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Effects of olive oil-based propolis, caffeic acid phenethyl ester, and methylprednisolone on differentiation of human acute myeloid leukemia cells

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ABSTRACT: Leukemia, originating from the hematopoietic system, is a malignant disease for the treatment of which various glucocorticoids are used. The use of natural products alongside conventional therapy has focused on increasing drug-drug interactions and reducing potential side effects. Based on the idea that propolis and caffeic acid phenethyl ester (CAPE) can stimulate cancer cells to differentiation and enhance the effect of chemotherapeutic drugs, we conducted this study. In this study, we investigated whether the synergistic effect of olive oil-based propolis and CAPE is effective with metylprednisolone (MP), which is used for supportive therapy in AML. In the study, OEP (Olive Oil-Based Propolis), CAPE, MP and their combinations were applied to HL -60 cells for 1-3 days. Then, CD11b, CD14 and CD68 antigens were detected on HL -60 cells using flow cytometry techniques to determine the cellular differentiations. As a result, HL -60 cells were found to be significantly directed to CD11b differentiation. OEP and CAPE will enhance the effect of chemotherapy by differentiating cells and ensure treatment completion by minimizing damage to normal tissues and cells.

KEYWORDS: Acute myeloid leukemia; differentiation; Caffeic acid phenethyl ester; Metylprednisolon; Olive Oil-Based; Propolis.

1. INTRODUCTION

Hematopoiesis, the formation of blood cells, originates from pluripotent stem cells that can self-renew and differentiate into various cell lineages (1). Pluripotent stem cells are directed to develop into cells of the myeloid or lymphoid lineage in the bone marrow (2,3). Systemic hormones play an important role in regulating hematopoietic stem cells, which are recognized by almost all hormone receptors (4). Inactivation of many genes in the metabolic pathways that regulate cellular functions can cause leukemia (5). Leukemia is a clonal and neoplastic blood disease that originates from the hematopoietic system (6).

The search for new drugs that can act as chemotherapeutic agents and have low side effects, especially from natural products, has increased in recent years (7). Many researchers have turned to natural and high-quality products believed to have antioxidant, antimicrobial, anti-inflammatory, and similar properties to treat and prevent disease. Treatment with bee products (Apitherapy) and treatment with herbal products are the most studied supportive treatments (8). Bee products have been used to treat many diseases since ancient times. Turkey has several biogeographical features due to its location on the earth, and therefore it is able to practise all types of beekeeping (9). Natural bee products such as honey, propolis, pollen and royal jelly are very important for human life and health. These products are not only consumed as food but also used in alternative medicine (10). Numerous biological effects have been demonstrated depending on the polyphenol content of bee products (11,12). The chemical composition and pharmacological effects of propolis, obtained by modifying special resins and waxy substances collected from honey bees (*Apis mellifera* L.) with salivary enzymes, may vary (13-16). Propolis is effective against various cancers by preventing metastasis, interrupting the cell cycle, inducing apoptosis, and mitigating the harmful side effects of chemotherapy (17). In many studies, propolis has been used by extraction with various solvents (18). One of

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the most potent antioxidant substances contained in propolis, whose antioxidant capacity may vary depending on the type of polyphenols, is CAPE (19).

In this study, the flow cytometric differentiation status of AML cells exposed to MP, OEP and CAPE was determined using the cell markers CD11b, CD14 and CD68.

2. RESULTS

The summaries of flow cytometric data on the degree of differentiation of HL-60 cells administered OEP, CAPE, MP, and their combinations for 24 hours are shown in Table 1. The flow cytometric images of CD11b, CD14 and CD68 are shown in Figure 1, and their comparative evaluations are shown in the graph in Figure 2. Compared to the control group, CD11b differentiation was statistically highest in the group receiving OEP at 24 hours (p=0.001, p≤0.05). Statistically higher CD11b differentiation was seen in the groups treated with MP+CAPE (p=0.006) and CAPE (p=0.039) compared to the control group (p≤0.05). The CD11b differentiation level in cells treated with OEP was also higher than in cells treated with the combination MP +OEP (p=0.006, p≤0.05). Although there was a statistical difference between groups in CD14 and CD68 differentiation at 24 hours, no significant increase was observed compared to the control group (p≥0.05).

Table 1. The degree of differentiation of CD11b/CD14 and CD68 in OEP, CAPE, MP, MP+OEP and MP+CAPE treated HL -60 cells after 24 hours.

Cell Markers	Control	OEP	CAPE	MP	MP+OEP	MP+CAPE
CD14	0,36[f]	0,55[d,e,f]	0,32[f]	0,18[b,f]	0,17[a,b,f]	0,70[c,d,e]
CD14+CD11b	0,10	0,21	0,05	0,14	0,20	0,23
Non-differentiating cell	95,75	90,33	95,58	95,66	95,67	94,45
CD11b	3,79[b,c,f]	**8,91[a,d,e]**	**4,06[a]**	4,02[b]	3,96[b,f]	**4,62[a,e]**
Unstained cell	0,03	0,03	0,06	0,05	0,01	0,00
Unstained cell +CD68	0,07	0,19	0,10	0,08	0,08	0,03
Undifferentiated cell	99,78	99,36	99,14	99,78	99,87	99,97
CD68	0,12[f]	0,43[e,f]	0,70[d,e,f]	0,09[c]	0,03[b,c]	0,00[a,b,c]

*The meaning values are as follows: [a]p<0.05 vs control, [b]p< 0.05 vs OEP group, [c]p<0.05 vs CAPE group, [d]p<0,05 vs MP group, [e]p<0.05 vs MP+OEP, [f]p<0.05 vs MP+CAPE.

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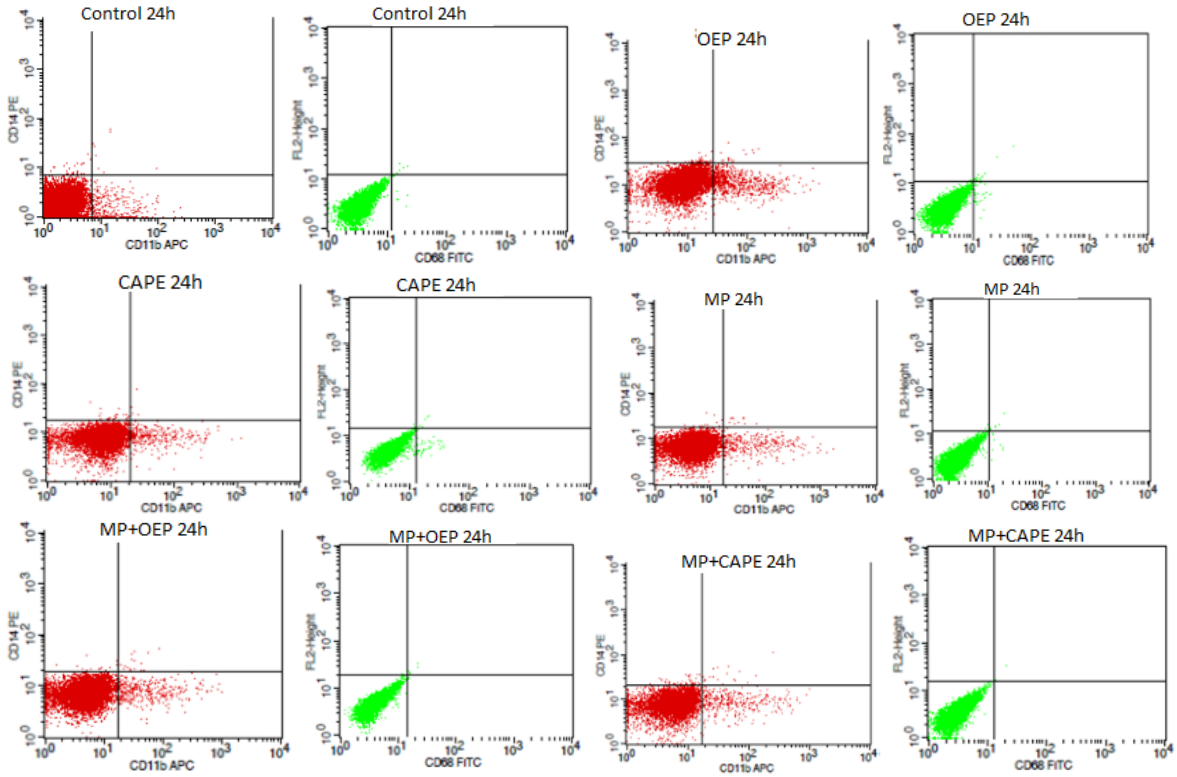

Figure 1. Flow cytometric measurement data of differentiation level in OEP, CAPE, MP, MP+OEP and MP+CAPE treated HL -60 cells after 24 hours.

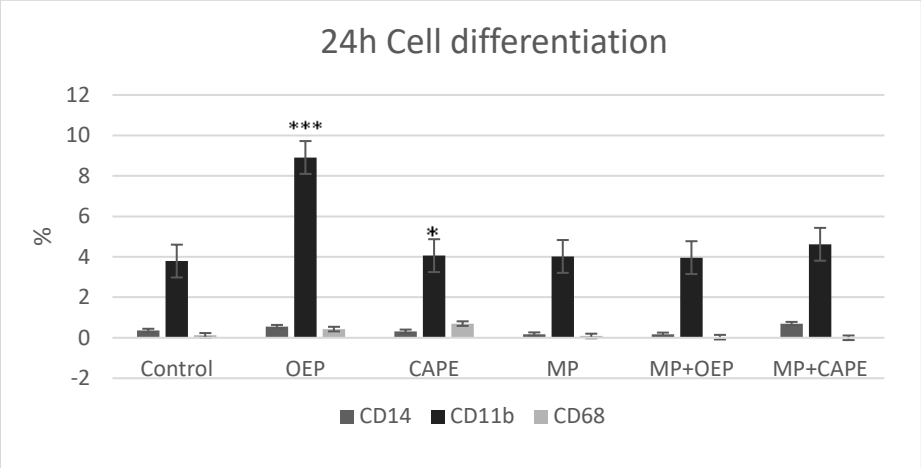

Figure 2. Graph of differentiation level in HL -60 cells in the 24-hour treatment groups. ***p value less than 0.001; **p value ranging from 0.001 to 0.01; *p value ranging from 0.01 to 0.05.

Flow cytometric data on the differentiation level of HL -60 cells after 48 hours exposed to OEP, CAPE, MP and their combinations are shown in Table 2. Flow cytometric images of CD11b/CD14 and CD68 differentiation are shown in Figure 3 and a graph showing the difference between the groups is shown in Figure 4. We can see the highest expression of CD11b in HL -60 cells treated with OEP compared to the control group (p=0.001, p≤0.05). In cells treated with the combination of MP (p=0.006) and MP+OEP (p=0.039), the CD11b differentiation level statistically increased compared to the control group (p≤0.05). When CD14 differentiation was statistically examined after 48 hours, it was found that the OEP (p=0.012), CAPE (p=0.022) and MP+OEP (p=0.001) groups had statistically higher CD14 differentiation than the control group (p≤0.05). In addition, no significant increase in CD68 differentiation was observed compared to the control group (p≥0.05).

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Table 2. The degree of differentiation of CD11b/CD14 and CD68 in OEP, CAPE, MP, MP+OEP and MP+CAPE treated HL -60 cells after 48 hours.

Cell Markers	Control	OEP	CAPE	MP	MP+OEP	MP+CAPE
CD14	0,02[b,c,e]	0,70[a,d]	0,68[a]	0,22[b,d]	0,82[a,d,f]	0,24[e]
CD14+CD11b	0,00	0,25	0,03	0,07	0,26	0,01
Non-differentiating cell	97,72	70,27	96,44	92,58	94,27	96,10
CD11b	2,26[b,d,e]	**19,78[a,c,e]**	2,84[b,d]	**7,13[a,c]**	**4,65[a]**	3,65[b]
Unstained cell	0,08	0,32	0,04	0,04	0,12	0,10
Unstained cell +CD68	0,16	0,50	0,19	0,05	0,22	0,10
Undifferentiated cell	99,27	95,67	99,58	99,88	99,41	99,63
CD68	0,50[d,f]	3,51[c,d,f]	0,19[b]	0,03[a,b,e]	0,25[d]	0,18[a,b]

*The meaning values are as follows: [a]$p<0.05$ vs control, [b]$p< 0.05$ vs OEP group, [c]$p<0.05$ vs CAPE group, [d]$p<0,05$ vs MP group, [e]$p<0.05$ vs MP+OEP, [f]$p<0.05$ vs MP+CAPE.

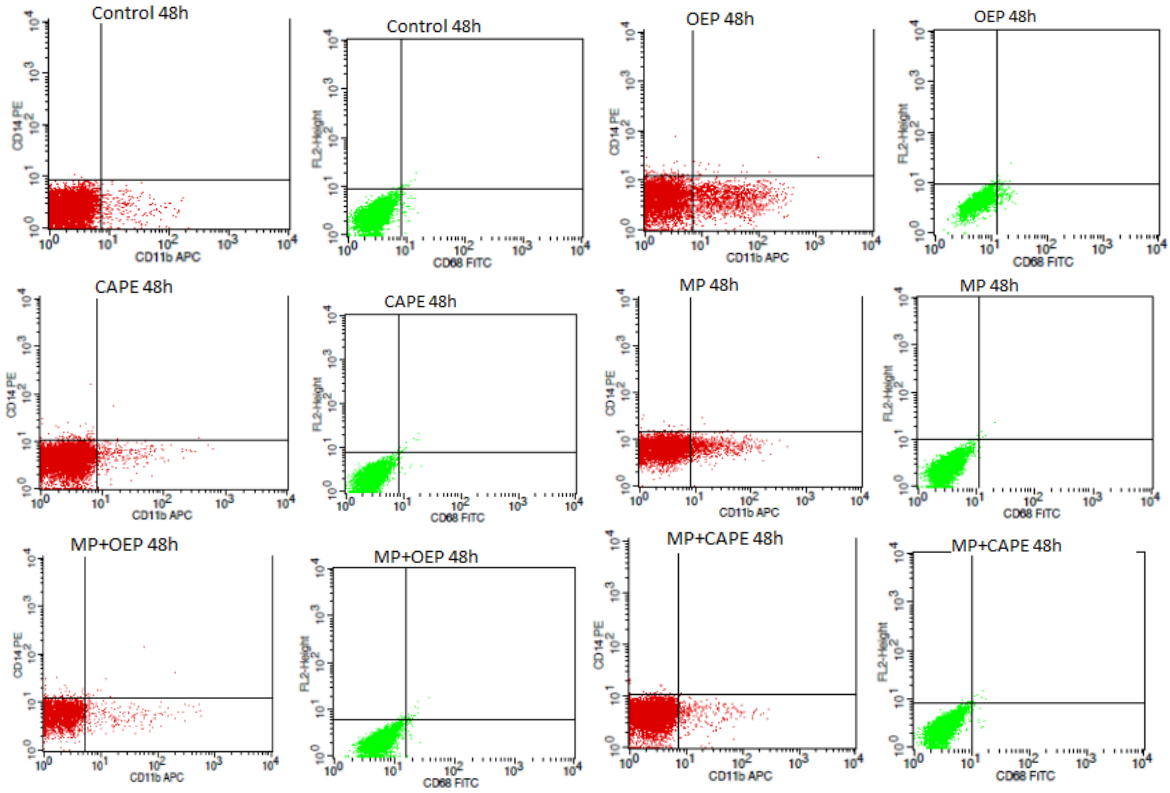

Figure 3. Flow cytometric measurement data of differentiation level in OEP, CAPE, MP, MP+OEP and MP+CAPE treated HL -60 cells after 48 hours.

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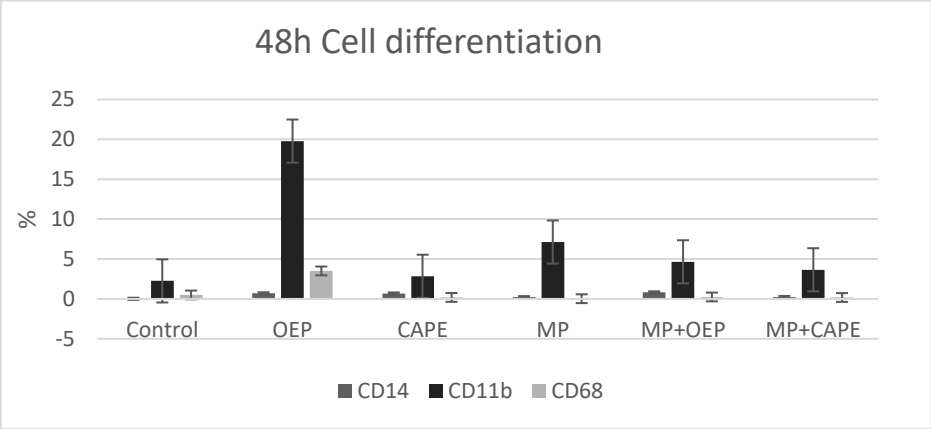

Figure 4. Graph of differentiation level in HL -60 cells in the 48-hour treatment groups. ***p value less than 0.001; **p value ranging from 0.001 to 0.01; *p value ranging from 0.01 to 0.05.

The data obtained after 72 hours of application of OEP, CAPE, MP, MP+OEP and MP+CAPE to HL -60 cells are shown in Table 3. Flow cytometric images of CD11b/CD14 and CD68 differentiation are shown in Figure 5, and a comparison plot is shown in Figure 6. When the 72-hour data were examined, the highest CD11b differentiation was observed in the OEP group (p=0.000, p≤0.05). CD11b differentiation was significantly increased in the MP+OEP group (p=0.022) compared to the control group (p≤0.05). It was observed that CD14 differentiation was not significant at 72 hours compared to the control group (p≥0.05). CD68 differentiation is observed, albeit slightly, in the OEP and combination groups (p≤0.05).

Table 3. The degree of differentiation of CD11b/CD14 and CD68 in OEP, CAPE, MP, MP+OEP and MP+CAPE treated HL -60 cells after 72 hours.

Cell Markers	*Control*	*OEP*	*CAPE*	*MP*	*MP+OEP*	*MP+CAPE*
CD14	3,13^{d,e}	3,82^{c,d,e}	1,04^{b}	0,27^{a,b,f}	0,58^{a,b}	1,14^{d}
CD14+CD11b	0,00	0,76	0,12	0,06	0,31	0,25
Non-differentiating cell	96,26	76,91	98,26	90,31	84,70	88,57
CD11b	0,60^{b,e}	**18,50^{a,c,d}**	0,58^{b,e,f}	9,36^{b}	**14,41^{a,c}**	10,05^{c}
Unstained cell	0,38	0,35	0,00	0,15	0,05	0,18
Unstained cell +CD68	0,00	1,05	0,13	0,07	0,20	0,16
Undifferentiated cell	99,62	97,91	99,48	99,74	99,37	99,48
CD68	0,00^{b,c,e}	0,70^{a,d,f}	0,39^{a}	0,05^{b}	0,39^{a}	0,18^{b}

*The meaning values are as follows: ^{a}p<0.05 vs control, ^{b}p< 0.05 vs OEP group, ^{c}p<0.05 vs CAPE group, ^{d}p<0,05 vs MP group, ^{e}p<0.05 vs MP+OEP, ^{f}p<0.05 vs MP+CAPE.

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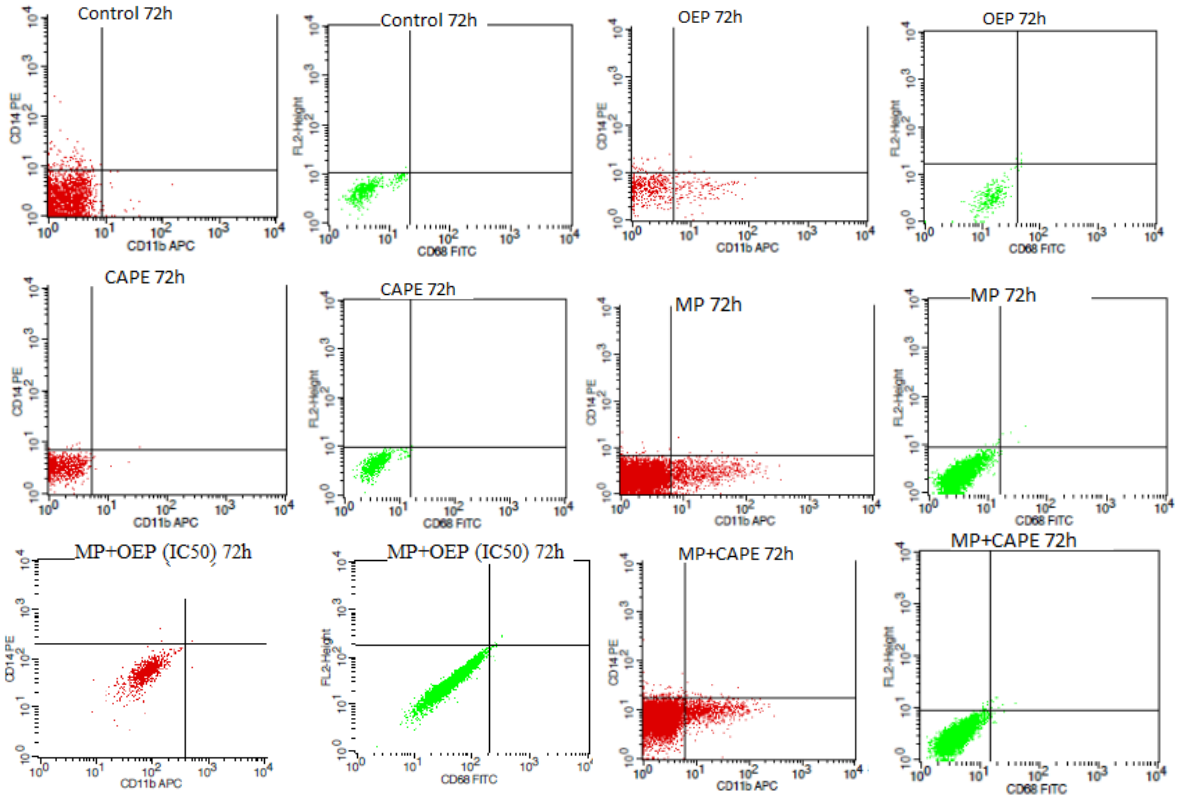

Figure 5. Flow cytometric measurement data of differentiation level in OEP, CAPE, MP, MP+OEP and MP+CAPE treated HL -60 cells after 72 hours.

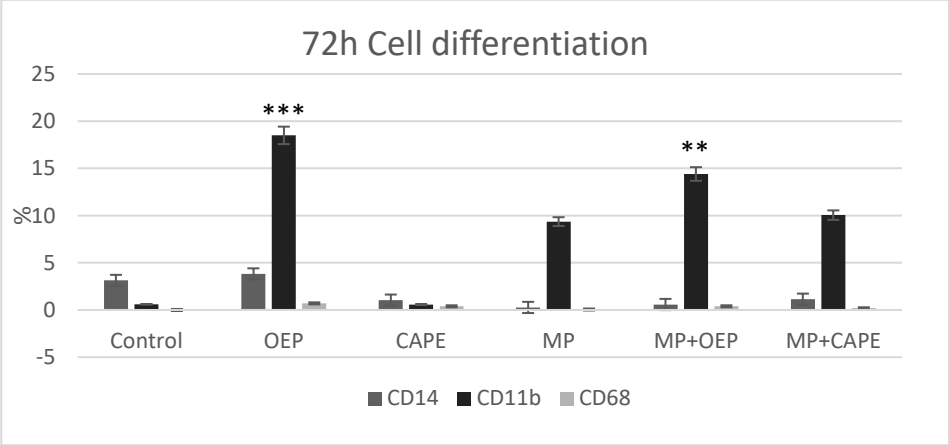

Figure 6. Graph of differentiation level in HL -60 cells in the 72-hour treatment groups. ***p value less than 0.001; **p value ranging from 0.001 to 0.01; *p value ranging from 0.01 to 0.05.

3. DISCUSSION

Many studies on the support of drug therapy by natural products focus on the elucidation of their mechanism of action. In this context, apitherapy and phytotherapy are the most studied areas. Propolis, an apitherapeutic product, is a natural product that should be highlighted in terms of its content of highly effective compounds. One of the most studied compounds of propolis is CAPE (20,21). It is known that there is a synergistic effect between propolis and anticancer agents, and that blast cells are directed to apoptosis and cell differentiation due to this effect (22,23). Various chemotherapeutic agents used for treatment can damage normal tissues and cells while healing damaged cells and tissues (24). In this study, we focused on propolis, which is known to prevent tissue and organ damage, and CAPE, the most active component of

Turkish propolis. In this direction, OEP and CAPE were applied to HL -60 cells alone or in combination with MP, which can be used during supportive chemotherapy.

Researchers have found that MP, one of the most important members of the steroid hormone family, causes granulocytic and monocytic differentiation of cells (25-28). In a study we conducted in recent years, the effect of MP' various doses in HL -60 cells on differentiation was measured by flow cytometry using cell surface antigens (29). The previous study was confirmed by the data of the current study. While CD11b expression increased in the MP -treated groups, there was no change in CD14 and CD68 gene expression. In a study comparing extraction of Brazilian propolis with water and ethanol with ATRA, phenolic compound residues extracted from propolis resulted in granulocytic differentiation in HL -60 cells (30). In parallel with these data, a significant increase in the concentration of the granulocytic differentiation marker CD11b was observed in all hours when we administered olive oil-based propolis. In a study by Kitamuro et al, CD11b differentiation was induced in mice by an ethanolic extract of Brazilian propolis (31). In a study in which the level of the granulocytic differentiation marker CD11b/CD14 was determined by flow cytometry by applying CAPE, ATRA and their combinations to HL -60 cells, the combinations significantly increased the level of CAPE, CD11b differentiation with decreasing/increasing dose (32). In our study, there was a significant increase in CAPE and its combination in the first 24 hours compared with the control group, whereas the increase in the other hours was not statistically significant. According to the data of the study, although the CD14 level was low in percentage at the 48th hour, it increased statistically in the groups treated with OEP, CAPE, and MP +CAPE. In addition, it is observed that the effect of OEP inducing CD11b differentiation at all hours is reduced in the combination groups. While CAPE did not play an active role in inducing differentiation, it was observed that its combination with MP slightly enhanced the effect of MP. While CAPE did not play an active role in triggering differentiation, it was observed that its combination with MP slightly enhanced the effect of MP. The CD14 and CD68 differentiation levels observed in some groups are confounding and should be substantiated by additional experiments. In all groups administered OEP, the cell count tended to decrease significantly, especially in the following hours.

4. CONCLUSION

The content of propolis, which may vary depending on the biogeographical regions where it is collected, contains many active compounds. It is known that extracts and compounds of propolis prepared with various solvents have numerous biological activities, such as antimicrobial, antioxidant and anticancer properties. Propolis originating from Turkey also has a unique composition. The fact that propolis is made with an organic solvent such as olive oil provides an alternative method. Based on the data from this study, it was quite effective in terms of OEP differentiation, so we expect parallel efficacy with CAPE. Although many properties of propolis are already known, there are still not all mechanisms of action clarified. MP Propolis, which is used as a supportive treatment to prevent the side effects of chemotherapy and cancer, also has common side effects. Propolis has been found to have tissue- and cell-protective properties. The induction of CD11b differentiation by propolis in HL -60 cells, a cell line of acute myeloid leukemia, suggests that propolis can be used as a supportive treatment product in the treatment of AML. This study should be supported by research into the anticancer mechanism of action.

5. MATERIALS AND METHODS

5.1. Olive Oil-Based Propolis (OEP)

Standardization of natural products is one of the most important requirements for research. The product used in the research is a standardized product produced in the Technopark of Erciyes University Technopark (Nutral Therapy Company, TR). In addition, residue and quality analysis (GC-MS and HPLC) of the product were performed. CAPE was also purchased commercially (Cat. No.: C8221, Sigma-Aldrich, MO, USA).

5.2. Cell Culture

HL -60 cells, an acute myeloblastic leukemia (AML) cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The HL-60 cells were grown in RPMI 1640 medium

containing 10% fetal bovine serum, 2 mM L-glutamine, 10,000 units penicilin perm L, 10 mg/mL streptomycin at 37°C in humidified air with 5% CO_2. The effective dose of MP and CAPE was determined in our previous study (33). To determine cellular differentiation, HL -60 cells were exposed to MP ($5x10^{-4}$ M), CAPE ($1x10^{-6}$ M), and OEP (containing the active dose of CAPE =0.19 mg) for 24, 48, and 72 hours. The experimental groups were designed as control, OEP (0.5 µL/mL), CAPE ($1x10^{-6}$ M), MP ($5x10^{-4}$ M), MP ($5x10^{-4}$ M)+OEP(0.5 µL/mL and MP ($5x10^{-4}$ M)+ CAPE ($1x10^{-6}$ M).

5.3. Determination of differentiation by cell markers

OEP, CAPE, MP, and their combinations were applied to AML cells in a time- and dose-dependent manner for 24, 48, and 72 hours, and then differentiation was determined by flow cytometric measurement of granulocyte cell surface antigens (CD11b, CD14 and CD68). HL-60 cells were treated with APC anti-human CD11b (5 µL; cat. no.: 301310), PE anti-human CD14 (5 µL; cat. no.:367104) and FITC anti-human CD68 (5 µL; cat. no.: 333806) using APC Mouse IgG1 (Cat. no.: 400120), PE Mouse IgG1 (Cat. no: 400114), and FITC Mouse IgG2b (Cat. no.: 400310) isotypic controls (Biolegend, San Diego, CA, USA). Cells with and without OEP, MP, and their combinations were collected at 24, 48, and 72 hours and centrifuged for 5 minutes; 2 tubes were prepared for each group at a cell density of 1 million cells in 100 µL. For surface staining, 5 µL of APC Mouse IgG1 and 5 µL of PE Mouse IgG1 isotypic controls were added to the first tubes, and 5 µL of APC CD11b and 5 µL of PE CD14 conjugated fluorescent primary antibodies were added to the second tubes and incubated in the dark for 15-20 minutes. For intracellular staining, 0.5 mL of 1X BD FACS Permeabilizing Solution 2 was added to cells prepared to contain 1 million cells in 100 µL and incubated in the dark for 10 minutes. 2 mL of FACS flow was added and centrifuged at 350 g for 5 minutes. 5 µL of the isotypic FITC-mouse IgG2b control was added to the first tube and 5 µL of the primary fluorescent antibody conjugated to FITC-CD68 was added to the second tube. All stained cells were centrifuged at 350 g for 5 minutes by adding 2 mL of FACS flow. Finally, 0.5 mL of FACS Flow was added to perform the flow cytometric measurement. The stained cells were analyzed by flow cytometry using the FACS-Diva software program.

5.4. Statistical analysis

Data measured by flow cytometry are expressed in "%". Statistical comparison of data obtained from each group was performed using the Kruskal-Wallis test for independent samples in the software program IBM SPSS 26. Asymptotic significances (2-sided tests) were indicated. A statistically significant value was taken as $p \leq 0.05$.

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Conflict of Interest Statement: Complete this section according to the signed conflict of interest statement when you submit your article. If none of the authors has a conflict of interest to declare, write "The authors have declared no conflict of interest" in the manuscript.

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