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Research Article

Modulation of Cytokine Production and Transcription Factors Activities in Human Jurkat T Cells by Thymol and Carvacrol

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Abstract

Purpose: Thymol and carvacrol, two main components of thyme, have shown anti-inflammatory effects. The aim of this study was to assess the effects of these components on Jurkat leukemia cells as an in vitro T cell model and their molecular mechanisms of activity.

Methods: Cells were cultured in the presence of components and subsequently stimulated with phorbol-12-myristate-13-acetate (PMA)/calcium ionophore for evaluating interleukin (IL)-2 and interferon (IFN)-γ production. The activation of T cell transcription factors that included nuclear factors of activated T cells (NFATs), activator protein-1 (AP-1; c-Jun/c-Fos), and nuclear factor (NF)-κB were examined by Western blot analysis.

Results: Thymol and carvacrol at 25 µg/ml significantly reduced IL-2 levels from 119.4 ± 8pg/ml in control cells treated only with PMA/Calcium ionophore and the solvent to 66.9 ± 6.4pg/ml (thymol) and 32.3 ± 3.6pg/ml (carvacrol) and IFN-γ from 423.7 ± 19.7pg/ml in control cells to 311.9 ± 11.6pg/ml (thymol) and 293.5 ± 16.7pg/ml (carvacrol). Western blot analyses of nuclear extracts showed that the same concentrations of components significantly reduced NFAT-2 to 44.2 ± 2.7% (thymol) and 91.4 ± 2.3% (carvacrol) of the control ($p<0.05$), and c-Fos to 31.2 ± 6.2% (thymol) and 27.6 ± 3.1% (carvacrol) of the control ($p<0.01$). No effects on NFAT-1, c-Jun and phospho-NF-κBp65 levels were observed.

Conclusion: Thymol and carvacrol could contribute to modulation of T cell activity by reducing IL-2 and IFN-γ production possibly through down regulation of AP-1 and NFAT-2 transcription factors suggesting their potential usefulness for reduction of T cell overactivity in immune-mediated diseases.

Introduction

An overactive immune system can cause autoimmune disorders, allergies and numerous other immune-mediated diseases. Immune suppressive drugs are now available for the treatment of immunological disorders; however they have side effects and are expensive.

Throughout history, herbs have been used for curative purposes. Herbal remedies have become increasingly popular and are often safe and effective alternative treatments because of their decreased side effects and cost-effectiveness.[1,2] A large number of plant species contain a range of bioactive compounds that possess beneficial health properties.[3-5] Many of these natural products have been already evaluated for improvements to new immunomodulatory drugs. Additional products remain that deserve more investigations for their possible therapeutic usefulness in immune-related diseases.[6]

Thymol and carvacrol are two important natural terpenoid products present in the essential oil fractions of aromatic plants such as thyme.[7] These products have broad antimicrobial, antioxidant and anti-inflammatory effects. They have been shown to inhibit inflammatory edema and leukocyte migration in animal models, reduce key mediators of inflammation such as cyclooxygenase-2 and inducible nitric oxide synthase, and inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β.[8] In other studies administration of carvacrol or thymol has reduced TNF-α levels in pleural lavage, decreased the lipopolysaccharide (LPS)-induced nitrite production and suppressed autoimmune arthritis in

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experimental models.[9] The combination of carvacrol with methotrexate has also suppressed complete Freund's adjuvant induced synovial inflammation with reduced hepatotoxicity in rats.[10]

Inflammatory responses are mainly controlled by T lymphocytes. These cells play a key role in the initiation and maintenance of inflammation. T lymphocytes recognize antigens on the surface of antigen-presenting cells such as dendritic cells or macrophages, which leads to their subsequent activation and proliferation. A major feature of T cell activation is the production of cytokines such as IL-2 and interferon (IFN)-γ that can cause proliferation and differentiation of T cells and trigger different effector mechanisms of inflammatory responses that are characteristic for certain immune-mediated diseases.[11] The activation and secretion of cytokines by T cells is under the control of several transcription factors. The most important transcription factors for the induction of cytokine expression in T cells are activator protein-1 (AP-1), nuclear factors of activated T cells (NFAT) and nuclear factor-κB (NF-κB).[12] AP-1 is composed of c-Jun, c-Fos, and activating transcription factor (ATF) proteins in different combinations of hetero- or homodimers. The composition of AP-1 defines the genes that it regulates. C-Jun and c-Fos as major components of AP-1 are involved in several cellular processes such as differentiation, proliferation, transformation, and apoptosis. Their interaction with NFAT proteins induces transcription of several cytokine genes.[13,14]

The NFAT family of transcription factors, first identified in T cells as a quickly inducible nuclear factor, can bind to the human IL-2 promoter. NFAT-1 and NFAT-2, two prevalent members of this family expressed in immune cells, play an important role in regulating a large number of inducible genes through the immune responses.[15]

NFAT proteins are involved in regulating transcription of numerous inducible genes in immune cells including IL-2, IFN-γ, IL-4, IL-5, TNF-α and CD40L that regulate cell differentiation, proliferation, apoptosis and survival.[16] NFAT/AP-1 cooperation is necessary for the majority of these genes to undergo expression.[17]

NF-κB plays a central role in inflammatory processes and is a main transcription factor responsible for regulating both the innate and adaptive immune response genes.[18] The NF-κB complex is retained in an inactive form in the cytoplasm by binding to its inhibitor IκB (inhibitor of kappa B). Upon activation, IκB is ubiquitinated and degenerated, allowing phosphorylation of the NF-κB complex and promoting its transportation into the nucleus where it can bind to promoter sites and regulate transcription of target genes.[19] Similar to AP-1, NF-κB regulates numerous immune response genes, many of which require the simultaneous and cooperative activation of AP-1.[20]

Recently, the molecular basis of the anti-inflammatory action of thymol has been studied in LPS-stimulated mouse mammary epithelial cells. It was shown that thymol could decrease phosphorylation of several signaling molecules such as NF-κB p65, IκBα, c-Jun NH(2)-terminal protein kinases (JNK), extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinases (MAPK) in these cells.[21] Considering the central role of T cells in inflammatory processes, in the present study we aimed to investigate the effects of these components on T cells in order to derive a better understanding of the mechanisms underlying their anti-inflammatory effects. In addition, as altered T cell function is often a key component associated with numerous pathologies, discerning how they may affect T cells can help scientists build upon our findings and take better advantage of these natural products. For this purpose we have used Jurkat human leukemia cells, a T cell leukemia cell line. This cell line has been used as an alternate for T cells in numerous studies that included evaluation of T cell signaling and the immune response. The production of important cytokines such as IL-2 and IFN-γ and the activation of key transcription factors involved in T cell signaling which included NFATs, AP-1 and NF-κB were also investigated in these cells.

Materials and Methods

Materials

Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cell culture grade dimethylsulphoxide (DMSO), RPMI 1640 medium, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, thymol, carvacrol, phosphatase inhibitor cocktail 3, phenylmethylsulfonyl fluoride (PMSF), luminal, bovine serum albumin (BSA) and coumaric acid were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco (Kentucky, USA), Sodium dodecyl sulfate (SDS) and polyacrylamide gel was from BioRad (Carlsbad, CA, USA). Nuclear extraction reagents were from Thermo Scientific (CA, USA). IL-2 and IFN-γ were measured using enzyme-linked immuosorbent assay (ELISA) kits (eBioscience, USA). Histone H3, phospho-NF-κB p65 (Ser536), c-Fos, c-Jun, NFAT-1 and NFAT-2 rabbit monoclonal antibodies (mAb) and anti-rabbit IgG horse radish peroxidase (HRP)-linked antibody were obtained from Cell Signaling Technology® (Beverly, Massachusetts, USA). Cyclosporine A (CSA) was obtained from Zahravipharma (Tabriz, Iran). Other chemicals and solvents were of reagent grade and available.

Cell culture and preparation of components

The human T lymphocyte Jurkat cells were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated FBS, at a density of 1×10^6 cells/ml in a humidified 5% CO_2 incubator at 37°C. Viability of the cells were evaluated by trypan blue (0.4% trypan blue in phosphate buffer saline, PBS) exclusion method. Thymol and carvacrol were prepared in DMSO at a stock solution of 20mg/ml. DMSO as the solvent at the highest

concentration used in the tests was added to all control cultures that received none of the test compounds.

Viability assay

The viability of Jurkat T cells was determined by MTT reduction assay in the presence or absence of the compounds as previously described.[22] Briefly, 15×10^3 cells/well were seeded in 96-well plates (Nunclone, Thermo Scientific, CA, USA) and then were treated with different concentrations of the components (1, 10, 25, 50, 100, 200µg/ml) in a final volume of 100 µl. Negative control was cells treated with DMSO as the solvent at the highest concentration used in the tests (e.g., 1%) and positive control was cells treated with a cytotoxic drug e.g., cisplatin (50µg/ml). After 24 h of incubation, 10µl MTT solution (5 mg/ml in PBS) was added to each well and cells were further incubated for 4 h. After removing medium from each well, produced formazan in the cells was dissolved by adding 150µl DMSO. The optical density (OD) of the solubilized formazan in the wells was measured with a microplate reader (Biotek, Nevada, USA) at 570 nm with a background subtraction at 630 nm. The absorbance of solubilized formazan in negative control cells was taken as 100% viability. Experiment was carried out in triplicate and repeated at least three times.

ELISA assay

IL-2 and IFN-γ production were measured using ELISA kits with the sensitivity of 2 and 4pg/ml, respectively (eBioscience, USA). Briefly, 5×10^5 cells/well were cultured in 24-well plates (Nunclone), then cells were treated with PMA (10nM) and calcium ionophore (1 µM) and two concentrations of compounds (10 and 25µg/ml) in triplicate. Negative control cells were those treated only with DMSO at the highest concentrations used in the tests (0.1%) and positive control were those treated with DMSO and PMA/calcium ionophore without the compounds. After 24 h, supernatant of cell cultures were collected and stored at −80°C until used. ELISA assay was performed as described by the manufacturer. Briefly, 96-well micro plates (Nunclone) were coated an overnight at 4°C with 100µl capture antibody. The plates were blocked and 100µl/well of different standards and samples were added to the appropriate wells and incubated for 2 h at room temperature (RT). 100µl of kit-provided detection antibody was added to each well and the plate incubated at RT for 1 h. After washing, 100µl of Avidin-HRP (30 min at RT), 100µl of tetramethylbenzidine (TMB) substrate (15 min at RT) and then 50µl of stop solution was added. The absorbance of reaction was measured at 450 nm with a background subtraction at 570 nm using a microplate reader (Biotek, Nevada, USA).

Nuclear protein extraction

Jurkat cells were cultured at concentration of $2x10^6$ cells/2ml in 6-well culture plates (Nunclone). The cells were treated with 10 and 25µg/ml of thymol and carvacrol and 2µg/ml of CSA as an NFAT inhibitor for 24h and then PMA (10nM) and calcium ionophore (1 µM) was added. Negative control cells were those treated only with DMSO at the highest concentrations used in the tests (0.1%) and positive control were those treated with DMSO plus PMA/calcium ionophore without adding the compounds. After 30 min, cells were washed with cold PBS and a total of 10×10^6 cells centrifuged at 500×g for 2-3 min. The resultant supernatant then carefully removed and the cell pellet was left to dry as possible. Then 200µl ice-cold cytoplasmic extraction reagent-1 (CER I) was added. After a short vortexing, 11µl of ice-cold CER II was added and then the suspension was centrifuged for 5 min at ~16000×g. The resultant supernatant was removed and the remaining pellet was suspended in100µl ice-cold nuclear extraction reagent (NER) plus 1mM PMSF and phosphatase inhibitor cocktail-3. After sufficient vortexing on ice, the tube was centrifuged at ~16000×g for 10min. The supernatant (nuclear extract) was immediately transferred to a clean pre-chilled tube and stored at -80°C until use for Western blot analysis.

Western blot analysis

Twenty five microgram nuclear proteins from each sample was separated over 10% SDS-polyacrylamide gels and then electrotransferred to nitrocellulose membranes. Membranes were blocked in 2% BSA/Tris-buffered saline with 0.1%Tween 20 for overnight and then incubated with the histone H3 (1:1200), c-Fos (1:1000), c-Jun (1:1000), NFAT-1 (1:1000) and NFAT-2 (1:1000) and phospho-NF-κB p65 (1:600) rabbit mAbs followed by anti-rabbit IgG HRP-linked secondary antibody (1:2000). Immunoreactive bands were visualized using the chemiluminescence reaction. Quantification of the transcription factors levels were normalized to histone H3 using ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA).

Statistical analysis

All experiments were performed in triplicate and repeated at least 3 times. Significant differences between groups were evaluated by Graphpad prism software (San Diego, CA, USA) and appropriate statistical tests e.g., one-way analysis of varience (ANOVA) and a Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

The effects of thymol and carvacrol on Jurkat human T cell viability

The cytotoxic effect of different concentrations of thymol and carvacrol on Jurkat cells were determined by MTT colorimetric assay. As shown in Figure 1 A and B, thymol and carvacrol at concentration of 50µg/ml decreased the viability of Jurkat cells to 51.35 ± 5% and 73.1±2%, respectively. The cell viability at concentrations up to 25µg/ml of these components did not change compared to the control, therefore we used 10 and 25µg/ml of thymol and carvacrol for further experiments on this cell line.

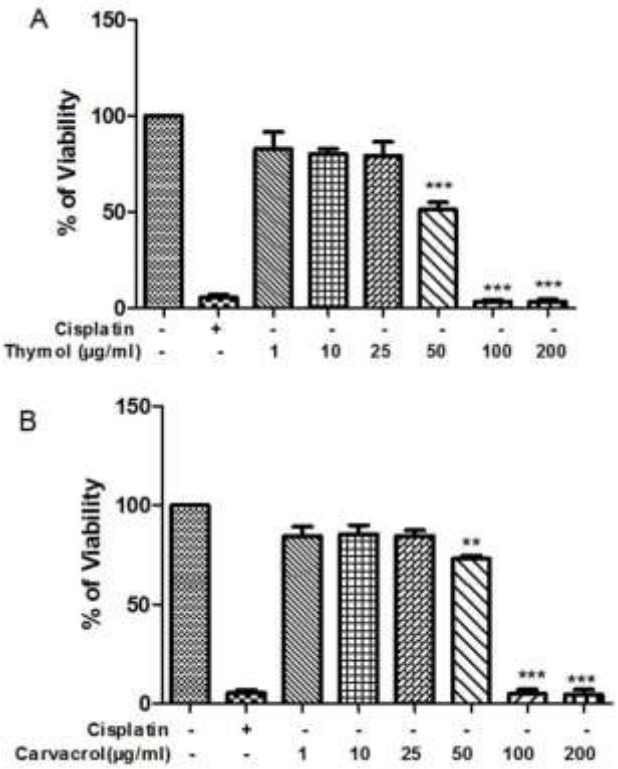

Figure1. Effects of thymol (A) and carvacrol (B) on Jurkat cell line viability. Jurkat cells were treated with different concentrations of thymol and carvacrol for 24 h. Viability was determined by MTT colorimetric assay. Negative control values were obtained in the absence of components and positive control in the presence of cisplatin (50µg/ml). DMSO as the solvent was added to the controls at the highest concentration used in the tests (1%). The values show mean ± SD of three independent experiments in triplicate **p<0.01, ***p<0.001 compared to negative control.

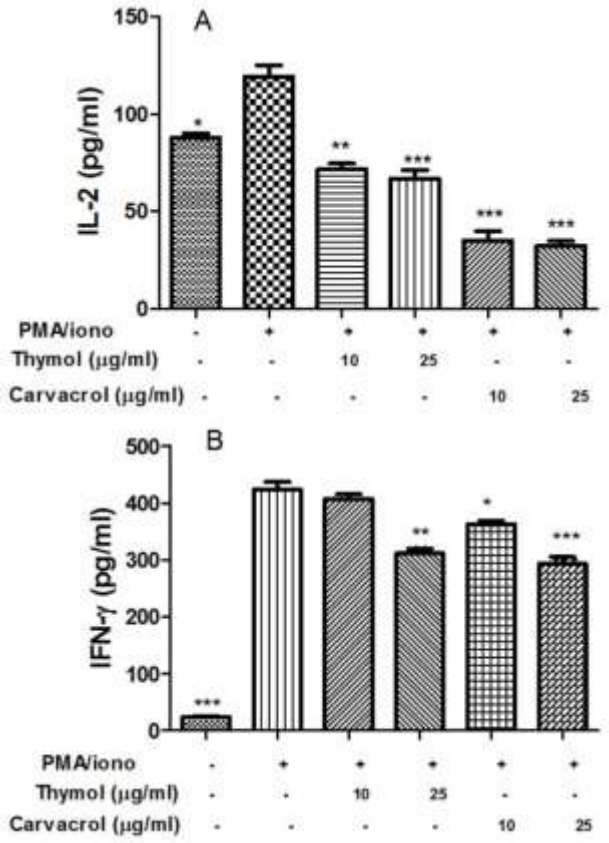

Figure 2. Effects of thymol and carvacrol on phorbol-12-myristate-13-acetate (PMA)/calcium ionophore-induced cytokine production in Jurkat cells. Cells were treated with thymol and carvacrol (10 and 25µg/ml) in the presence of PMA (phorbol 12-myristate 13-acetate) and calcium ionophore (iono) for 24 h. Negative control values were obtained in the absence of PMA/calcium ionophore and compounds and positive control was cells treated just with PMA/calcium ionophore. DMSO as the solvent was added to the controls at the highest concentration used in the tests (0.1%). The values represent mean ± SD of three independent experiments in triplicate. *p<0.05, **p<0.01, ***p<0.001 compared to positive control.

ELISA assessment of IL-2 and IFN-γ

The influence of thymol and carvacrol on the production of IL-2 and IFN-γ cytokines in PMA/calcium ionophore-stimulated cells were detected using ELISA method. As shown in Figure 2 A and B, there were significantly increased IL-2 and IFN-γ cytokine levels after the PMA/calcium ionophore challenge (IL-2 from 88±2.8 pg/ml to 119.4±8 pg/ml and IFN-γ from 23.7±3.2 pg/ml to 423.5±19.7 pg/ml). Treatment of cells with thymol at concentrations of 10 and 25µg/ml significantly reduced IL-2 from 119.4 ± 8 pg/ml in the positive control to 71.7± 4.2 pg/ml ($p<0.01$) and 66.9 ± 6.4 pg/ml ($p<0.001$) respectively. Concentrations of 10 and 25µg/ml of carvacrol also decreased IL-2 levels to 35.2± 6.7 pg/ml ($p<0.001$) and 32.3 ± 3.6 pg/ml ($p<0.001$), respectively. IFN-γ secretion reduced from 423.7 ± 19.7 pg/ml in the positive control to 407.4 ± 11.4 pg/ml for 10 µg/ml thymol and 311.9 ± 11.6 pg/ml ($p<0.01$) for 25µg/ml thymol and to 362.6 ± 8.9 pg/ml ($p<0.05$) for 10 µg/ml carvacrol and 293.5 ± 16.7 pg/ml ($p<0.001$) for 25µg/ml carvacrol. These data indicated the dose-dependent reducing effect of these components on IL-2 and IFN-γ cytokines secretion.

Western blot analysis of transcription factors

We evaluated the influence of thymol and carvacrol on NFAT-1, NFAT-2, c-Fos, c-Jun, and NF-κB activation by treating the cells overnight with thymol and carvacrol, after which they were subsequently activated with PMA/calcium ionophore for 30 min. The nuclear cell extract was then prepared for Western blot analysis. As demonstrated in Figure 3 A and B, CSA as an NFAT inhibitor drug at a concentration of 2 µg/ml significantly decreased NFAT-1 to 57.1 ± 14.7% ($p<0.01$) and NFAT-2 to 43.3 ± 0.9% ($p<0.001$) of the positive control. Thymol and carvacrol did not influence NFAT-1 transcription factor, but both components at concentrations of 10 and 25 µg/ml significantly reduced nuclear NFAT-2 to 52.7 ± 2.5% ($p<0.001$) and 37.2 ± 2.7% ($p<0.001$) for thymol and 81.1 ± 3.9% ($p<0.05$) and 60.9 ± 3% ($p<0.01$) of the positive control, for carvacrol. As shown in Figure 3 C and D, pretreatment of cells with 10 and 25 µg/ml of components significantly reduced nuclear c-Fos to 51.2 ± 10.5%

($p<0.05$) and 31.2 ± 6.2% ($p<0.01$) for thymol and to 35 ± 4.1% ($p<0.01$) and 27.6 ± 3.1% ($p<0.001$) of the positive control, for carvacrol. None of the components significantly decreased nuclear c-Jun and phospho-NF-κB p65 levels (Figure 3 E).

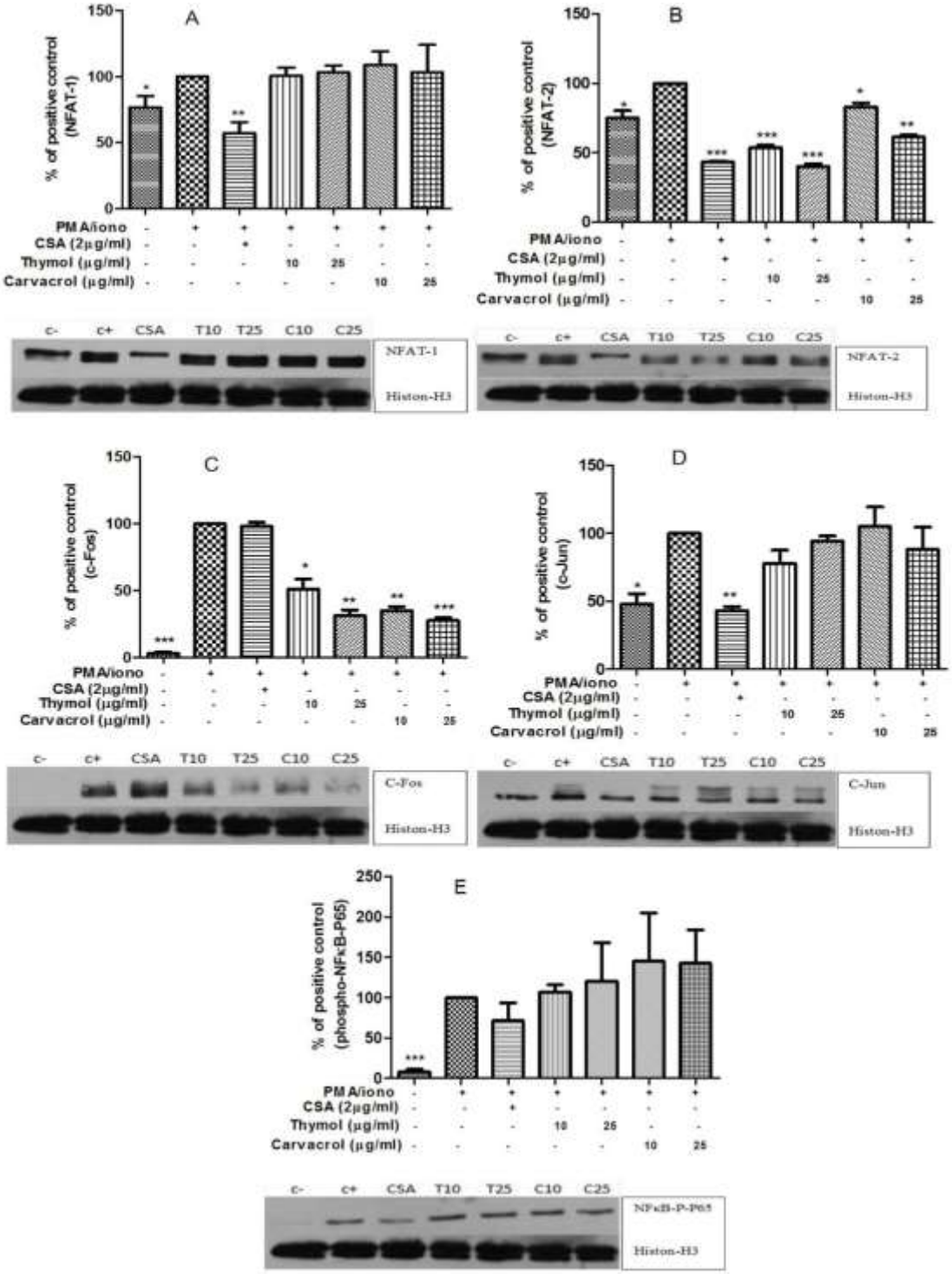

Figure 3. Effects of thymol and carvacrol on transcription factors in stimulated Jurkat cells. The cells were treated with 10 and 25µg/ml of thymol (T) and carvacrol (C) for overnight and then activated with PMA (phorbol 12-myristate 13-acetate) and calcium ionophore (iono) for NFAT-1, NFAT-2, c-Fos, c-Jun and phospho-NF-κB p65 measurement in the nuclear cell extract by Western blotting. Quantification of these transcription factors levels were normalized to Histone H3 using a densitometer. Negative control (c-) values were obtained in the absence of PMA/calcium ionophore and compounds and positive control (c+) was cells treated just with PMA/calcium ionophore. DMSO as the solvent was added to the controls at the highest concentration used in the tests (0.1%). Cyclosporine A (CSA) was used as an NFAT suppressant drug. Data are representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to positive control.

Discussion

The aim of the current study was to examine the effects of thymol and carvacrol on Jurkat cells and to investigate their molecular mechanisms of action. The human T-leukemia cell line, Jurkat, is an alternate for T cells in T cell signaling and immune response evaluation. We have first examined the effect of these components on this cell line's viability after which the non-cytotoxic concentrations of the compounds were used in further examinations. IL-2 and IFN-γ are produced by activated T cells and a variety of immune disorders have been related to imbalanced production of these cytokines. Various immunosuppressive drugs have prevented the IL-2 and IFN-γ secretion in activated T cells and by this mechanism diminished immune system overactivity in autoimmunity and allergies.[23,24] Therefore, determining the production of these cytokines in biological studies is a useful tool to assess immunomodulatory activity of the compounds on T cells. In the current study, we have evaluated the effects of carvacrol and thymol on IL-2 and IFN-γ production in PMA/calcium ionophore-stimulated Jurkat cells. As the results showed, both compounds significantly decreased the levels of these cytokines in the treated cells. IL-2 production displays T cell activation and IFN-γ is produced by activated T cells and has been shown to play critical roles in central immunological processes such as cell-mediated immunity, inflammatory reactions and autoimmunity.[25] The secretion of these cytokines is under the control of various transcription factors. The most important transcription factors for the induction of cytokines expressions in T cells are AP-1, NFAT and NF-κB.[26] The IL-2 promoter contains multiple binding sites for AP-1, NFAT and NF-κB, which coordinately activate the IL-2 promoter.[23,24] Also, the IFN-γ promoter region has an AP-1 site expected to support cooperative interactions of NFAT and AP-1.[26] Whether thymol and carvacrol could inactivate these transcription factors was investigated by Western blot analysis of the nuclear cell extract of stimulated Jurkat cells. As the results showed, both compounds significantly reduced nuclear NFAT-2 but had no influence on NFAT-1. The compounds influenced AP-1 transcription factor activation by reducing nuclear c-Fos level. We found that pretreatment of cells with thymol and carvacrol did not change nuclear c-Jun level. The activity of AP-1 is dependent on its dimer composition such as c-Jun homodimers, or heterodimers with other c-Jun, c-Fos or ATF proteins. It has been shown that and c-Jun/c-Fos heterodimers are more stable than c-Jun/c-Jun homodimers. Thus the decline in c-Fos levels, as observed in this study could decrease the stable c-Fos/c-Jun heterodimer and reduce AP-1 activity. Balanced activation of AP-1 and NFAT is essential for proficient immune responses; pharmacological interference with NFAT/AP-1 interaction may be suitable in selective manipulation of the immune response.[27]

NF-κB is a main transcription factor responsible for regulating both the innate and adaptive immune response genes.[28] Many of these genes need the cooperative activation of AP-1. Both components have shown no significant changes in phospho-NF-κB p65 levels. Since the studied compounds did not inhibit NF-κB and NFAT-1 transcription factors, it could be assumed that the reduced cytokine production by these compounds might be mediated through their suppressive effects on AP-1 and NFAT-2 levels. These data are in line with our previous study in which reduced inflammatory responses through modulation of the expression of AP-1, and NFATs in lipopolysaccharide-treated macrophages was shown.[29] In a study by Chan et al., carvacrol dose-dependently increased activation of p38 and ERK transcription factors in Jurkat T cells.[30] In another study carvacrol decreased the phosphorylation of ERK and activated phosphorylation of p38 but did not affect JNK phosphorylation in a hepatocellular carcinoma cell line.[31] These data indicated that thymol and carvacrol had diverse effects on different transcription factors and suggested that they might modulate the functions of immune cells via different intracellular signaling pathways. The net effect of activation of the transcription factors might determine the final outcome.

Conclusion

Thymol and carvacrol decreased IL-2 and IFN-γ production in the Jurkat T cell line in part due to inhibition of AP-1 and NFAT2 and perhaps other signaling pathways rather than NF-κB and NFAT-1. These components are proposed to be potential therapeutic agents for inflammatory and immunological disorders associated with T cell overactivation.

Acknowledgments

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Ethical Issues

Not applicable.

Conflict of Interest

Authors declare no conflict of interest in this study.

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