ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Protocol

Anti-Viral Activity of Thymol against Influenza A Virus

Tanusri Nandi and Madhu Khanna*

Department of Microbiology (Virology), Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India

***Corresponding Author:** Madhu Khanna, Professor, Department of Microbiology (Virology), Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India.

Received: July 20, 2022; **Published:** July 28, 2022

Abstract

Thymol is an established anti-parasitic drug against Trypanosoma brucei rhodesiense. Thymol is characterized as small molecule drug mainly isolated from Thymus vulgaris and Trachyspermum ammi. The active compound of thymol, 2-isopropyl-5-methylphenol, is a natural monoterpenoid phenol shows potent anti-microbial action against numerous gram positive and negative bacteria as well as fungal species. The objective of the study was to elucidate the anti-influenza effect of thymol in A549 cells. The data represents the effect of thymol on influenza A virus replication. Assessment of cytotoxicity of thymol was checked by MTT assay and 50% of minimal toxic concentration was found at 49.4 µg/ml. The inhibitory effect of thymol was analyzed by quantitative RT-PCR and western blotting. When treated with thymol at different concentrations only 50 and 25 µg/ml showed inhibition in expression compared to control at 24hr in both RNA and protein level. This is the first study which showed that thymol exhibited significant anti-influenza efficacy in A549 cells.

Keywords: *Thymol; Influenza A Virus; Anti-Viral; Quantitative RT-PCR and Western Blot*

Abbreviations

IAV: Influenza A Virus; DMEM: Dulbecco's Modified Essential Medium; BSA: Bovine Serum Albumin; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; DMSO: Dimethyl Sulfoxide; PBS: Phosphate Buffer Saline; MOI: Multiplicity of Infection; PVDF: Polyvinylidene Difluoride; HRP: Horseradish Peroxidase; RT: Room Temperature

Introduction

The therapeutic application of plant derived molecules has been quoted in ancient cultures and traditions in many countries as traditional medicine. Thymol known as 2-isopropyl-5-methylphenol, is a natural monoterpenoid phenol derivative of cymene, found in oil of thyme and extracted from *Thymus vulgaris*, *Trachyspermum ammi*. Thymol, a white crystalline substance has potent antiseptic properties including anti-bacterial, anti-fungal, anti-oxidative, expectorant, anti-spasmodic and anti-helminthic. The lethal dose (LD_{50}) value of thymol in rat was 980 mg/kg/body weight; hence forth thymol based formulations likely devoid of toxic effects on humans [1]. The phenolic monoterpenoids of thymol an effective agent of killing arthropod vectors responsible for Yellow Fever, Dengue, Malaria, West Nile, Rift Valley fever, Chikungunya, Japanese encephalitis and Zika virus [2]. Nanoemulsions of thymol-eugenol conjugate showed larvicidal effect on *Aedes aegypti*, the main causative agent of Zika, Chikungunya and Dengue transmission to human worldwide [3].

Thymol displayed anthelmintic activity to pig roundworm, *Ascarissuum* in *in-vitro* system [4]. Thymol isolated from Turkish *Origanum onites* demonstrated anti-protozoan efficacy in *in-vitro* and in mouse model against *Trypanosoma brucei rhodesiense*, *T. cruzi*, *Leishmania donovani* and *Plasmodium falciparum* and also increase the survival rate of animal model after treatment [5]. Further combination therapy of carvacrol and thymol demonstrated synergistic anti-bacterial effect on *Staphylococcus aureus in-vitro* method [6]. Thymol also possesses anti-microbiocidal effect on some antibiotic resistance gram positive and negative bacteria and fungal strain [7]. Thymol also has virucidal efficacy against hepatitis C virus (HCV) causes bovine viral diarrhea in cattle [8].

Influenza A viruses (IAV) belongs to the family of *Orthomyxoviridae*, a major respiratory pathogen and responsible for causing remarkable public health threat also number of pandemics and epidemics. However, IAV infections still persists and challenges clinically especially in high-risk patients such as children, elderly and immuno-compromised person. Antiviral drugs are important for treatment, especially with emergence of novel viruses which may not prevent infections by seasonal vaccines [9]. Traditional use of anti-viral drug such as Oseltamivir, Rimantadine, Zanamivir and Amantadine are limited due to its side effects and evolution of new viral strains [10]. The main prevention from influenza is vaccination and individuals are at greater risk of developing complications at the time of infection like pregnant women, children, elder groups and people with medical conditions. The composition and time of administration of vaccine are crucial to provide adequate immunity against influenza virus [11]. Thus, development of an anti-viral drug with minimal toxicity may combat the burden of disease progression by suppressing influenza infection. The study shed lights on the inhibitory effect of thymol against IAV in A549 cells. Our results showed reduction of virus infectivity and anti-viral efficacy of thymol. The purpose of the study was to elucidate and establishment of a naturally derived, low toxic anti-viral compound. Data obtained from the hypothesis demonstrated potential anti-influenza efficacy of thymol in A549 cell line (Human lung epithelial cells) by significant reduction of viral replication in dose dependent manner.

Materials and Methods

Cells, virus, reagents, antibodies and drug

A549 cells were maintained in Dulbecco's Modified Essential medium (DMEM; Invitrogen) containing 10% Fetal Bovine Serum (Invitrogen) at 37˚C and 5% CO_2. Influenza A virus (strain A/Puerto Rico/8/1934 H1N1) was used for the study. Thymol (Sigma) was dissolved in DMSO (dimethyl sulfoxide, Sigma). Stock solution of 100 mg/ml was prepared and stored at -20˚C until use. Anti-PB1 antibody was purchased from Thermo Scientific. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was procured from Cell Signaling Technology. Primers were synthesized from Eurofins.

MTT assay

A549 cells were seeded in 96 wells plate and incubated at 37˚C and 5% CO_2. Varying concentration of thymol (100 µg/ml to 3.9 µg/ml) was added to the cells and incubated for 24h at 37˚C. Following 24h incubation, 20 µl (5 mg/ml) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma) was added to the wells and incubated for 3h at 37˚C in dark. The media containing MTT reagent was removed and 100 µl of DMSO was added to dissolve the formazan crystals and kept in shaker for 10 minutes in dark. The absorbance was recorded at 570 nm using Epoch 2 micro plate spectrophotometer (Biotek).

Virus infection and quantitative real time PCR

A549 cells were plated in 6 wells plate and maintained as mentioned above. The cells were washed 2 times with 1X phosphate buffer saline (PBS). The cells were infected with influenza A virus (IAV) at multiplicity of infection (MOI) of 1 for 1hr at 37˚C. Cells were washed with 1X PBS. Serially diluted thymol (50 µg/ml to 6.25 µg/ml) in incomplete DMEM was added to the cells and incubated for 1h at 37˚C in gentle shaking. After treatment, the cells were washed again with 1X PBS and replenished with 1% FBS containing DMEM and incubated

for 24hr. Total RNA was isolated using Trizol reagent (Invitrogen) as per manufacturer's protocol (Qiagen). One microgram of RNA was used for cDNA synthesis (Promega) as per manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. Relative expression of viral gene was monitored by CFX96 real time PCR (Biorad) using 2X SYBR green master mix (Affymatrix). The following thermal cycling profile was used for the PCR analysis: 95°C for 10 minutes, 95°C for 15 seconds, and 55°C for 15 seconds, 60°C for 1 minute 72°C for 30 seconds for 35 cycles.

The following primers were used:

HA: 5′- CCCAGGGYATTTCGCYGACTATGAG 3′ and 3′- CATGATGCTGAYACTCCGGTTACG 5′.

GAPDH: 5′- TGCACCACCAACTGCTTAGC 3′ and 3′- GGCATGGACTGTGGTCATGAG 5′.

Immunoblotting

Whole protein was isolated using RIPA buffer and protease inhibitor cocktail (Cell Signaling Technology) and quantified using BCA method, 50 µg of protein was separated onto 12% SDS-PAGE gel. The protein was transferred in polyvinylidene difluoride (PVDF) membrane, blocked using 5% bovine serum albumin (BSA, Himedia) for 1h at room temperature (RT) in gentle shaking. The membrane was then incubated with anti-PB1 antibody (1: 2500; Thermo Scientific) overnight at 4˚C. The membrane was washed with 0.1% Tween in 1X phosphate buffer saline (PBST) and incubated with horseradish peroxidase (HRP) conjugated anti-rabbit IgG (1: 1000; Cell Signaling Technology) for 1hr at room temperature. The PVDF membrane was washed using 0.1% PBST for 10x3 minutes at RT. Expression was detected using chemiluminescence detection kit and chemidoc imaging system (Biorad).

Statistical analysis

Graph Pad Prism 9.0v was used for statistical analysis. The result obtained was expressed in mean ± SEM. One-way ANOVA was used to analyze the data and p value < 0.05 were considered significant. All the experiments were done in triplicates and three independent sets were performed.

Results and Discussion

Cytotoxicity of thymol in A549 cells was assessed by MTT assay. The result displayed dose dependent cell viability and 50% inhibitory concentration at 49.4 µg/ml (Figure 1). Thus, concentration of 50 µg/ml to 6.25 µg/ml used for all the experiment. Anti-viral activity of thymol at transcriptomic level demonstrated inhibitory effect at both 50 µg/ml and 25 µg/ml compared to virus infected cells. Significant inhibition was observed at 25 µg/ml of thymol treatment (Figure 2). In western blotting, similar inhibition of viral protein was also observed further validating our hypothesis. The band intensity was calculated and normalized against GAPDH (Figure 3A and 3B). Our study revealed that thymol has potent anti-influenza action in A549 cells at minimal concentration. Quantitative RT-PCR and immunoblotting suggested thymol hindered with influenza A virus replication and progression. RC-37 cells showed antiviral activity by plaque reduction assay upon treatment with essential oil contain monoterpenoids, the main component of thymol. HSV-1 was inhibited about > 80% by monoterpenoids [12]. The essential oil of *Thymus guyonii* contains 21.2% of thymol also demonstrated anti-bacterial efficacy on blood agar at 99.9% against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and also possess anti-viral activity in Vero non-tumoural cell line at 90 - 100% against Coxsackievirus B3 virus [13]. Electron microscopy on Vero cells showed disruption of virus particles 100% after 1 hr treatment at 100 µM by thymol as compare to Acyclovir, a drug of choice for HSV-1 virus infection. The morphological changes are consistent with antiviral activity by thymol [14]. Overall, from this study we can interpret thymol, a natural monoterpenoids has ability to suppress influenza A virus infection in *in-vitro* model.

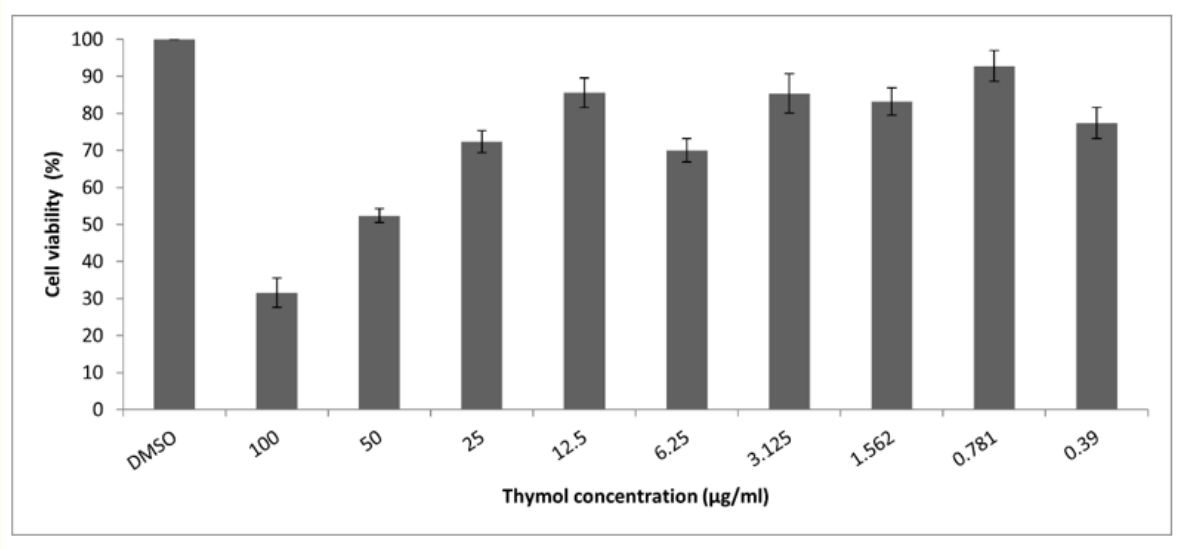

Figure 1: *Cytotoxicity assay. Cell viability assay in presence of thymol in A549 cells at 24hr. The absorbance was recorded at 570 nm and the IC50 value was found to be at 49.4 µg/ml.*

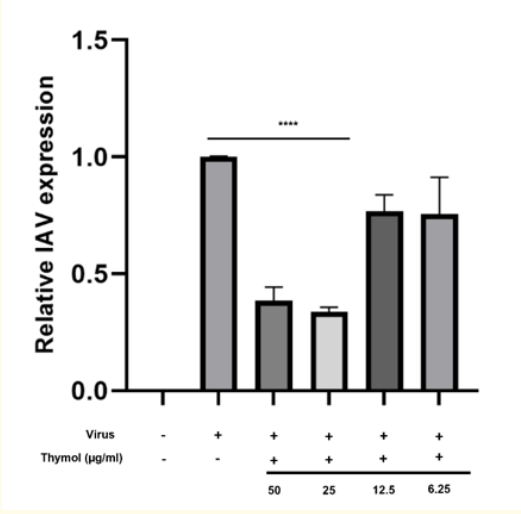

Figure 2: *Real-time quantitative PCR analysis of anti-viral activity. Relative expression of influenza A virus RNA by quantitative RT-PCR when treated with thymol in A549 cells at 24hr. In control group, A549 cells were treated with equal volume of solvent vehicle (DMSO, < 0.1%). In virus group, cells were infected with IAV (MOI 1). In other groups, cells were infected with IAV followed by treatment with thymol for 24hr. Error bars indicated the standard deviation of the mean from three independent experiments *** p < 0.001.*

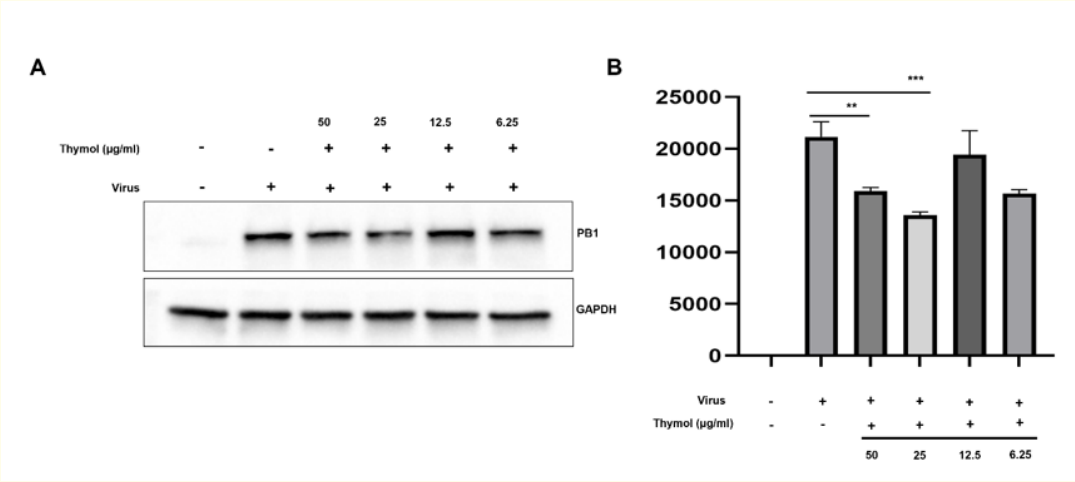

Figure 3: *Inhibitory effect of thymol on influenza A virus at 24hr in A549 cells. A) Western blot of viral protein PB1. B) Densitometric analysis (IDV) of western blot. In control group, A549 cells were treated with equal volume of solvent vehicle (DMSO, < 0.1%). In virus group, cells were infected with IAV. In other groups, cells were infected with IAV followed by treatment with thymol for 24hr. Error bars indicated the standard deviation of the mean from three independent experiments *** p < 0.001, ** p < 0.01.*

Conclusion

Thymol, a natural compound consists of monoterpenoids has various anti-microbiocidal effects. Our study demonstrated that, thymol exhibited anti-viral activity by reducing virus infectivity in dose dependent manner. Thymol might be acceptable for the treatment of influenza virus infections as long as there is no better treatment available. However, appropriate preclinical studies will further strengthen the data. To the best of our knowledge, this is the first data which demonstrated significant anti-viral activity of thymol against influenza A virus. Therefore, we believe this investigation may be useful in initiating further research to develop thymol as novel therapeutic intervention for treatment of IAV.

Acknowledgements

Authors wish to acknowledge Vallabhbhai Patel Chest Institute for financial support to carry out the study.

Conflict of Interest

Authors declare no conflict of interest.

Bibliography

1. Nagoor Meeran MF., *et al*. "Pharmacological Properties and Molecular Mechanisms of Thymol: Prospects for Its Therapeutic Potential and Pharmaceutical Development". *Frontiers in Pharmacology* 26.8 (2017): 380.

2. Youssefi MR., *et al*. "Efficacy of Two Monoterpenoids, Carvacrol and Thymol, and Their Combinations against Eggs and Larvae of the West Nile Vector Culex pipiens". *Molecules* 15.2410 (2019): 1867.

3. Lucia A., *et al*. "Nanoemulsions based on thymol-eugenol mixtures: characterization, stability and larvicidal activity against *Aedes aegypti*". *Bulletin of Insectology* 73.1 (2020): 153-160.

4. Garcia-Bustos JF., *et al*. "An appraisal of natural products active against parasitic nematodes of animals". *Parasites and Vectors* 17.121 (2019): 306.

5. Tasdemir D., *et al*. "Antiprotozoal Activity of Turkish Origanum onites Essential Oil and Its Components". *Molecules* 3.24.23 (2019): 4421.

6. Rúa J., *et al*. "Combination of Carvacrol and Thymol: Antimicrobial Activity against *Staphylococcus aureus* and Antioxidant Activity". *Foodborne Pathogens and Disease* 16.9 (2019): 622-629.

7. Marchese A., *et al*. "Antibacterial and antifungal activities of thymol: a brief review of the literature". *Food Chemistry* 1.210 (2016): 402-414.

8. Kubiça TF., *et al*. "In vitro inhibition of the bovine viral diarrhoea virus by the essential oil of *Ocimum basilicum* (basil) and monoterpenes". *The Brazilian Journal of Microbiology* 11.451 (2014): 209-214.

9. Makau JN., *et al*. "A Quinolinone Compound Inhibiting the Oligomerization of Nucleoprotein of Influenza A Virus Prevents the Selection of Escape Mutants". *Viruses* 12 (2020): 337.

10. Dou D., *et al*. "Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement". *Frontiers in Immunology* 9 (2018): 1581.

11. Caini S., *et al*. "The epidemiological signature of influenza B virus and its B/Victoria and B/Yamagata lineages in the 21st century". *PLOS ONE* 14.9 (2019): e0222381.

12. Astani A., *et al*. "Comparative Study on the Antiviral Activity of Selected Monoterpenes Derived from Essential Oils". *Phytotherapy Research* 24.5 (2010): 673-679.

13. Zeghib A., *et al*. "Antibacterial, Antiviral, Antioxidant and Antiproliferative Activities of Thymus guyonii Essential Oil". *Natural Product Research* 16 (2019): 1-4.

14. Lai WL., *et al*. "Inhibition of Herpes Simplex Virus Type 1 by Thymol-Related Monoterpenoids". *Planta Medica* 78.15 (2012): 1636-1638.

Volume 18 Issue 8 August 2022