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Antifungal Effect of Flavonoid Extract of Trachyspermum Ammi Plant on the Gene Expression of Pro-inflammatory Cytokines Such as IL-18 and TNF-α in Articular THP-1 monocyte/ Macrophages Cells

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The Trachyspermum ammi plant has anti-fungal properties. The aim of this study is to examine the anti-fungal effects of this plant on the pro-inflammatory bio-markers, which are important in the course of fungal diseases. At first the ethanol extract of the Trachyspermum ammi plant was prepared from the Iranian biological resource center. We used monocyte/macrophage cells as samples because they were similar to the cells with fungal infection. Then THP-1 monocyte/macrophages (5×10^5 cells/well) were incubated at a humidity of 90%, 5% CO2, 37ÚC for 72 h with control media alone or Trachyspermum ammi at concentrations of 10 μg/ml. One set of cells was activated for 1 h with mannose (3 mg/ml) for both reverse-transcriptase Polymerase chain reaction and real-time Polymerase chain reaction analysis of Tumor necrosis factor alpha (TNF-α), Interleukin-18 (IL-18) expression. The activated cells by mannose with a plant extract redused the Interleukin-18 and Tumor necrosis factor alpha expression as activated cell by mannose with fluconazole (p-value=0.91 for IL-18, p-valu=0.25 for TNF-α). Based on the PCR and realtime methode to analysis of gene expression, Trachyspermum ammi plant such as the flavonoids has noticeable anti-fungal properties.

Key words: Trachyspermum ammi, THP-1 Monocyte/Macrophage, Fungal diseases

In the early 1990's the increase of fungal infections due to pathogenic fungi and opportunistic fungus, especially in patients who had a weak immune system, was one of the most important reasons for death in patients who were kept in hospitals[1]. pathogen-associated molecular patterns, [PAMPs] that are recognized by germ line-encoded proteins (pattern recognition receptors [PRRs]) present in different cells of the organism, mainly monocytes, macrophages, dendritic cells, B-cells, T-cells and endothelial cells. PRRs include the Toll-like receptors (TLRs), a protein family of cellular receptors that mediate recognition of microbial pathogens and subsequent inflammatory response in vertebrates[2,3].

Interleukine-18 has a role in regulating the innate and acquired immune system and als plays a key role in auto-immune, inflammatory, infectious and fungal diseases[4]. TNF-α is a polytrophic factor which belongs to the cytokine

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family. TNF-α is a strong pro-inflammatory cytokine which belongs to the poly-peptide group, which consists of several cytokines and growth factors[5]. Anti-fungal medications, with various formulas, are abundantly available in drugstores. However sometimes they are unable to provide a cure or must be used for long periods of time which may result in the appearance of diverse side effects[6,7]. *Trachyspermum ammi* is a native of Egypt and is cultivated in Iraq, Iran, Afghanistan, Pakistan, and India[8], *Trachyspermum ammi* L. belonging to family Apiaceae is a highly valued medicinally important seed spice. Trchysper ammi, commonly referred as Bishop„s weed, Carom seed (English names) and ajowani or ajwain or omum in Indian languages[9]. Ajwain seed analysis has revealed it to contain fiber (11.9%), carbohydrates (38.6%), tannins, glycosides, moisture (8.9%), protein (15.4%), fat (18.1%), saponins, flavone and mineral matter (7.1%) containing calcium, phosphorous, iron and nicotinic acid[10]. Higher phenolic and flavonoids content showed high anti-oxidant activity in ajowain seed extracts[11]. In general all the parts of this active ingredient are made up of fenols, which have both anti-oxidant and anti-microbe properties. Therefore it can be used as a preservative in food[12]. The aim of this study is to compare the effect of the Trachyspermum ammi plant and the widely used fluconazole medication. In order to reach aim, firstly the monocyte cells were actived by using a suitable concentration of mannose and forcing the cell to produce infectious cytokines, which consist of IL-18 and TNF-a, the effect of the Trachyspermum ammi plant on decreasing gene expression of the infectious intermediates is examined. The results are then compared with and the fluconazole medication.

METHODS

Cultivating THP-1 Monocyte/Macrophage cells

Human THP-1 monocyte/macrophage-like cells (Pasteur Institute of Iran) were proliferated in controlled media containing; RPMI-1640 medium (GIBCO, USA) supplemented with 1.5 g.l sodium bicarbonate (Sigma-Aldrich), 10% FBS (Invitrogen), 2 Mm L-Glutamine(GIBCO, USA), 10 Mm 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, GIBCO, USA), 4.5 g/l glucose (GIBCO, USA), 0.05 Mm β-mercaptoethanol (GIBCO, USA) and 1.0 Mm sodium pyruvate (GIBCO, USA). Cells were pelleted via centrifugation, which occurred at 2000 cycles per minute and was carried out for 3 minutes, and assessed for viability using the Trypan-blue exclusion method. Viable cells were plated in six-well plates at a density of 5×10^5 cells/well and treated the same day[13].

Determining LC50 in THP-1 cells by using the Zenyan extract

The ethanol extract of Tracyspermum ammi were prepared at concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg per ml and using the RPM-1640 cultured environment. These concentrations were added to 12 pocketed flat bottom plates, which were kept in an incubator at a temperature of 37 C, 5% $co_{2,}$ and 90% humidity (14). Thus LC50 has a concentration of 30 µg/ml and its average was calculated to be 15 µg/ml.

Expressing the Human TNF-α, Human GAPDH, Human IL-18 genes by treating (Human THP-1) with mannose

After determining LC50, the ethanol extract at a consistency of 10 µg/ml was added. The plates were incubated at a temperature of 37°C, 5% $CO_{2,}$ and 90% humidity and a duration of 72 hours. At the end of this stage the cells were treated with a 3mg/ml [15] concentration of mannose. They were then incubated at a temperature of 37°C, 5% $CO_{2,}$ and 90% humidity and a duration of 1 hour in order to express the Human TNF-α, Human GAPDH, Human IL-18 genes. Furthermore in the next stage of this experiment a 30 mg/ml concentration of fluconazole was added to plates containing monocyte and mannose. In order to measure the amount of gene expression the infected cytokines were kept in an incubator for 8 hours and under suitable conditions[13].

Total RNA isolation

The cells which were treated and controlled were collected in order to examine RNA. Total cellular Rna was isolated by lysing the cells with TRIzol reagent (Invitrogen) and extracted with chloroform (Sigma-Aldrich). Following vigorous agitation and a 3-min

incubation at room temperature, the samples were centrifuges and the aqueous phase containing RNA was collected. The RNA was precipitated with isopropyl alcohol (GIBCO, USA) and resusupended in Rnease-free water. In order to work out the concentration of the RNA the electroforez gel agaroze 1% method was used[13].

Complementary DNA synthesis

For each sample, 1 µg of total RNA was converted to complementary DNA (cDNA) using Moloney-Murine Leukemia Virus reverse transcriptase from the Advantage RT-for PCR kit (CinnaGen, IRAN). RT was carried out at 42°C for 60 min followed by heating at 94°C for 5 min to stop direction of the reaction of the cDNA synthesis reaction and to destroy any Dnase activity. Semiquantitative RT-PCR (Model Bio-Rad, Made in Thermo Cycler, USA) was performed using primers specific to human TNF-± (forward, GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC; reverse, GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T), human IL-18 (forward, AGG TCA GTC TTT GCT ATC ATT CCA GG; reverse, CTG CAA CAG AAA GTA AGC TTG CGG AGA GG) and human GAPDH (forward, TGA AGG TCG GAG TCA ACG GAT TTG GT; reverse, CAT GTG GGC CAT GAG GTC CAC CAC) as the housekeeping gene were aiso used (Takapouzist, IRAN) using 2 µl Cdna template and reagents from the SuperTaq Plus Kit (Takapouzist, IRAN). All the samples together were measured to be 25 µl. The samples were instantly put in the PCR, and the following standard program was used: 5 minutes separation of two strands at 95 °C, 45 seconds of denaturation at a temperature of 95 °C (this stage was repeated for 40 cycles), 45 seconds of connecting the primer to the sample strand at a temperature of 59°C, 30 seconds of expanding the new strand at 72°C (this stage was repeated for 40 cycles), and infinite time at the final stage of strand expansion at a temperature of 72°C. In this way the genes that were needed for our investigation were cultivated[13].

Quantity of Real-time PCR

Real-time PCR was used with the primer sequences as it was mentioned earlier and kit evagreen from the Cinocolon company was used to bring about a Real-Time reaction.

In addition by using a standard program, which consisted of the primary stage: one cycle of denaturation for a duration of 15 minutes at a temperature of 95°C, then the denaturation stage: denaturation for 15 seconds in 40 cycles at a temperature of 96°C, then the connection stage: 60 seconds of connection in 40 cycles at a temperature of 60-65°C, and finally the elongation stage: elongation for 20 seconds in 40 cycles at a temperature of 72°C, the scale of specific mRNA for each gene was evaluated[13].

Statistical Tests

After cultivating the THP-1 cells in suitable conditions they were then treated in the following groups: monocyte cells without mannose (Cell group), Monocyte cells accompanied with 3 mg/ml of mannose (Cell+ mannous group), Monocyte cells accompanied with 3 mg/ml of mannose and 10 µg/ml of the ethanol extract of Trachyspermum ammi (Cell+ mannous+ extract group) and THP-1 cells along with 3 mg/ml of mannose, and 30 µg/ml of fluconazole chemical medication (Cell + Mannose + Fluconazole group). To compare groups, one-way ANOVA and bioferri were used because the distribution of variables was normal. The SPSS 21 was used and p-value under 0.05 was consider for significancy.

RESULTS

The mean and SD of IL-18 and SD TNF-α cytokines are shown in the table 1.

The THP-1 monocyte/macrophage cell activated with 3 mg/ml of mannose showed significantly a large scale of expression of the genes TNF and IL-18 in comparison with the control cells.

The effect of Trachyspermum ammi on the gene expression of IL-18

ANOVA showed that to compare to the mannous cells , Trachyspermum ammi and Fluconazole both separately decreased the expression of the gene IL-18 ($F = 78.18$, p-value < 0.0001). Bonferroni analysis showed there was no significant different between the cells treated by Trachyspermum ammi extract and Fluconazole (p-value = 0.91) (figure I).

The effect of Trachyspermum ammi on the gene expression of TNF-α

ANOVA showed that to compare to the

mannous cells , Trachyspermum ammi and Fluconazole both separately decreased the expression of the gene TNF-α (F =49.38, p-value < 0.0001). Bonferroni analysis showed there was no significant different between the cells treated by Trachyspermum ammi extract and Fluconazole (p-value = 0.25) (figure 2).

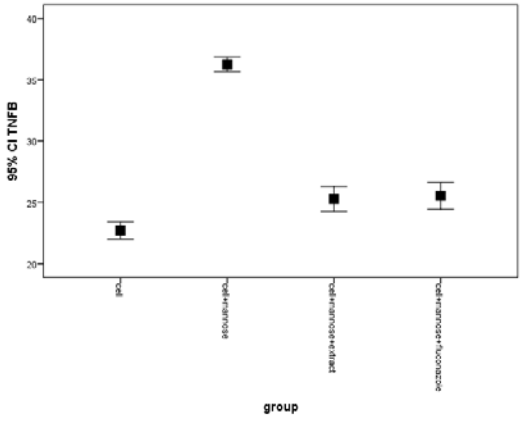

Fig. 1 . Error plot showed the mean (SD) of IL-18 in all groups: There is significant Difference between groups (P value < 0.003), but no significant difference between Cell + Mannose + Extract group and Cell + Mannose + Fluconazole

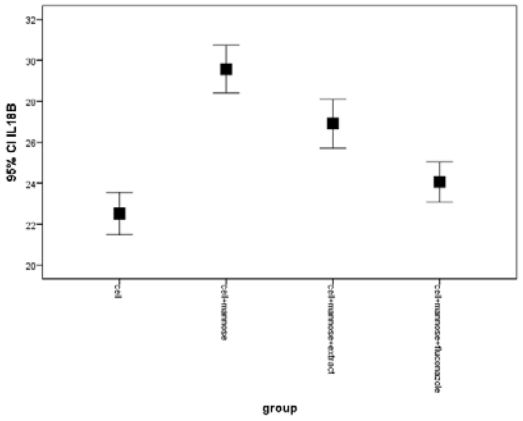

Fig. 2. Error plot showed the mean (SD) of TNF- α in all group: There is significant difference between groups (p-value < 0.001), no significant difference between Cell + Mannose group and Cell+ Mannose + Fluconazole

DISCUSSION

In this study we have investigated the effects of the Trachyspermum ammi plant on human monocytes/ macrophages. It will be proven that the anti-fungal effects of the flavonoid extract of Trachyspermum ammi fights some of the effective pre-infectious gene expression of cytokines, IL-18 and TNF-±. This extract is used in a cellular model similar to monocytes/ macrophages activated with mannose. Creating an infectious model with mannose, which exists in the fungi cell walls, starts off infectious replies in the tissues. In addition since the increase of the production of pre-infectious cytokines via mannose plays a role in the tissue damage caused, the infectious model was used.

In order to allow the human THP-1 cells to work they are used as a model substitutes of mocytes/macrophages, which in researches are jointly used as pre-infectious intermediaries in safety responses and provide good evidence that this model is suitable. These cells increase rapidly and produce an adequate amount of living cells for investigation. The Trachyspermum ammi extract can act as the main intermediary and as an anti-fungal factor.

In the present study, it has been proven that the Trachyspermum ammi plant extract suppresses the gene expression of IL-18 and TNF-α in the THP-1 cell which is cultivated and activated with mannose like as Fluconazole. Moreover by adding a two-fold or three-fold consistency of amphotericin B the duration of cell division decreases and this investigation showed that it reduced from 69-72 hours to 24-36 hours. Furthermore, for the first time, the level of expression of pre-infectious cytokines was determined via mRNA. Instead of using in vitro cells we were able to use laboratory (in vivo) mice and investigate the pathology of the tissues. The study of fungal infectious disease is perhaps a more challenging task than viral or bacterial diseases, because fungi are eukaryotic organisms and therefore harder for the immune system to distinguish from the self-cells. On the other hand, the paradox is that most fungal diseases mainly affect individuals with impaired immune systems, such as after strong chemotherapy or bone marrow transplant patients, but not healthy ones. This puts

fungal diseases somewhere between viral/ bacterial infectious diseases and cancer and autoimmune diseases[16]. Iacobellis and its active partner, the antibacterial Trachyspermum ammi essence, were investigated via the Agar Diffusion method, and a high level of controlling effects were found. They controlled the growth of Rodotorula, Erwinia, Agrobacterium and Xanthomonas[17]. Devasankaraiah, Singh, Rani, Srivastava, Navarro and their partners have also confirmed the anti-bacterial activity of the Trachyspermum ammi essence and have investigated its effects on several microbes resistant to medication[18,19]. Saksena and their partners have similarly confirmed the anti-fungal activity of Trachyspermum ammi against the dermatophytes[20]. Likewise Pattanki and partners[21] and Ahmad and partners have carried out different investigations on the controlling effect of the Trachyspermum ammi essence on Candida albicans and have attested that the Trachyspermum ammi essence possesses this controlling effect[22]. The limitations of this study include a lack of in vivo experiments and using an animal serum that had a high probability of contamination. It is recommended that for future studies the effect of the extract on monocytes in in vivo conditions be investigated. In addition to the factors examined in this study, it is also advised that the effect of the extract on the production of prostaglandins be studied.

CONCLUSION

Based on the active ingredients found in the Trachyspermum ammi plant such as the flavonoids, it seems that this plant has noticeable anti-fungal properties. Therefore Trachyspermum Ammi also reduced IL-18 and TNF-α expression in mannose-activated THP-1 monocyte/ macrophage-like cells. Consequently if we take into account the effect the Trachyspermum ammi plant has on the afore-named cytokines, it can be used as an anti-fungal medication. It is advised that the effect of Thymol on fungal arthritis is investigated. Thymol is a phenol chemical compound which possesses anti-bacterial qualities and is used as an anti-fungal and an anti-parasitic.

Competing interests

Authors declared that they had no competing interests'

Authors' contributions

Maryam sadat Abtahi carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. Dr.Hossein maghsoudi participated in the design of the study and participated in the sequence alignment .Dr. Boshra hatef performed the statistical analysis and revised the manuscript. Pegah danyaly conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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