RESEARCH ARTICLE
Bull Biotechnol (2024) 5(2):51-55
https://doi.org/10.51539/biotech.1582575 Journal homepage: www.dergipark.org.tr/biotech

Bulletin of Biotechnology

Investigation of antiproliferative and apoptotic effects of *Rosmarinus officinalis* essential oil obtained by hydrodistillation on neuroblastoma cells

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Received : 10/11/2024
Accepted : 08/12/2024

To Cite: Yumrutaş P, Pehlivan M, Korkmaz M, Kahraman D, Yumrutaş Ö (2024) Investigation of antiproliferative and apoptotic effects of *Rosmarinus officinalis* essential oil obtained by hydrodistillation on neuroblastoma cells. Bull Biotechnol 5(2):51-55 https://doi.org/10.51539/biotech.1582575

Abstract: This study was designed to determine the potential antiproliferative and apoptosis inducing activities of *Rosmarinus officinalis* essential oil (RE) on neuroblastoma cancer cells. For this purpose, different concentrations of RE were applied to SH-SY5Y cells for 24 hours and cell viability was determined by MTT. In addition, the percentage of early, late and non-apoptotic cells was determined by AnnexinV/propodium iodide staining to determine the induction of apoptosis. In addition, the composition of RE was determined by GC-MS. In MTT assay, it was determined that the viability of SH-SY5Y cells decreased dose-dependently as a result of the application of different concentrations of RE. Moreover, 200 mg/ml RE treatment increased the percentage of cells in the late apoptotic phase. The main compounds of RE were determined as (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene, Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl, 1,8-Cineole, Camphor by GC-MS. In conclusion, RE is thought to be an important source of anti-proliferative and apoptosis inducing activity on neuroblastoma cells.

Keywords: Neuroblastoma; *Rosmarinus officinalis*; Apoptosis

1 Introduction

Neuroblastoma is a malignant tumour of neuroblasts, immature nerve cells found in various parts of the body. It usually affects children under 5 years of age. The tumour has the ability to grow rapidly and expand greatly, progressing to death (Quadir et al. 2024). NB is the most common extracranial solid tumour in children. It accounts for 8% of childhood cancers and 15% of childhood cancer deaths. NBs are neural crest developmental tumours arising from the sympathetic nervous system and most tumours occur in the adrenal medulla or along the paraspinal chain in the sympathetic ganglia. The clinical behaviour of NBs varies from spontaneous regression to relentless progression. These neuroblastic tumours show various histologies ranging from undifferentiated neuroblastoma to ganglioneuroblastoma or mature ganglioneuroma (Kamihara et al. 2024).

The most prominent features of malignant cell transformation and tumourisation are cell proliferation or irregularities in the apoptotic pathway. Apoptosis is programmed cell death and many physiological events are controlled by apoptosis (Letai 2017). Briefly explained, cells are eliminated by activating the apoptotic pathway as a result of mutations that occur in cells and cannot be repaired. However, as a result of irregularities in the apoptotic pathway, cells can escape from apoptosis and proliferate rapidly by entering the pathway of cancerisation. Moreover, it is thought that cells can be suppressed by activation of apoptosis in neuroblastoma cells (Fulda and Debatin 2003).

Previous studies have shown that plant materials play important roles in the activation of apoptosis and suppression of cell proliferation in different cancer cells. Especially essential oils have antiproliferative and apoptosis inducing activities due to important phytochemicals in their structure (Dhifi et al. 2016; Nieto 2017). Rosmarinus officinalis L. (Salvia rosmarinus Schleid), a plant belonging to the Lamiaceae family, is popularly known as Rosemary. It originates from the Mediterranean region, but can be found

all over the world. It is a shrubby perennial and aromatic herb with green leaves emitting a characteristic odour. R. officinalis can be used as a spice, as a natural preservative in the food industry and as an ornamental and medicinal plant. (González-Trujano et al. 2007; Pérez-Fons et al. 2010; Brewer 2011; Rašković et al. 2014). It exhibits important biological activities due to many phytochemicals in its structure (Hussain et al. 2010). It has been reported to have antioxidant (Hendel et al. 2024), anticancer (Wang et al. 2012), antibacterial (Bajalan et al. 2017), antifungal (Ozcan and Chalchat 2008) activities, especially in studies conducted with the essential oil of R. Officinalis. Although there are studies on the suppression of different cancer cells, studies showing the proliferative and apoptotic effects of this oil on neuroblastoma cells are very limited. Therefore, in this study, it was aimed to determine the antiproliferative and apoptosis inducing activities and chemical content of RE on neuroblastoma cells.

2 Materials and Method

2.1 Collection and extraction of *R.officinalis*

R.officinalis was collected from campus area of Gaziantep University. Then, washed with distilled water and was dried on blotting paper in the open air and in a room away from sunlight. Then, it was ground in a mortar before the extraction. 100 g of *R.officinalis* was placed in a Clavenger flask and extracted by hydrodistillation for 3 h. The *R.officinalis* essential oil (RE) was stored in the refrigerator (+4 C) until the experiments were started.

2.2 Maintenance and growth of SH-SY5Y cells

SH-SY5Y cells were grown with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic (Gibco, USA) supplemented in DMEM. Cells were maintained in an incubator with a 5% CO_2 supply at 37 °C. After cultivation, SH-SY5Y (6 x 10^4 cells/mL) cells were seeded onto 96 well plates and cultured for 24 h on DMEM medium with supplements. After 24 h incubation, the old medium was aspirated and replenished with a new medium containing different concentrations (50, 100, 150 and 200 µg/mL) of RE with serum-free medium and exposed for 24 h under specified conditions. RE was dissolved in DMSO (at concetration of 10% of dimethyl sulfoxide).

2.3 Determination of anti-proliferation activity

After preserving SH-SY5Y cells in DMEM for 24 hours, 96-well plates containing 70–80% confluent of lung cancer cell cultures were treated for 24 hours with various dilutions of RE (50, 100, 150 and 200 µg/mL). MTT was utilized to evaluate cell viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). Replaced the culture media with DMEM containing 1 mg/mL MTT (Sigma) and incubated at 37 °C for 15 minutes. The cells were then treated with MTT solution and dimethyl sulfoxide (DMSO, Sigma). The density of the cells will be measured at 550 nm with a colorimetric reader (BioTek instrument, USA).

2.4 Determination of apoptosis induction by AnnexineV and Propodium iodide (PI)

For the determination of apoptosis induction activity of RE, SH-SY5Y cells were seeded with a $1x10^6$/mL density to 6-well plates and lowest (50µg/mL) and highest (200 µg/mL) concentrations of RE were applied for a period of 24 hours. Annexin V/PI apoptosis detection kit was used to measure the apoptosis according to manufacturer' recommended protocol. Results were measured in Becton-Dickinson flow cytometer.

2.5 Statistically analysis

Statistical analysis was carried out using GraphPad Prism 8.0.2. program. Dunnett's test was used for statistical evaluation for antiproliferation, apoptosis and the mRNA levels. In evaluation of statistical, the terms "*", "**" and "***" were meant $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

3 Results

3.1 Determination of antiproliferative activity

It was observed that the viability of SH-SY5Y cells decreased as a result of RE treatment for 24 hours (Figure 1). Figure 1 shows that 50 µg/ml RE concentration had no effect on cell viability, but 100 ($p<0.05$), 150 ($p<0.01$) and 200 ($p<0.01$) µg/ml RE concentrations inhibited cell proliferation in a concentration-dependent manner. After the application of 100, 150 and 200 µg/ml concentrations, 71.31%, 61.53% and 25.58% of SH-SY5Y cells remained viable, respectively.

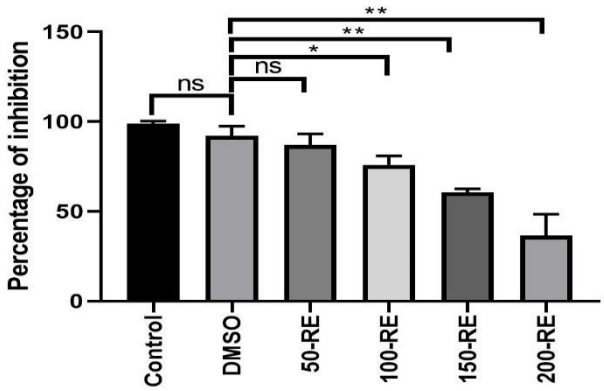

Fig. 1 The antiproliferative activity of RE on SH-SY5Y cells

3.2 Induction of apoptosis

In this experiment, cells were exposed to the lowest and highest dose of RE for 24 hours and then stained with AnnexinV/PI and early apoptotic, late apoptotic and non-apoptotic cell percentages were determined by flow cytometry. When Figure 2 was analysed, it was determined that 50 µg/ml RE concentration did not affect cell percentages in non-apoptotic early apoptosis and late apoptosis phases compared to control. On the other hand, after the application of 150 µg/ml RE concentration, the amount of viable cells decreased, the percentage of cells in the non apoptotic phase increased from 0.3% to 7.0%, the percentage of cells in the early apoptotic phase increased from 0.1% to 1.5% and the percentage of cells in the late apoptotic phase increased from 2.2% to 16.5%.

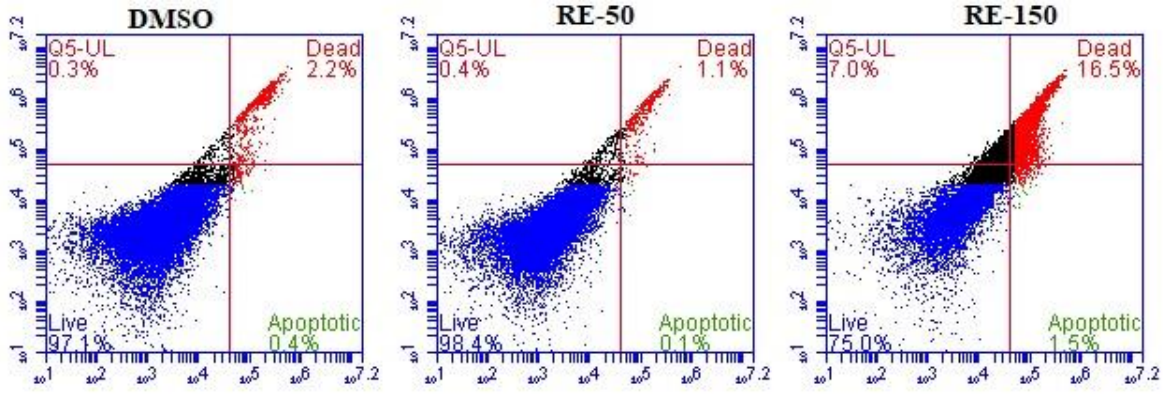

Fig. 2 Determination of apoptosis induction after RE application

3.3 Screening of RE content by GC-MS

As a result of screening the content of RE by GC-MS, 57 compounds were determined (Table 1). The identified compounds constitute 100 % of the total oil. However, the main compounds of RE were (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (24.66%), Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl (13.48%), 1,8-Cineole (11.21%), Camphor (6.71%).

Table 1 Content of RE by GC-MS

No	R.Time	Name	%
1	1.901	1,3-Pentadiene, 2-methyl-, (E)-	0.09
2	3.494	Tricyclene	0.14
3	3.719	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	24.66
4	4.109	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1R)-	0.06
5	4.215	Camphene	3.27
6	4.765	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	1.64
7	4.955	Sabinene	0.05
8	5.006	Verbenene	0.44
9	5.060	2-Pentanone, 4-hydroxy-4-methyl- (CAS)	0.02
10	5.586	.beta.-Myrcene	4.58
11	5.801	.alpha.-Terpinene	0.87
12	6.088	D-Limonene	4.17
13	6.221	1,8-Cineole	11.21
14	6.553	Furan, 2-pentyl-	0.03
15	6.611	trans-.beta.-Ocimene	0.18
16	6.771	.gamma.-Terpinene	1.85
17	7.122	Benzene, 1-methyl-4-(1-methylethyl)- (CAS)	1.27
18	7.324	.Alpha.-Terpinolene	1.38
19	8.947	.Alpha.-Fenchone	0.04
20	9.510	Benzene, Methyl(1-Methylethenyl)-	0.06
21	9.558	Filifolone	0.07
22	9.846	Trans-Sabinene Hydrate	0.13
23	9.895	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans-	0.22
24	10.246	.Alpha.-Campholene Aldehyde	0.09
25	10.492	Chrysanthenone	0.21
26	10.629	Camphor (CAS)	6.71
27	10.834	trans-3(10)-Caren-2-ol	0.34
28	10.954	1,6-Octadien-3-ol, 3,7-dimethyl-	2.85
29	11.025	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-, (1.alpha.,2.alpha.,5.alpha.)-	0.89
30	11.189	p-menth-2-en-1 –ol	0.08
31	11.321	Pinocarvone	0.24
32	11.517	Bornyl acetate	3.64
33	11.647	Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl-	0.10
34	11.722	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	2.36
35	11.801	Caryophyllene	0.23
36	11.906	Spiro [bicyclo[3.3.0] octan-6-one-3 - cyclopropane]	0.17
37	12.027	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	0.05
38	12.344	Cyclohexanone, 5-methyl-2-(1 - methylethylidene)-	0.71
39	12.416	(S)-cis-Verbenol	0.20
40	12.622	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, (S)- (CAS)	0.24
41	12.726	(S)-cis-Verbenol	0.71
42	12.885	Cyclopentane, 1 -acetoxymethyl-3-isopropenyl-2-methyl-	0.09
43	12.980	.Alpha. Terpineol	2.89
44	13.079	endo-Borneol	1.17
45	13.208	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	13.48
46	13.395	p-Mentha-1,5-dien-8-ol (CAS)	0.10

47	13.448	Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl- (CAS)	0.22
48	13.606	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)- (CAS)	0.20
49	14.140	.alpha.-Campholenal	0.19
50	14.439	2-Heptene, 5-ethyl-2,4-dimethyl- (CAS)	0.27
51	14.579	Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl- (CAS)	1.38
52	15.342	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	0.18
53	15.416	cis-Myrtanol	0.40
54	15.577	trans-Geraniol	2.69
55	17.142	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)- (CAS)	0.14
56	17.611	2-Cyclopenten-1-one, 3-methyl-2-(2-pentenyl)-, (Z)-	0.25
57	19.209	Benzene, 1,2-dimethoxy-4-(2-propenyl)- (CAS)	0.10
		Total (%)	100.00

4 Discussion

Millions of people pass away from cancer every year. Medicinal plants are used in cancer research by many researchers due to the different chemicals they contain and the low possible side effects of these chemicals. Rosmarinus officinalis is one of the most well-known plants among medicinal plants and its oil and other extracts have been tested for the suppression of cancer types. Significant cytotoxic effects have been reported in many anticancer studies with essential oil and extracts of R. officinalis. Santos et al. (2016) reported that RE had no cytotoxic activity on Hela cells. However, Jardak et al. 2017 reported that 0.01 and 0.253 µl/ml doses exhibited potent cytotoxic activity on cervix (HeLa) and breast (MCF7) cancer cells, respectively. In a different study, Hussain et al. (2010) reported that RE suppressed the proliferation of MCF7 cells. In addition, Dolghi et al. 2022 determined that the viability and proliferation of HCT-116 colorectal cancer cells decreased significantly, especially at doses of 100-500 µg/ml. In a different study, the proliferation of HepG2 and ECV304 cells was determined at doses of 508.7 µg/ml and 525.7 µg/ml, respectively (Becer et al. 2023). In our study, possible antiproliferative effects of RE on neuroblastoma cells, one of the childhood tumours, were tested. In the light of the data obtained, it was determined that especially 100 and 200 mg/ml doses suppressed neuroblastoma cells. In this context, it can be said that RE has agents that can reduce the proliferation and viability of different cancer cells. In addition, in previous studies, the number of studies showing the cytotoxic activity of RE on neuroblastoma cells is very limited and therefore the results obtained in our study are important in terms of cytotoxicity.

It was stated that neuroblastoma cells would be suppressed by inducing apoptosis. With the activation of caspases involved in both mitocontrial and extrinsic apoptotic pathways, the DNA of neuroblastoma cells can be fragmented and the cells are removed (Fulda and Debatin 2003). However, it has also been shown that neuroblastoma cells are suppressed by non-apoptotic cell death pathways such as necrosis (Fulda and Debatin 2003). In our study, it was determined that early and late apoptotic and non-apoptotic neuroblastoma cell percentages increased at 200 µg/ml concentration. It can be concluded that RE induces apoptotic cell death and non-apoptotic cell death.

It has been shown in previous studies that RE collected from different geographies contain different types and amounts of chemicals (Santos et al. 2016). They identified a total of 19 compounds in RE and 1,8-cineole (52.2%), Camphor (15.2%) and alpha-pinene (12.4%) were the main compounds of the oil. In another study, the main components of RE were 1,8-cineole (23.56%), camphene (12.78%), camphor (12.55%) and β-pinene (12.3%). Dolghi et al (2022). They determined 23 compounds in RE. However, the main components of the oil were eucalyptol (33.592%), α-pinene (12.239%), L-camphor (12.2%), β-Thujene (9.709%), β-pinene (9.435%), camphene (5.723%). Husaain et al. 2010 determined the main compounds of RE as 1,8-cineol (38.5%), camphor (17.1%), -pinene (12.3%), limonene (6.23%), camphene (6.00%), and linalool (5.70%). However, Becer et al. 2023 found the main compounds of RE as camphor (15.1%), verbenone (14.3%), α-pinene (13.6%), 1,8-cineole (11.8%), and borneol (7.9%). In addition, the main compounds of RE in our study were (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (24.66%), bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl (13.48%), 1,8-Cineole (11.21%), Camphor (6.71%). It is thought that the different effects on different cancer cells may be due to the type, amount and interaction of these chemicals with each other.

5 Conclusion

In conclusion, antiproliferative and apoptotic effects of RE on neuroblastoma cells SH-SY5Y were determined in this study. However, it is recommended to determine the levels of caspases, BCL2, caspase inhibitors and necrotic factors such as RIPK and MLK1 for non-apoptotic effects in order to understand the apoptotic effects more clearly.

Acknowledgements

The authors would like to thank Adiyaman, Gaziantep and Gaziantep Islam Science and Technology Universities for their support.

Authors' contributions:

PY: Methodology, Data curation, writing draft. MP: Methodology, Material collection, Data curation. MK: Writing, editing draft. DK: Methodology, Data curation. ÖY: Supervision, Data curation, Writing, editing draft.

Conflict of interest disclosure:

The authors declare that there is no conflict of interest.

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