

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 Chondrocyte Cells

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DOI: <http://dx.doi.org/10.13005/bbra/1876>

(Received: 26 July 2015; accepted: 19 September 2015)

The *Trachyspermum ammi* plant has anti-fungal properties. Some fungi such as a yeast two *Sporothrix schenckii* produces the arthritis in pack animals. 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 provide by the Iranian biological resource center. We used chondrocyte cells as samples because they were similar to the cells with fungal infection. Then chondrocytes (5×10^5 cells/well) were incubated at a humidity of 90%, 5% CO₂ and 37°C for 72 h with control media alone or with *Trachyspermum ammi* at concentrations of 10 μ g/ml. One set of cells was activated for 1 h with mannose (3 mg/ml) for both reverse-transcriptase PCR and real-time PCR analysis of Tumor necrosis factor alpha (TNF- α) and Interleukin-18 (IL-18) expression. The activated cells by mannose treated by the *Trachyspermum ammi* extract reduced TNF- α expression as activated cells by mannose with fluconazole. However the IL-18 gene expression was reduced by both treatments but fluconazole had better effect. Based on the PCR and Real-time PCR methods analysis of inflammatory gene expression, *Trachyspermum ammi* plant such as the flavonoids has noticeable anti-fungal properties on chondrocyte cells.

Key words: *Trachyspermum ammi*, fluconazole, chondrocyte cells, Anti-fungal Infection

Background

There are only 100 species between 200,000 to 50,000 fungal species that cause infectious diseases in humans (kobayashi, 1990). The one cause of Arthritis could be some fungi like sporotrichosis that is a cause of *sporothrix schenckii*. Fungal arthritis damages the bone of wrists and ankles, fingers, ribs and vertebrae

following the release of cutaneous and subcutaneous lesions and causes the occurrence of fungus (rippon, 1988). The hospital infections increases the fungal disease (Kantarciohla & Yucel, 2002). pathogen-associated molecular patterns (PAMPs) that are recognized by germ line-encoded proteins and pattern recognition receptors (PRRs), are present in different cells of the organism. Their presence is mainly seen in monocytes, macrophages, dendritic cells, B-cells, T-cells and endothelial cells. PRRs include the Toll-like receptors (TLRs), a protein family of cellular

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receptors that mediate recognition of microbial pathogens and subsequent inflammatory response in vertebrates (Latge', 2010 and Levitz, 2010). TLRs and other PRRs confer PAMP recognition and their signaling triggers synthesis followed by release of pro-inflammatory cytokines, and induces expression of co-stimulatory molecules for promoting activation of adaptive immunity during antigen presentation. The simultaneous activation of multiple PRRs by one fungal pathogen endows the immune system with a broad range of possibilities for a specific and effective immune response (Valent, 2002)

Interleukine-18 (IL-18) has a role in regulating the innate and acquired immune system and also plays a key role in auto-immune, inflammatory, infectious and fungal diseases (Harishan, 2007). Tumor necrosis factor alpha (TNF- α) is a polytrophic factor which belongs to the cytokine family. TNF- α is a strong pro-inflammatory cytokine which belongs to the polypeptide group, which consists of several cytokines and growth factors (Carswell, 1975). Anti-fungal medications, with various formulas.. 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 (Rippon, 1988 and kown-chung, 1992).

Trachyspermum ammi is a native of Egypt and is cultivated in Iraq, Iran, Afghanistan, Pakistan, and India(Prakash Nadda, 1999-2011), Trachyspermum ammi L. belongin to family Apiaceae is a highly valued medicinally important seed spice. Trachysper ammi, commonly referred as Bishop's weed, Carom seed (English names) and ajowani or omum in Indian languages (Wadicar *et al.*, 2012). 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 (Pruthi, 1992). Higher phenolic and flavonoids content showed high antioxidant activity in ajowani seed extract (Saxena *et al.*, 2012). 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 (bown, 1996). studies in Iran showed that the extract of this plant has better anti-fungal effects compared

to anti-bacterial effects (khowje karam dini, 1988).

The aim of this study is to compare the effect of the Trachyspermum ammi plant and the common medication, fluconazole on chondrocyte cells. firstly the chondrocyte cells (Cartilage-forming cells) were activated by using a suitable concentration of mannose and forcing the cell to produce infectious cytokines, which consist of IL-18 and TNF- α , the effect of the Trachyspermum ammi plant on decreasing gene expression of the infectious intermediates is examined and compared with fluconazole.

METHODS

Cultivating THP-1 Monocyte/Macrophage cells

Articular chondrocytes were isolated from the radiocarpal joints of mature Holsteins by digestion of the tissue with typeII collagenase (220U/ml, GIBCO, USA) for 12-18 h at 37 °C. The isolated cells were filtered through a wire mesh screen to remove debris, and rinsed four times with Hank's Balanced Salt Solution (GIBCO, USA). Cells were pelleted by centrifugation and resuspended in control media containing: Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 50 μ g/ml ascorbic acid (GIBCO, USA), and 50 μ g/ml gentamycin (GIBCO, USA). Chondrocyte were counted and assessed for viability using a hemacytometer and the Trypan – blue exclusion method. Articular chondrocytes were plated in six-well plates (Costar; Cambridge, MA, USA) at a density of 5×10^5 cells/well and maintained at 37°C, 5% CO₂ for 5 days prior to use (Au B *et al.*, 2007).

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 μ g per ml and using the DMEMF-12 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% co₂ and 90% humidity. Thus LC50 has a concentration of 30 μ g/ml and its average was calculated to be 15 μ g/ml (Claude, 2005).

Expressing the Bovine TNF- α , Bovine GAPDH, Bovine IL-18 genes by treating

(chondrocyte cells) 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% CO₂ and 90% humidity and a duration of 72 hours. At the end of this stage the cells were treated with a 3mg/ml (Astarie-Dequeker *et al.*, 1999) concentration of mannose. They were then incubated at a temperature of 37°C, 5% CO₂ and 90% humidity and a duration of 1 hour in order to express the Bovine TNF-α, Bovine GAPDH, Bovine 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 (Au B *et al.*, 2007).

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 resuspended in Rnease-free water. In order to work out the concentration of the RNA the electroforez gel agaroze 1% method was used (Au B *et al.*, 2007).

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 Bovine TNF-α (forward, TAA CAA GCC GGT AGC CCA CG; reverse, GCA AGG GCT CTT GAT GGC AGA), Bovine IL-18 (forward, AGACCT GGA ACT AGATCA CTT TGG; reverse, TTG TTC TCA CAG GAG AGA GTA GAC) and Bovine GAPDH (forward, ATT CCA CCC ACG GCA AGT T; reverse, CGC TCC TGG AAG ATG

GTG AT) as the housekeeping gene were also 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 (Au B.A *et al.*, 2007).

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 (Au B *et al.*, 2007).

Statistical tests

After cultivating the chondrocyte cells in suitable conditions they were then treated in the following groups: chondrocyte cells without mannose (Cell group), chondrocyte cells accompanied with 3 mg/ml of mannose (Cell+mannous group), chondrocyte cells accompanied with 3 mg/ml of mannose and 10 µg/ml of the ethanol extract of *Trachyspermum ammi* (Cell+mannous+extract group) and chondrocyte 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 chondrocyte cells 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.

Table 1. Represents the mean (SD) of IL-18 and TNF- α in groups (Cell, Cell+Mannose, Cell+Mannose+Extract, Cell+Mannose+Fluconazole)

Cytokinins	Cell (N=8)	Cell + Mannose (N=8)	Cell + Mannose + Extract (N=8)	Cell + Mannose + Fluconazole (N=8)
IL-18	22.52 (1.2)	29.56 (1.3)	26.9 (1.4)	24.05 (1.1)
TNF- α	22.72 (0.83)	36.25 (0.7)	25.29 (1.2)	25.54 (1.3)

0.7) and P-value= 0.002. between Cell+ mannous and Cell+ mannous+ Fluconazole: mean difference (95% confidence interval was 5.5 (3.6 to 7.3) and P-value was P-value <0.0001 . But the Fluconazole showed better effect than extract: mean difference (95% confidence interval was 2.8 (0.98 to 4.8) and P-value<0.002). the IL-18 was significantly lower than all groups expect Cell+ mannous+ Fluconazole group (P-value = 0.158) (figure 1).

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

ANOVA test and Bonferroni analysis showed that both *trachyspermum ammi* and

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

ANOVA test and Bonferroni analysis showed that both *trachyspermum ammi* and Fluconazole decreased the expression of the gene IL-18 to compare the mannous cells. between Cell+ mannous and Cell+ mannous+ extract: mean difference (95% confidence interval was 2.6 (4.5 to

Fluconazole decreased the expression of the gene TNF- α to compare the mannous cells. Bonferroni test showed that between Cell+ mannous and Cell+ mannous+ extract: mean difference (95% confidence interval was 10.9 (9.4 to 12.4) with P-value< 0.0001, between Cell+ mannous and Cell+ mannous+ Fluconazole: mean difference (95% confidence interval was 10.7 (9.2 to 12.2) with P-value<0.0001 . But the Fluconazole showed no difference with extract: mean difference (95% confidence interval was 0.25 (-1.2 to 1.7) with P-value=1. (figure 2).

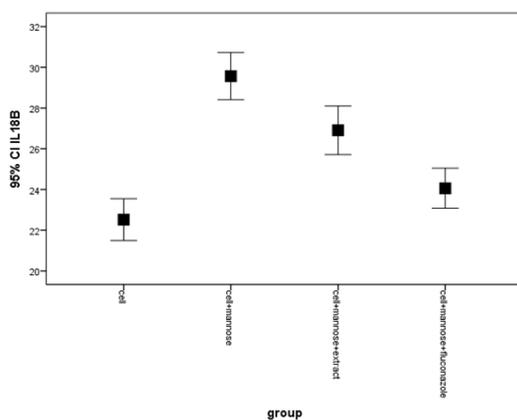


Fig. 1. Error plot showed the mean (SD) of IL-18 in all groups: There is significant Difference between groups (P value < 0.003), but no significant difference between Cell + Mannose + Extract group and Cell + Mannose + Fluconazole.

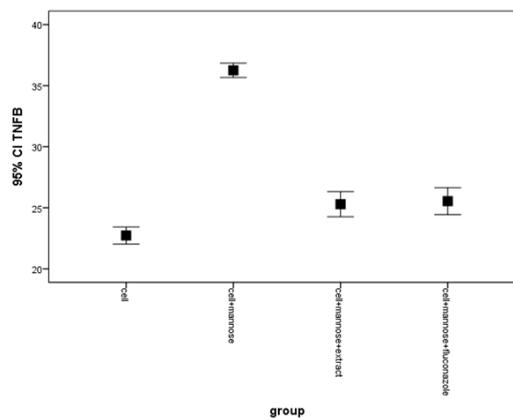


Fig. 2. Error plot showed the mean (SD) of TNF- α in all group: There is significant difference between groups (p-value < 0.001), no significant difference between Cell + Mannose group and Cell+ Mannose + Fluconazole.

DISCUSSION

In this study we have investigated the effects of the *Trachyspermum ammi* plant on Chondrocyte cells. 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- α . Chondrocyte cells activated with mannose that is a common for infectious model. mannose exists in the fungi cell walls, starts off infectious replies in the tissues. Chondrocyte 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 chondrocyte cell which is cultivated and activated with mannose like as Fluconazole. But these treatment could not reach the suppression level of gene expression like as Cell group expect Cell+ mannous+ Fluconazole group in IL-18 factor. However previous study showed that both treatment could suppress the gene expression of both inflammatory factors in monocyte cell like as cell group (Abtahi *et al.*, 2015)

The study of fungal infectious disease is perhaps a more challenging task than viral or bacterial diseases, because fungi are eukaryotic organisms and therefore immune system hardly distinguish them 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 (Kitano, 2007). Infectious arthritis is a common cause of joint destruction. The release of inflammatory mediators, joint and cartilage damage it, so that 48 hours after infection, the cells demolition begins (Robert *et al.*, 1997). Various types of plants, ethanol and water extracts and essential oils have been successful in stopping the growth of fungi in experimental conditions (Refaei, 2001).

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* (Iacobellis *et al.*, 2005).

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 (Navarro *et al.*, 1996 and Devasanker *et al.*, 1974,). Saksena and their partners have similarly confirmed the anti-fungal activity of *Trachyspermum ammi* against the dermatophytes (Saksena, 1984). Likewise Pattanki and partners (Pattanki, 1996) 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 (Ahmad, 2001).

In the present study, for the first time, the level of expression of pre-infectious cytokines was determined via mRNA. Moreover by adding a two-fold or three-fold consistency of amphotericin B, the duration of cell division decreased and showed that it reduced from 69-72 hours to 24-36 hours. we attempted to isolate chondrocytes through separation a layer of cartilage cells without the addition of collagenase type II only three times with wash and put in a medium enriched with a cocktail of antibiotics.

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 suggested that for future studies the effect of the extract on chondrocyte cells in vivo conditions and production of prostaglandins be investigated too..

CONCLUSIONS

The study showed that IL-18 and TNF- α expression were suppressed in mannose-activated chondrocyte cells treated by *trachyspermum ammi* plant like as fluconazole. Thymol is a phenol chemical compound which possesses anti-bacterial qualities and is used as an anti-fungal and an anti-parasitic is suggested to investigation.

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 and participated in the design of the study and participated in the sequence alignment . Dr. Boshra hatef performed the statistical analysis and revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We would like to thank Zahra Mousavi khalkhali for native language revision of the manuscript.

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