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Research Article
Autophagy Anticancer Properties of *Rosmarinus officinalis* L. Essential Oils on The SH-SY5Y Neuroblastoma Cell Line *in vitro*

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Abstract

Background and Objective: Despite aggressive multimodal therapy, neuroblastoma patients have a poor prognosis. The goal of this study was to look into the effects of different concentrations of rosemary oil on reducing drug resistance in the SH-SY5Y neuroblastoma cell line. Furthermore, to investigate autophagy as a mechanism for tumor and cell survival as well as chemoresistance. **Materials and Methods:** Cold-pressing rosemary oil was diluted with the cultivation medium to test against SH-SY5Y cell lines. The MTT cell proliferation assay was used to assess cell survival in SH-SY5Y cell lines treated with varying concentrations of rosemary oil (50-200 μg mL^{-1}). Over 30 compounds were identified using GC-Mass. A panel of antioxidants and proinflammatory markers were measured as well as LC3 as a specific marker for autophagy, using a flow cytometer. **Results:** Rosemary oil reduces the pro-inflammatory cytokines and free radicals in SH-SY5Y treated cells in a dose and time dependent manner. Furthermore, LC3 expression was significantly higher in the SH-SY5Y-treated cell line (200 μg mL^{-1}) compared to the untreated cells. Elevation in the LC3 autophagy marker reveals a partially different process rather than one that is dependent on mTOR inhibition. **Conclusion:** Rosemary has the potential to diminish SH-SY5Y drug resistance through antioxidant and anti-inflammatory properties together with autophagy.

Key words: SH-SY5Y, rosemary oil, antioxidants, pro-inflammatory markers, LC3

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a global health problem showing increasing and life-threatening prevalence. Medicinal plants are in the top positions whenever we discuss anticancer products. Herbal medicines have an important role in the prevention and treatment of cancer. These herbs are a source of ingredients with possible anticancer potency, which can stop or minimize the progression of carcinogenesis at different stages. These substances exhibit a wide range of properties that influence several pathways by regulating the activities of multiple transcription factors[1]. A complex and multifaceted molecular and cellular process, autophagy is critical for preserving cells under nutritional, metabolic and pathogenic stress and for ensuring cellular homeostasis[2]. Additionally, autophagy, a self-degradative mechanism in cancer cells, may be linked to chemoresistance and may be activated by chemotherapy[3]. Autophagy has two distinct functions in cancer: It can either stimulate cell death and stop the growth of the tumor or it can help cells survive[4]. Neuroblastoma (NB) is the most prevalent extracranial solid tumor frequently diagnosed in childhood. Regardless of the intense efforts to develop an effective treatment, currently available remedies are still challenged by high rates of resistance, recurrence and progression, most notably in advanced cases and highly malignant tumors. Emerging evidence proposes that this might be due to a subpopulation of tumor-initiating cells (TICs) or cancer stem cells (CSCs) found in the bulk of the tumor[5].

Rosmarinus officinalis L. or rosemary grows in the Mediterranean Region and is commonly used as flavoring food additives approved by the European Union legislation and permitting food corporations. It is reported with anti-inflammatory, anti-infective or anticancer action[6-8]. Regulation of the immune and anti-inflammatory response, alteration of specific metabolic pathways and increased expression of onco-suppressor genes are the main mechanisms behind the anticancer potency of rosemary[9-14]. The active metabolites present include phenolic diterpenes and triterpenes described by the presence of hydroxylated aromatic rings[15]. Its oil contains rosmarinic and carnosic acid, camphor, limonene, linalool and myrcene.

The brain is especially vulnerable to oxygen free radicals, which have been linked to the pathophysiology of various neurological illnesses. The antioxidant enzyme system of the brain may play a significant role in oxidative stress prevention. While IL-1α can protect cells from the harmful effects of reactive oxygen species (ROS), IL-4, IL-6, TNF-α and IL-1β appear to be capable of regulating the effects of IL-1. Increase of hydrogen peroxide (H_2O_2) as a product of superoxide dismutase (SOD), an enzyme that converts superoxide anion to H_2O_2 results in increased resistance to oxidative damage as a character of neuroblastoma cell line[16,17].

Autophagy, a prevalent self-degradation process, involves the dismantling and recycling of cellular cytoplasmic components through the lysosomal pathway. In the context of cancer, autophagy exhibits a dual role, contributing either to cell death induction and inhibition of tumor advancement or facilitating cell survival[18]. During the initial phases of carcinogenesis, autophagy acts as a primary tumor suppressor, impeding tumor progression[19]. Nevertheless, autophagy can also empower tumor cells with the capability to resist ionizing radiation and chemotherapy[20,21]. This study aims to test the anticancer effects of different concentrations of rosemary oil on SH-SY5Y neuroblastoma cell line in an attempt to amend the challenge of resistance, recurrence and progression of neuroblastoma (NB) as the most prevalent solid tumor in children.

MATERIALS AND METHODS

Study area: The study was conducted at the National Research Center, Egypt from June, 2022 to August, 2022.

Chemicals and materials: Chemicals and reagents Dulbecco's Modified Eagle's Medium (DMEM) culture medium containing 4.5 g L^{-1} glucose was purchased from AppliChem, Darmstadt, Germany. Fetal bovine serum (FBS), 0.25% Trypsin-EDTA solution and antibiotic/ antimycotic solution were purchased from GIBCO® Invitrogen, Life Technologies, USA. Other chemicals and reagents were of cell culture grade and were purchased from Sigma-Aldrich Chemical Company, St. Louis, Missouri, USA. Disposable culture ware and consumable materials were procured from Corning, New York, USA.

Preparation of seed oils: *Rosmarinus officinalis* L. was purchased from the special unit of refining oils, National Research Center, Cairo, Egypt. The oil was extracted by cold pressing in order to retain all the aromatic, chemical and nutritional characteristics of the oil. The purified oil was used to prepare a stock solution of 1 mg mL^{-1} in Dimethyl Sulfoxide (DMSO). The working seed oil solutions were prepared by serially diluting the stock oil solution with the cultivation medium. Finally, the working solution sets were filtered using 0.22 µm sterile syringe filters (Millipore, USA).

GC/MS analysis of rosemary oil: The GC/MS analysis of rosemary consists of 38 compounds. The total peak areas of the detected compounds is 100% and the probabilities of the structures of the detected compounds were listed in Table (1): The major compounds 2-α-Pinene (28.81%), 1,2-Diethyl,

benzene (10.11%), 1-(But-2'-phenylsulfonyl)-3-methylbuta-1,2-diene (12.06%), 1-(Methoxymethoxy)-3-methyl-3-hydroxybutane (10.56%) and 4-(2-Propynyl) cyclo-hex-2-en-1-one (14.57%), are for which represented 76.11% of the total peak areas. The identification of these compounds was accomplished using computer search user-generated reference libraries, incorporating mass spectra. Peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass spectral fragmentation. Reference compounds were co-chromatographed, when possible, to confirm GC retention times.

MTT assay: Cultured from the human neuroblastoma cell strain SH-SY5Y, acquired from the National Cancer Institute in Cairo, Egypt, the cells underwent digestion, suspension in DMEM medium supplemented with 10% FBS and subsequent quantification. They were then placed in a 96-well plate at a density of 1×10^4 cells per well. One day after inoculation, the adherent cells were shifted to serum-free DMEM and exposed to rosemary essential oil at concentrations of 50, 100, 150 or 200 µM for 24 and 48 hrs. The culture media were collected and stored at -20°C for future use and the cells were reacted with 5 mg mL^{-1} 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) solution for 4 hrs. The reaction was terminated by adding 100 µM L DMSO and the absorbance at 570 nm was determined using an enzyme-linked immunosorbent assay reader.

Cell viability: Cells were seeded in a 96-well culture plate at a concentration of 5×10^3 cells per well overnight. Following overnight incubation, the cells were subjected to various conditions for 24 hrs and their proliferation was assessed using the MTT cell proliferation assay (Cell Titer 96 Non-Radioactive Cell Proliferation Assay, Promega) as per the manufacturer's guidelines. The absorbance at 570 nm was measured using the Spectra Max 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, California). The assays were conducted in triplicate and the relative cell viability (expressed as a percentage of the control) was determined using the formula:

$$\frac{\text{Mean OD of treated cells}}{\text{Mean OD of control cells}}\times100$$

At each time point, the treated cells were compared with control cells that had undergone treatment with the vehicle only.

Biochemical analysis

Glutathione (GSH) level: The GSH level was estimated using the kit provided by Randox Company according to the manufacturer's instructions[19].

Malondialdehyde (MDA) level: The MDA, as an index of lipid peroxidation, was measured using the kit provided by Randox Company according to the manufacturer's instructions[20].

Superoxide oxide dismutase (SOD) level: The SOD level was estimated using the kit provided by Randox Company according to the manufacturer's instructions.

Determination of IL-1β and IL-10: The IL-1β and IL-10-containing supernatants (BD Bioscience Pharmingen, Franklin Lakes, New Jersey, USA) were analyzed using ELISA kits following the prescribed protocol of the manufacturer. In summary, the capacity of cells to release IL-1β in response to cytokines was assessed through a sandwich ELISA. A flat-bottom 96-well microtiter plate (Greiner Bio-One, Kempten, Germany) was coated with 100 µL/well of anti-human IL-1β mAb (2 mg mL^{-1} in a mixture of sodium carbonate and sodium bicarbonate, pH 9.5) overnight at 4°C. Following washes with phosphate-buffered saline (PBS; pH 7.0) and 0.05% Tween-20, the plate was blocked with 10% fetal calf serum (FCS). The IL-1β standards (rHu IL-1β) were prepared in a solution of PBS (pH 7.0) and 10% FCS using serial dilutions. Standards or supernatants (100 µL/well) were plated in triplicate and incubated at room temperature for 2 hrs. After three washes, 100 µL/well of biotinylated anti-human IL-1β mAb (100 ng mL^{-1} in PBS, pH 7.0 and 10% FCS) was added, followed by 100 µL/well of streptavidin–peroxidase conjugate. The chromogen substrate (100 µL/well) was applied; after 30 min, 10% H_2SO_4 was added to terminate the reaction. Absorbance was read at 450 nm using an automated microplate reader (BioTek Instruments, Richmond, California, USA). The quantification of IL-10 was also conducted via ELISA using a dedicated ELISA kit.

Determination of TNF-α: The collected supernatants from each treatment were incubated at room temperature and were used to measure TNF-α level using specific Enzyme-Linked Immunosorbent Assay (ELISA) kits, according to the manufacturer's instructions. The TNF-α cytokine levels are shown as the Mean±SD (picograms of each cytokine per milliliter).

Flow cytometric detection of autophagy (LC3): Detection of Autophagy marker LC3 was done according to the

manufacturer's instructions using accuri c6 Becton Dickinson flow cytometer. Briefly, one hundred microliter of cell suspension were blocked with 200 µL of protein block solution (2% BSA, cat. No. 810652; Merck KGaA) for 20 min, at room temperature. Then samples were fixed using 200 µL (1% paraformaldehyde) for 20 min, at room temperature as directed in the instructions for the IntraPrep permeabilization reagent (cat. No. GAS003; Invitrogen; Thermo Fisher Scientific, Inc.). Samples were incubated with 10 µL of primary antibody (rabbit anti LC3A/II (1:100, cat. no. 4108; Cell Signaling Technology, Inc.) was added to the sample in the dark at room temperature for 15 min and washed twice with PBS/BSA and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and 10 µL of secondary polyclonal antibody (IgG) labeled with flour chrome Fluorescein Isothiocyanate (FITC) were added to the resident cells at room temperature in the dark for 15 min. Then cells were washed twice with PBS/BSA as mentioned above. Finally, the labeled cells were fixed with 200 µL of 0.5% paraformaldehyde at 37°C overnight and prepared for flow cytometric analysis. The control cells were incubated with 10 µL secondary antibody alone at room temperature for 15 min and immediately analyzed on an Accuri C6 flow cytometer (Becton Dickinson, Sunnyvale, California, USA). Histogram derived from flow cytometry was obtained with the computer program AccuriC6 software. Staining values were calculated as the percentage of the total number of cells counted. All experiments were repeated three times.

Statistical analysis: All the data were normally distributed; therefore, in instances of single mean comparisons, Levine's test for equality of variances followed by a student's t-test for independent samples was used to assess significance. Level of significance was set at 0.05 for all the analyses. The statistical package for the social sciences release 19 (SPSS, SN: 5087722) was used for all the data analyses).

RESULTS

Table 1 and Fig. 1 presented the GC- MS phytoanalysis of rosemary essential oil, which shows the compounds detected in the rosemary essential oil sample.

The anti-inflammatory and antioxidant effects of rosemary essential oil ex-tract on the SH-SY5Y neuroblastoma cell line compared to normal cells were shown in Fig. 2(a-d) and Fig. 3(a-d). It can be easily noticed that the rosemary oil exhibited dose-independent anti-inflammatory and antioxidant effects against the SH-SY5Y neuroblastoma cell line. Figure 3 demonstrated much lower levels of TNF, vIL-10, IL-6 and IL-1B compared to untreated cells. Figure 3 demonstrated more or less the same trend of anti-oxidant effects of rosemary oil in a dose and time-independent manner. Figure 4(a-b) represented the cell viability of *R. officinalis* L. essential oils against the SH-SY5Y neuroblastoma cell line compared to treated normal cells. Figure 5 presented the remarkable increase of

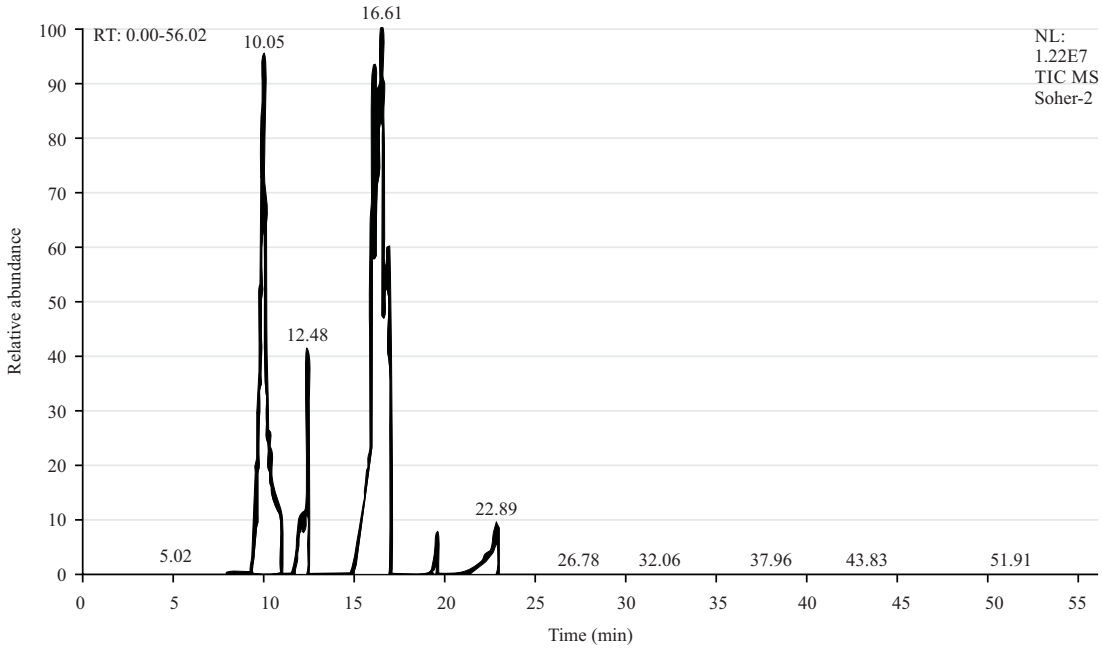

Fig. 1: GC-MS chromatograms of rosemary essential oil

Table 1: GC/MS Phyto analysis of rosemary oil

Identified compounds	R_t (min)	MW	MF	Area (%)
3,3a,6,6a-Tetrahydro-3,6-methano-2H-cyclopenta(b)furan	8.22	122	$C_8H_{10}O$	0.18
Bicyclo[2.2.1]heptane, 2-(1-methylethenyl)-	9.52	136	$C_{10}H_{16}$	0.06
Pseudolimonene	9.60	136	$C_{10}H_{16}$	0.08
α-Thujene	9.76	136	$C_{10}H_{16}$	0.76
2-α-Pinene	10.05	136	$C_{10}H_{16}$	28.81
6-BenzyloxyhexylBromide	10.38	270	$C_{13}H_{19}BrO$	0.70
δ.3-Carene	10.46	136	$C_{10}H_{16}$	0.49
Exo-bicyclo[4.1.0]hept-3-en-7-Isocyanate	10.54	135	C_8H_9NO	0.18
2-(Ethoxypropyl)-1,3-cyclo pentadiene	10.63	154	$C_{10}H_{18}O$	0.09
1-(p-nitrobenzyl)-5-nitro-2H-Indazole	10.77	298	$C_{14}H_{10}N_4O_4$	0.08
(1S)-2,6,6-Trimethylbicyclo[3.1.1] hept-2-ene	11.00	136	$C_{10}H_{16}$	3.26
1,2-Diethyl, benzene	12.17	134	$C_{10}H_{14}$	10.11
Camphor	14.99	152	$C_{10}H_{16}O$	0.06
9-Dimethyl-7,8-dihydroretinal	15.35	272	$C_{19}H_{28}O$	0.04
4-Benzyloxy-2-methylhexa-2,5-Diene	15.48	202	$C_{14}H_{18}O$	0.03
dl-Limonene	15.56	136	$C_{10}H_{16}$	0.04
(+)-6-EXO-HYDROXYCAMPHENE	15.61	152	$C_{10}H_{16}O$	0.12
4-Ethyl-3,5-dimethylpyrrole-2-carboxaldehyde	15.71	151	$C_9H_{13}NO$	0.08
9,12,15-Octadecatrienoic acid, methyl ester,(Z,Z,Z)-	15.88	292	$C_{19}H_{32}O_2$	0.13
3-Butenamide,4-(4-chlorophenyl)-N-(1,1-dimethylethyl)-3-methyl-4-phenyl-, (Z)-	16.04	341	$C_{21}H_{24}ClNO$	6.84
1-(But-2'-phenylsulfonyl)-3-methylbuta-1,2-diene	16.11	186	$C_9H_{14}O_2S$	12.06
(Benzeneacetic acid,4-hydroxy-	16.29	152	$C_8H_8O_3$	2.08
7-Azatricyclo[4.2.2.0(2,5)]decane	16.38	137	$C_9H_{15}N$	2.27
1-(Methoxymethoxy)-3-methyl-3- hydroxybutane	16.61	148	$C_7H_{16}O_3$	10.56
Platydiol[2à,6à-Dihydroxybornane]	16.69	170	$C_{10}H_{18}O_2$	0.08
Cyclopentane, nitro-	16.74	115	$C_5H_9NO_2$	0.73
4-(2-Propynyl)cyclohex-2-en-1-one	17.00	134	$C_9H_{10}O$	14.57
Isobornyl Acetate	19.62	196	$C_{12}H_{20}O_2$	1.72
Bis(4-bromophenyl)(2,4-dibromophenyl)amine	21.41	557	$C_{18}H_{11}Br_4N$	0.04
4-(2-Methylphenyl)furan-2(5H)-one	21.79	174	$C_{11}H_{10}O_2$	0.11
1,2,3-Propanetriol, triacetate	22.13	218	$C_9H_{14}O_6$	0.16
Ethyl-N-methylthiazolidine-4-carboxylate	22.29	175	$C_7H_{13}NO_2S$	0.06
Cyclobutylamine	22.37	71	C_4H_9N	0.12
N-(s)-isobutyl urethane	22.60	145	$C_7H_{15}NO_2$	0.03
1H-1,2,4-Triazole,3-thiol-5-methyl-	22.69	115	$C_3H_5N_3S$	0.03
(3S,5R,6S)-6-Isopropyl-4-methyl-neopentyl-5-phenyltetrahydro-2H-1,4-oxazin-2-one	22.89	303	$C_{19}H_{29}NO_2$	2.01
1,2,3-Propanetriol, triacetate	22.96	218	$C_9H_{14}O_6$	1.23
(4-Bromophenyl)bis(2,4-dibromophenyl)amine	49.12	635	$C_{18}H_{10}Br_5N$	0.03

R_t: Retention time, MW: Molecular weight and MF: Molecular formula

autophagy following treatment with 200 µm as the highest concentration of *R. officinalis* L. essential oil compared to untreated controls.

DISCUSSION

Given the developed knowledge from traditional complementary and alternative medicine and the continuous curiosity in *R. officinalis* L. oil as a potential therapeutic agent, the present data demonstrates significant antioxidant and anti-inflammatory effects of *R. officinalis* L. against SH-SY5Y neuroblastoma cell line.

Almost all the measured pro-inflammatory cytokine levels were reduced upon treatment. There was also a significant decrease in H_2O_2, MDA, NO and ROS as oxidative stress markers which prove the antioxidant effects of the essential oils. The cytotoxic effects of rosemary reported in the current study could find support in the most recent study of Kakouri *et al*.[21] in which they proved the cytotoxic, antioxidant and anti-inflammatory effects of a hydroethanolic extract of *R. officinalis* on glioblastoma (A172) as an aggressive, malignant cancer of the CNS that originates from the glial cells, characterized by poor survival rate cancer cell lines. The major conflict between our study and Kakouri *et al*.[21] study is that they recorded the dose and time-dependent effects of *R. officinalis* extracts while our reported data was time and dose-independent. In an attempt to clarify this contradiction, it is interesting to highlight that in their study, for the first time, they observed that, up to certain concentrations, the extract exhibited comparable toxicity to the control sample. Beyond this threshold, however, the extract demonstrated effectiveness. This phenomenon

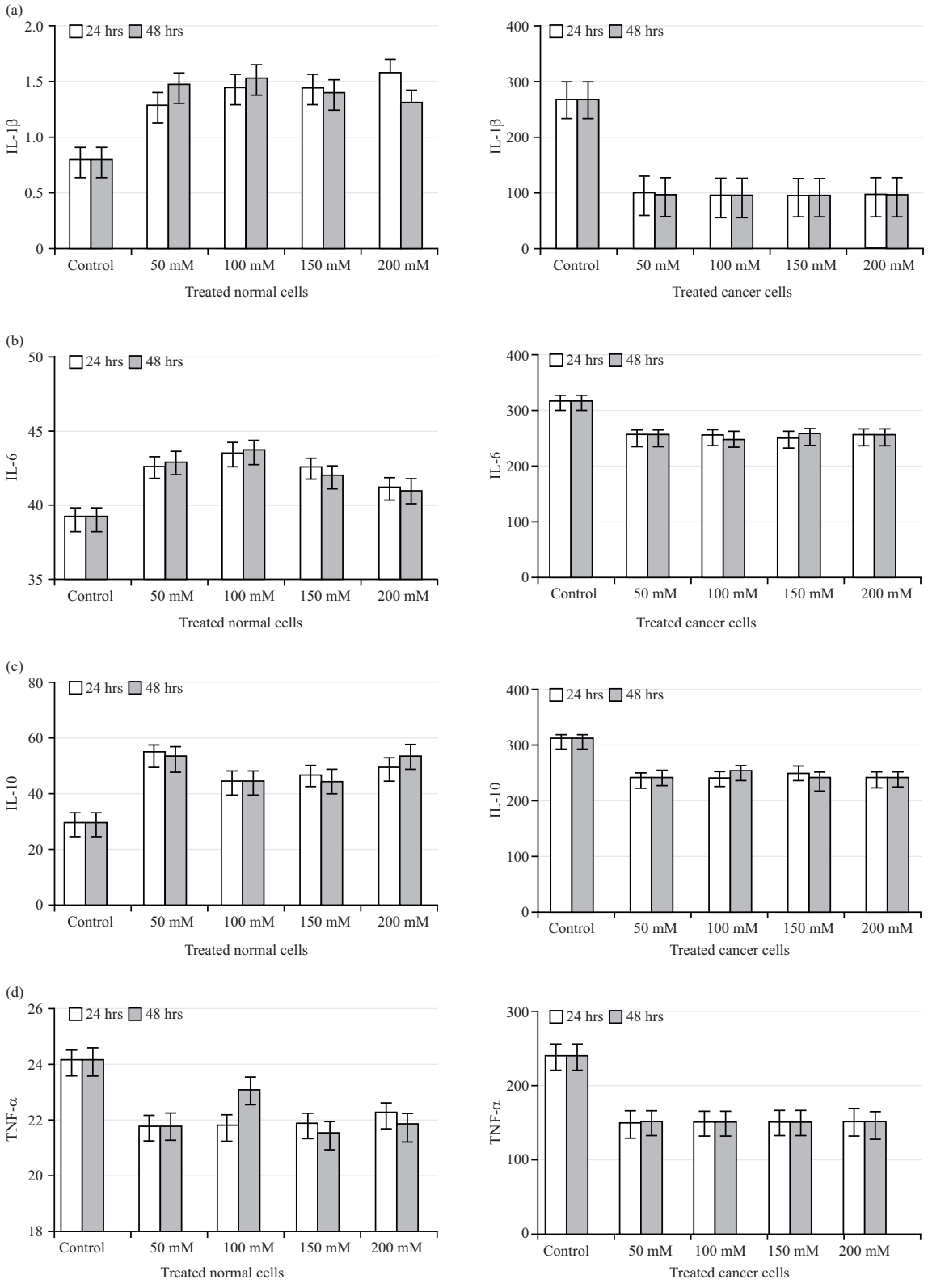

Fig. 2(a-d): Anti-inflammatory effect of serial concentrations of *R. officinalis* L. essential oils on selected cytokines, (a) IL1-1β, (b) IL-6, (c) IL-10 and (d) TNF-α of SH-SY5Y neuroblastoma cell line (right side) compared to treated normal cells (left side)

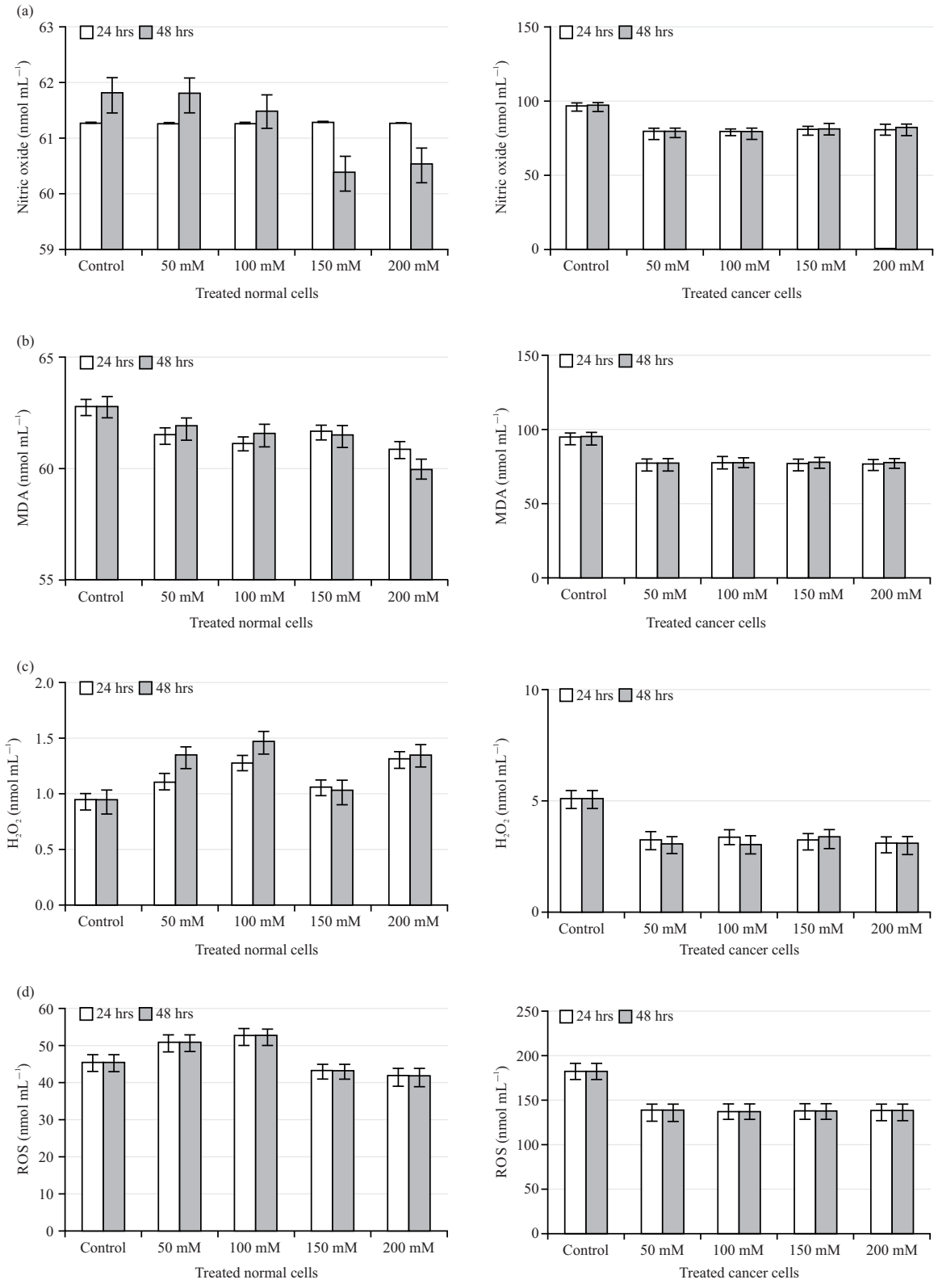

Fig. 3(a-d): Anti-oxidant effect of serial concentrations of *R. officinalis* L. essential oils on selected oxidative stress variables, (a) NO, (b) MDA, (c) H_2O_2 and (d) ROS of SH-SY5Y neuroblastoma cell line (right side)compared to treated normal cells (left side)

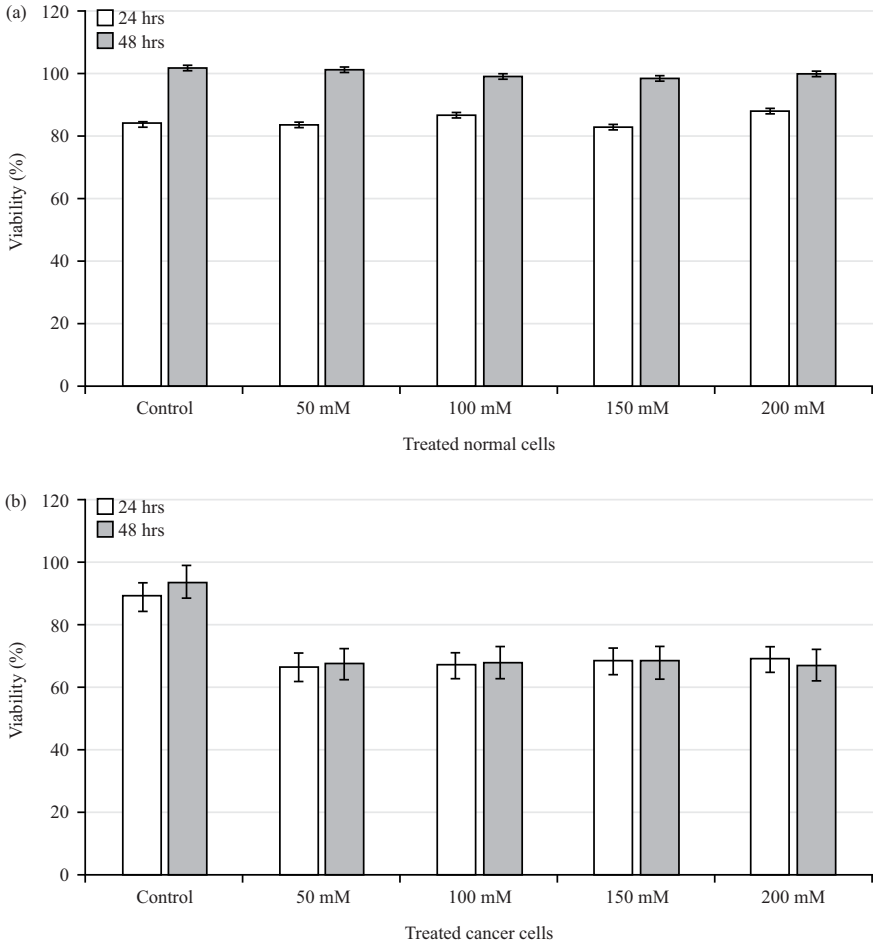

Fig. 4(a-b): Cell viability with of *R. officinalis* L. essential oils against compared to (a) Treated normal cells and (b) SH-SY5Y neuroblastoma cell line

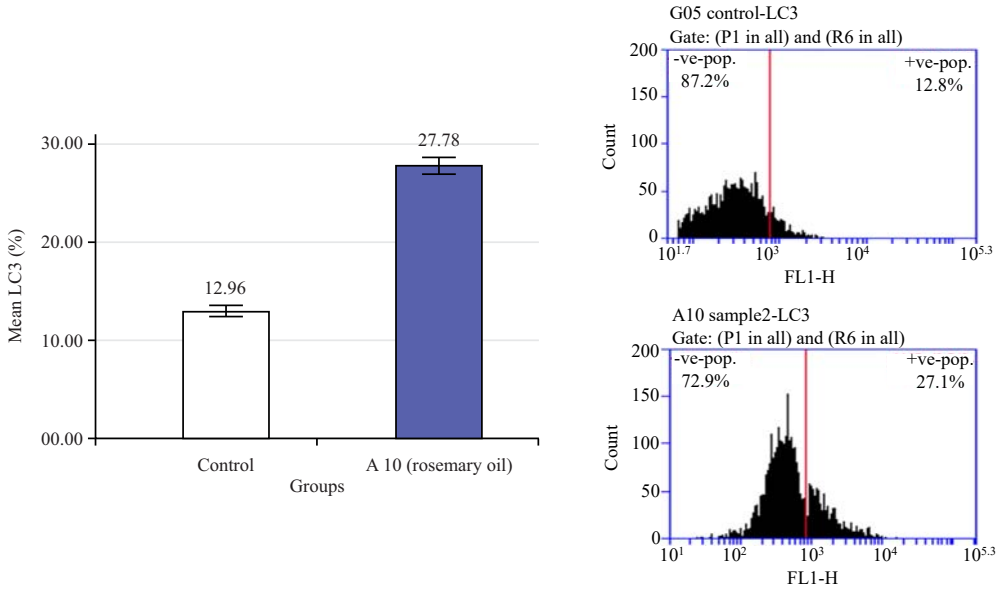

Fig. 5: Level of autophagic activity between control and rosemary oil-treated cells

exhibited both dose-dependent and time-dependent characteristics, remaining consistent at 24, 48 and 72 hrs. This deviated from findings in several studies investigating the impact of *R. officinalis* L. on various cancers such as prostate cancer cells[21,22], melanoma[23] and hematopoietic, epithelial and mesenchymal tumour cell types[24], where a gradual dose-dependent pattern was observed. It appears that Central Nervous System (CNS) cancer cell lines respond differently to drugs. Many brain cancers may inherently exhibit a multidrug resistance (MDR) phenotype, contributing to relapses or disease progression[25]. In an attempt to explain the reported dose and time independence of rosemary oils, the drug resistance of the neuroblastoma cell line was discussed. It is well documented that treatment failure of extracranial solid tumors in childhood is often attributable to drug resistance[26]. Neuroblastoma cell line resistance encompasses multiple cellular mechanisms, counting increased drug efflux through the expression of ATP-binding cassette transporters and the inability of cancer cells to activate an apoptotic response. Moreover, in a neuroblastoma cell line, the generation of sphingolipid species, such as ceramide, has an important role in drug resistance[27]. It has been progressively documented that reactive oxygen species (ROS) among which is Hydrogen Peroxide (H_2O_2) contribute to metabolic oxidative stress and may play a significant role in cytotoxicity, genotoxicity and the development of cancer[28]. Numerous studies suggest that cancer cells exhibit higher levels of ROS compared to normal tissue cells[29]. The surplus ROS can interact with various biomolecules, including DNA, proteins and lipids, generating other radicals or cytotoxic derivatives that may further contribute to the process of carcinogenesis. Increased ROS production also affects cell proliferation and carcinogenesis through an additional mechanism: Acting as a second messenger. This involvement in redox-regulated signaling and gene expression is implicated in enhancing the aggressiveness of cancer[30]. While much is known about ROS in adult solid tumors, little information is known about the role of ROS in pediatric tumors. In the current study, the remarkable reduction in the measured variables, markers of oxidative stress and neuroinflammation could be attributed to the therapeutic effects of rosemary essential oil components including 1,8-cineole, α-pinene, camphor, borneol, camphene and α-terpineol, D-limonene, β-pinene, β-caryophyllene and myrcene[31]. Despite the time and dose independence as a form of resistance of neuroblastoma cell line to *R. officinalis* L., it demonstrates considerable antioxidant effects presenting as lower H_2O_2, NO, MDA and ROS. The literature reveals conflicting findings regarding the dual effects of nitric oxide (NO) in carcinogenesis and tumor progression, emphasizing the complex nature of its role. Inducible NO Synthetase (iNOS) tends to be upregulated in response to inflammation, with elevated NO levels linked to pro-inflammatory cytokines like TNF-α[32,33]. The major inflammatory pathway involving Nuclear Factor kappa B (NF-κB) contributes to iNOS upregulation. Conversely, anti-inflammatory cytokines like IL-10 are associated with lower NO levels. Recent evidence suggests IL-10 is a predictor of severity and mortality in acute or post-acute infection, acting as an endogenous danger signal released by damaged tissues to protect against hyperinflammation. The bimodal effects of NO and its regulation by various cytokines underscore the intricate dynamics, posing challenges and opportunities for targeted treatment strategies in cancer therapy. Researchers and clinicians need to consider the specific conditions and stages of cancer when assessing the implications of NO and its associated pathways[34]. This was in good agreement with the reported data in which the used oil extract induced a remarkable decrease of the pro-inflammatory cytokines concomitant with significantly lower NO in the treated NB cell line (Fig. 2 and 3).

The biochemical results of the current study clearly demonstrated that rosemary activated an autophagic program in SH-SY5Y cells, whether differentiated or undifferentiated. It is easy to see in our study that autophagy is present in control-untreated cells to maintain homeostasis (Fig. 4). These findings could imply that SH-SY5Y cells induce autophagosome formation as an early survival mechanism. According to the phenomenon, autophagy could be a potential mechanism that promotes tumor cell survival and confers chemoresistance[4,35]. The observed increase in autophagy LC3 marker in the current study suggests that signaling events in rosemary oil-driven autophagy may be a partially separate mechanism from those produced by fasting and known to stimulate autophagy via mTOR inhibition[36]. These findings may be consistent with previous research that found autophagy in other types of cancers such as human hypopharyngeal squamous cell carcinoma (HSCC, melanoma and neurological impairment like).

Alzheimer's disease as well[4] where, expression of beclin 1 and LC3II correlates with poor prognosis[37]. Numerous studies in other cancer types and in response to other cancer therapies may provide more support for the observed increase in autophagy, confirming that autophagy is both induced by and confers resistance to the treatment[38]. Rosemary oil augmented LC3II levels in SH-SY5Y cells, indicating that it promoted rather than inhibited autophagosome formation. Although no autophagic-related proteins such as beclin-1 or p62 were investigated it concluded that the autophagic

process initiated by rose oil does not appear to involve the classical autophagy route dependent on beclin-1and not congregate on mTOR kinase[36,39]. These findings could be advantageous since they point to suggesting that the active compounds in rosemary may counteract the downregulation of autophagy in cells with impaired autophagic processes[39]. Additional experiments are essential to uncover the mechanisms by which rosemary influences autophagy and to attribute a potential role for this process in the reported pharmacological effects of rosemary oil in animal models of neurological diseases such as anxiety[40], cerebral ischemia[41] or pain[42]. The current findings are entirely compatible with prior research[36] which identified limonene, a rose fragrance component, as one of the molecules that enhance autophagic flow.

CONCLUSION

In the present study, the substantial rise in autophagy in rosemary-treated cells may indicate that these cell lines are drug-resistant and may support the idea that the reported decline in cell viability in rosemary oil-treated cells is primarily brought on by the antioxidant and anti-inflammatory effects rather than autophagy itself. Here, considering the inherent difficulties in dissecting the molecular mechanisms underlying the biological effects of rose oil mixtures, we engrossed in the whole synergetic effect of the plant. Each constituent of rose marry can subsidize the total effect of a phyto content, which may not be effortlessly simulated by any solitary ingredient given alone. So we have to report the synergetic mechanism of rosemary oil. Together, the evidence we have presented here shows that rose marry oil does as well and has an effect on autophagy *in vitro*. These findings may instigate supplementary investigations to pinpoint the precise.

SIGNIFICANCE STATEMENT

This study elucidates the mechanisms underlying neuroblastoma cell line drug resistance and identifies neuroinflammation and oxidative stress as major biological targets for rational medication development of this malignancy. In the present study, rosemary oil exhibited dose-independent anti-inflammatory and antioxidant effects against the SH-SY5Y neuroblastoma cell line. Rosemary oil use may benefit survivors of high-risk neuroblastoma who require continued multidisciplinary follow-up and lessen the long-term morbidity that commonly accompanies cure with the present therapy.

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