supplemented with 10% foetal calf serum (FCS) and 1% PSF (penicillin/streptomycin/fungizone) solution under 5%

CO_2 at 37ºC. The growing cells were split twice a week. The cells were subsequently seeded into a 96-well microtiter plate at 2 x 10^6 cells $well^{-1}$ (200 mL) prior to the commencement of each bioassay.

Cyclooxygenase (COX) activity: COX activity was assayed as per Walker and Gierse (2010) method. The cell lysate (100 µL) was incubated with tris-HCl buffer (pH 8), glutathione 5 mM L^{-1} and hematin 5 Mm L^{-1} for 1 min at 25°C. The sample (rosemary essential oil and 50 µL DMSO) was added to it. The reaction was initiated by adding arachidonic acid 200 Mm L^{-1} and terminated after 20 min incubation at 37°C by adding 200 µL 10% trichloroacetic acid (prepared in 1 N HCl). After centrifugal separation, 200 µL of 1% thiobarbiturate was added, the tubes were boiled for 20 min and cooled. The tubes were centrifuged for 3 min and COX activity measured by taking absorbance spectrophotometrically at 632 nm. Lipopolysaccharide was used as control. The percent inhibition in COX activity was calculated by the formula:

$$\text{Percent inhibition in COX activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \text{X100}$$

Lipoxygenase (LOX) activity: The LOX activity was assayed as per Bernald *et al.* (1992) method. The rosemary oil extracts [25, 50 and 100 µg mL^{-1}] prepared in 50 µL DMSO were taken separately and tris-HCl buffer (pH 7.4), 50 µL cell lysate, and sodium linoleate (200 µL) added. The rosemary EO concentrations were chosen because the concentrations < 25 µg mL^{-1} yielded insignificant inhibition and >1000 µg mL^{-1} value became too high. The LOX activity was measured as an increase in absorbance at 234 nm spectrophotometerically which reflected the formation of 5-hydroxyeicosapentaenoic acid. LPS was used as control. The percent inhibition was calculated by the formula:

$$\text{Percent inhibition in LOX activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \text{X 100}$$

Statistical analysis

All the above experiments were conducted in a completely randomised design and each treatment replicated three times. The data was analysed by one-way analysis of variance (Douglas, 2005) and the means with SE and critical difference ($CD_{0.05}$) at 5% level of significance was calculated. The statistical test used to compare variance was F-ratio test (or variance ratio test) which compares two variances to test whether they come from the same populations. SE measures the distance between values predicted from the estimated regression and the observed values of the dependent variable.

RESULTS AND DISCUSSION

Initially an attempt was made to determine the anti-inflammatory potency of essential oil *R. officinalis* (EO-RO) by protein denaturation and protein inhibitory assay. Dose-dependent inhibition was noticed in protein denaturation with 50 µg mL^{-1} essential oil showing a maximum of 67.77% inhibition (Table 1). The F ratio, SE and CD values were 989**, 0.0321 and 0.004, respectively. Similarly, the proteinase inhibitory assay showed 72.47% inhibition with F ratio, SE and CD values of 1047**, 0.014 and 0.02, respectively. The results suggested the possible role of EO-RO in promoting anti-inflammatory potential. Bhutia (2020) documented that *Citrus macroptera* EO possessed IC_{50} value of 54.6 µg mL^{-1}. Belkhodja *et al*. (2022) reported that essential oil of *Eucalyptus globulus* (250 µg mL^{-1}) and diclofenac inhibited the protein denaturation. The present results are at par with Habibur Rahman *et al.* (2012) who showed that the essential oil of *E. globulus* at the concentrations of 100, 250 and 500 µg mL^{-1} exhibited 16.98, 58.49, and 66.03% inhibition in protein denaturation. The mode of action of denaturation may involve the alteration of electrostatic, hydrogen, hydrophobic or disulfide bond. Therefore, the bioactive molecules in essential oil can participate in the protection of these different types of structural bonds (Kar *et al.*, 2012).

COX activity is involved in the prostaglandin synthesis which is responsible for the production of inflammatory signals (Ricciotti and Gerald, 2011). COX inhibition helps in down-regulation of prostaglandin synthesis, thus provides protection against the development of inflammation signals. The results revealed increased inhibition in COX activity by EO-RO in dose dependent manner i.e. 100 µg mL^{-1}concentration of EO-RO displayed 75.33% inhibition (Table 2). LOX is s responsible for the production of leukotrienes which can cause inflammation (Rudrapal *et al*., 2023). The study confirmed that EO-RO exhibited dose dependent inhibition in LOX activity. At higher concentration of EO-RO (100 µg mL^{-1}) 40.86% inhibition was observed with F ratio, SE and CD values of 671**, 0.036 and 0.0054, respectively. Hai Dang *et al.* (2020) reported that *Amomum aromaticum* fruit oil reduced the expression of two pro-inflammatory proteins iNOS and COX-2 in stimulated cells in a dose-dependent manner. Chen-Lung Ho *et al.* (2020) proved the anti-inflammatory power of *Eucalyptus* essential oils using LPS-induced inflammatory response in RAW264.7 macrophages through reducing COX, LOX, MAPK and NF-κB pathways. Elsayed Heba *et al.* (2023) screened *Psidium cattleianum* leaves and flower essential oil for their COX inhibitory activities, and found that the EOs exerted low COX-1 inhibitory activity (IC_{50} 45.96 µL mL^{-1}) in comparison to standard COX-1 inhibitor SC560. However, the EO from flowers showed moderate COX-1 activity (IC_{50}, 19.08 µL mL^{-1}) as compared to celecoxib (IC50, 11.34 µg mL^{-1}) and SC560 (IC50, 6.34 nM), while high IC50 in comparison to indomethacin (IC50, 1.067 µg mL^{-1}). The present results on EO-RO on LPS induced RAW 264.7 macrophage cells are in line with above observations.

Table 1: Effect of rosemary oil on protein denaturation and proteinase activity in LPS induced RAW 264.7 macrophage cells

Rosemary oil conc. (µg mL^{-1})	Inhibition in protein denaturation (%)	Inhibition in proteinase activity (%)
Control	0.00	0.00
6.25	11.98	15.03
12.5	48.01	57.88
25	51.06	70.73
50	67.77	72.47
F ratio	989**	1047**
S.E.	0.0321	0.014
$CD_{(0.05)}$	0.004	0.02

Cells were maintained in culture medium (control), or incubated with 1 µg mL^{-1} LPS or with LPS in the presence of different levels of oil (6.25-50.0 µg mL^{-1}) for 24 h. Results are expressed as percent inhibition in protein denaturation and proteinase activities by the cells treated with LPS. Each value represents the mean, F ratio, SE and $CD_{0.05}$ of three experiments.

Table 2: Effect of rosemary oil on COX and LOX activities in LPS induced RAW 264.7 macrophage cells

Rosemary oil conc. (µg mL^{-1})	Inhibition in COX activity (%)	Inhibition in LOX activity (%)
25	58.72	28.58
50	65.25	28.56
100	75.33	40.86
F ratio	1821**	671**
SE	0.009	0.036
$CD_{(0.05)}$	0.01	0.0054

Cells were maintained in culture medium (control), or incubated with 1 µg mL^{-1} LPS or with LPS in the presence of different concentrations of the oil (25-100 µg mL^{-1}), for 24 h. Results are expressed as percentage of inhibition in COX and LOX activities of the cells treated with LPS. Each value represents the mean, F ratio, SE and $CD_{0.05}$ of three experiments

In conclusion, it appears that natural herbs are safe, effective and better options as anti-inflammatory agents than synthetic ones. The phyto-constituents are as effective with the comparable mechanism of action as synthetic molecules. The present study revealed the anti-inflammatory potential of essential oil isolated from *R. offinalis* (EO-RO) in *in vitro* model of LPS activated RAW macrophage cell lines. EO-RO exhibited potent inhibition in proteinase activity, protein denaturation and COX and LOX activities. Further research should focus on exploring the molecular mechanisms of different beneficial applications of this essential oil in various inflammatory disease models which can aid in development of therapeutic strategies.

Acknowledgement: The facilities and infrastructure provided by the management of the Sree Narayana Trust are greatly acknowledged by the authors.

REFERENCES

Belkhodja, B., Meddah, K., Sidelarbi, D., Bouhadi, B., Medjadel, A., Brakna. 2022. *In vitro* and *in vivo* anti-inflammatory potential of *Eucalyptus globulus* essential oil. *Hamza Journal of Applied Biological Sciences*, **16**(1): 80-88.

Bernald, A., Steczko, J. and Dixon, G.A. 1991. Effect of ethanol and low temperature culture on expression of soybean lipoxygenase L-1 in *E. coli*. *Protein Expression and Purification*, 2(3): 221-227.

Bhutia, S. 2020. Evaluation of *in vitro* anti-inflammatory activity of *Citrus macroptera* Montr. *Asian Journal of Pharmaceutical and Clinical Research*, **13**(8): 101-103.

Chen-Lung, Ho, Lan-Hui, L., Yueh-Chun, Weng, Kuo-Feng, Hua and Tz-Chuen, Ju. 2020. Eucalyptus essential oils inhibit the lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages through reducing MAPK and NF-κB pathways. *BMC Complementary Medicine and Therapies*, **20**: 200. [*doi: 10.1186/s12906-020-02999-0*].

Douglas, C.M. 2005. *Design and Analysis of Experiments*. Chapter 3 p. 60. [*ISBN: 9780471661597*].

Elsayed Heba, E., El-Deeb Eman, M., Taha Heba, Taha Hussein, S., Elgindi Mohamed, R. and Moharram Fatma, A. 2023. Essential oils of *Psidium cattleianum* sabine leaves and flowers-anti-inflammatory and cytotoxic activities, *Frontiers in Chemistry*, **11**: 1-13.

Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., Ferrucci, L., Gilroy, D.W., Fasano, A., Miller, G.W., Miller, A.H., Mantovani, A., Weyand, C.M., Barzilai, N., Goronzy, J.J., Rando T.A., Effros R.B., Lucia, A., Kleinstreuer, N. and Slavich, G.M. 2019. Chronic inflammation in the etiology of disease across the life span. *Nature Medicine*, **25**: 1822-1832.

Habibur Rahman, M., Chinna, E., Kamala, V. and Madhavi, P. 2012. *In-vitro* studies suggest probable mechanism of *Eucalyptus* oil for anti-inflammatory and anti-arthritic activity. *International Journal of Phytopharmacy*, **2**(3): 81-83.

Hai Dang, N., Thi Van Anh, L. and Tien Dat, N. 2020. Anti-Inflammatory effects of essential oils of *Amomum aromaticum* fruits in lipopolysaccharide-stimulated RAW264.7 cells. *Journal of Food Quality*, Article ID 8831187: **5**. [*https://doi.org/10.1155/2020/8831187*].

Hao, X., Sun, W., Ke, C., Wang, F., Xue, Y., Luo, Z., Wang, X., Zhang, J. and Zhang, Y. 2019. Anti-inflammatory activities of leaf oil from *Cinnamomum subavenium in vitro* and *in vivo*. *Biomedical Research International*, 1823149. [*https://doi.org/10.1155/2019/1823149*].

Javanmardi, J., Khalighi, A., Kashi, A., Bais, H. and Vivanco, J. 2002. Chemical characterization of basil (*Ocimum basilicum* L.) found in local accessions and used in traditional medicines in Iran. *Journal Agriculture Food Chemistry*, **50**(21): 5878-5883

Kamelnia, E., Mohebbati, R., Kamelnia, R., El-Seedi, H.R. and Boskabady, M.H. 2023. Anti-inflammatory, immunomodulatory and anti-oxidant effects of *Ocimum basilicum* L. and its main constituents - A review. *Iranian Journal of Basic Medical Sciences*, **26**(6): 617-627.

Kar, B., Kumar, R.S., Karmakar, I., Dola, N., Bala, A., Mazumder, U.K. and Hadar, P.K. 2012. Antioxidant and *in vitro* anti-inflammatory activities of *Mimusops elengi* leaves. *Asian Pacific Journal of Tropical Biomedicine*, **2**(2): 976-980.

Kim, K., Ko, Y., Yang, H., Ham, Y., Roh, S.W., Jeon, Y., Ahn, G., Kanf, M., Yoon, W. and Kim, D. 2013. Anti-inflammatory effect of essential oil and its constituents from fingered citron (*Citrus medica* L. var. *sarcodactylis*) through blocking JNK, ERK and NF-κB signaling pathways in LPS-activated RAW 264.7 cells. *Food Chemistry Toxicology*, **14**(57): 126-131.

Lee, E., Shin, M. and Jung, S. 2017. Volatile compound analysis and anti-oxidant and anti-inflammatory effects of *Oenanthe javanica*, *Perilla frutescens*, and *Zanthoxylum piperitum* essential oils. *Asian Journal of Beauty and Cosmetology*, **18**(15): 355-366.

Leon-Mendez, G., Pajaro-Castro, N., Pajaro-Castro, E., Torrenegra-Alarcon, M. and Herrera-Barros, A. 2019. Essential oils as a source of bioactive molecules. *Revista Colombiana de Ciências Químico*. **28**(48): 80-93.

Mirke, N.B., Shelke, P.S., Malavdkar, P.R. and Jagtap, P.N. 2020. *In vitro* protein denaturation inhibition assay of *Eucalyptus globulus* and *Glycine max* for potential anti-inflammatory activity. *Innovations in Pharmaceuticals and Pharmacotherapy*, **8**(2): 28-31.

Ricciotti, E. and Gerald, G.A.F. 2011. Prostaglandins and inflammation *Arterioscler Thromb Vascular Biology*, **31**(5): 986-1000.

Rudrapal, M., Ali Eltayeb, W., Rakshit, G., Ahmed El-Arabey, A., Khan, J., Aldosari, S.M., Alshehri, B. and Abdalla, M. 2023. Dual synergistic inhibition of COX and LOX by potential chemicals from Indian daily spices investigated through detailed computational studies. *Scientific Reports*, **13**: 8656. [*https://doi.org/10.1038/s41598-023-35161-0*].

Sadgrove, N.J., Padilla-González, G.F. and Phumthum, M. 2022. Fundamental chemistry of essential oils and volatile organic compounds, methods of analysis and authentication. *Plants*, **4**(11): 789 [*https://www.mdpi.com/2223-7747/11/6/789*].

Sakat, S., Juvekar, A.R. and Gambhire, M.N. 2010. *In vitro* antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* Linn. *International Journal of Pharma and Pharmacological Sciences*, **2**(1): 146-155.

Walker, M.C. and Gierse J.K. 2010. *In vitro* assays for cyclooxygenase activity and inhibitor characterization. *Methods in Molecular Biology*, **644**: 131-44. [*doi: 10.1007/978-1-59745-364-6_11. PMID: 20645170*].

Yang, J., Choi, W.S., Kim, K.J., Eom, C.D. and Park, M.J. 2021. Investigation of active anti-inflammatory constituents of essential oil from *Pinus koraiensis* (Sieb. et Zucc.) wood in LPS-stimulated RBL-2H3 cells. *Biomolecules*, **11**(6): 10.3390/biom11060817. [*https://www.mdpi.com/2218-273X/11/6/817*].

Yang, J.; Choi, W.; Jeung, E.; Kim, K and Park, M. 2021. Anti-inflammatory effect of essential oil extracted from Pinus densiflora (Sieb. et Zucc.) wood on RBL-2H3 cells. *Journal of Wood Science*, **67**: Article No. 52. [*https://jwoodscience.springeropen.com/articles/10.1186/s10086-021-01982-8*].

Yoona, W., Moona, J., Kanga, J., Kima, G., Leeb, N. and Hyun Neolitsea sericea, C. 2010. Essential oil attenuates LPS-induced inflammation in RAW 264.7 macrophages by suppressing NF-κB and MAPK. *Activation Natural Product Communications*, **5**(8): 1312-1316.