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In-vitro evaluation of thymol derived from *Trachyspermum ammi* against acetaminophen-induced hepatotoxicity and its therapeutic applications for lymphatic filariasis

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ABSTRACT

Individuals with lymphatic filariasis (LF) show elevated levels of pro-inflammatory cytokines and altered expressions of inflammatory genes, and are prone to secondary bacterial infection. Also markers of liver dysfunction were reported to be high with increased filarial infection. Acetaminophen (Paracetamol), an anti-pyretic drug was prescribed to LF individuals along with diethylcarbamazine (DEC) and albendazole (ALB) to alleviate pain and filarial fever within the therapeutic dosage. It is unclear, whether parasite-secreted toxins augment the action of acetaminophen and enhance liver dysfunction. Hence, an agent which can attenuate the acetaminophen toxicity during filarial infection is warranted. *Trachyspermum ammi* (*T. ammi*) is the richest source of thymol, reported to have the anti-filarial lead molecule. In the present study we evaluated the effects of thymol against acetaminophen-induced hepatotoxicity in *in-vitro* settings. In addition, we examined the synergistic effects of thymol against bacterial infection, free radicals and cytokine production. Our results reveal that acetaminophen (250 µg/ml) induces significant reduction in the viability of WRL-68 liver cells compared to cells without treatment. However, thymol at the same concentration restores the cell viability significantly ($p = 0.031$) by attenuating the toxicity within 24 h. Thymol inhibits the expression of Interleukin-6 ($p = 0.043$) and Interleukin-8 ($p = 0.048$) in WRL-68 cells and thereby provides maximum protection to liver cells from inflammatory insults. Calorimetric analysis shows the ability of thymol in scavenging hydrazyl ($p = 0.004$) and hydrogen peroxide ($p = 0.008$) free radicals efficiently. Thus thymol derived from *T. ammi* may be a good therapeutic agent in reducing the toxicity of acetaminophen, and may also aid in LF treatment and management in addition to the existing drugs.

KEYWORDS: hepatotoxicity, acetaminophen, *Trachyspermum ammi*, lymphatic filariasis, thymol

INTRODUCTION

Lymphatic filariasis (LF) is a major parasitic infection caused by nematodes such as *Brugia malayi* and *Wuchereria bancrofti*. These parasites dwell in the lymphatic vessels and eventually lead to chronic lymphedema and elephantiasis [1]. Stagnation of lymph fluid encourages secondary bacterial and fungal infection on the dermal cells and tissues. Bacterial growth and multiple skin lesions attenuate lymph movement and the reduced ability of the lymph nodes to filter the bacteria leads to inflammation. Recurrent episodes of bacterial infections further damage the tiny lymphatic vessels in the skin, reducing their ability to drain fluid efficiently. Nevertheless LF is associated with the elevated levels of

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pro-inflammatory and pro-fibrotic cytokines [2]. Notably, the expression of genes involved in cellular adhesion, inflammation and lymphangiogenesis are altered. All these pathophysiological stimuli promote the progression of lymphedema from reversible to irreversible state [3].

DEC and ALB are the promising microfilaricidal drugs, least effective against microfilaria [4]. Paracetamol, a commonly used analgesic and anti-pyretic drug was given frequently to alleviate parasite-induced pain and filarial fever to the LF individuals within the therapeutic dose. The drug was considered to be safe, when taken within the therapeutic dose but higher dose leads to liver damage and acute liver failure [5, 6]. Pumford *et al*. reported that increased levels of alanine transaminase (ALT) and aspartate transaminase (AST) in serum, directly correlates with the appearance and formation of paracetamol-cytotoxic protein adducts, which is said to be a biomarker indicating the paracetamol toxicity [7]. Jaeschke and colleagues reported the plausible mechanisms pertaining to hepatotoxicity imparted by bile acids, adhesion molecules, cytochrome enzyme, peroxy-nitrite and mitochondrial dysfunction [8]. However the exact mechanism by which paracetamol induces toxicity in liver cells is yet to be studied.

India has a rich resource of medicinal herbs and aromatic spices with high potential abilities, whereas their synergetic effect in medical applications is not fully explored, which is the need of the hour and is highly warranted. Accumulated research reports highlight the beneficial and therapeutic role of *Trachyspermum ammi* [9, 10]; however research studies addressing the influence of thymol derived from *T. ammi* are necessary in understanding the mode of disease suppression. Pro-inflammatory cytokines namely Interleukin-6 (IL-6) and Interleukin-8 (IL-8) [2], play a key role in the initiation of irreversible swelling, fibrosis [11, 12], and regenerative processes of hepatic structure and function [13, 14]. Hence, we investigated the potential abilities of thymol against hepatotoxicity, bacterial infection; oxidative stress, elevated cytokines and inflammatory markers expression in response to paracetamol-induced effects in WRL-68 liver cells. The study warrants insights in addressing the strategies for lymphatic filarial treatment and management.

MATERIALS AND METHODS

Materials

HPLC grade methanol (Cat: AS059), hexane (Cat: AS097), chloroform (Cat: AS114), glacial acetic acid (Cat: AS001) and microbiological grade Mueller Hinton agar (Cat: M173) were purchased from Himedia. Molecular grade reagents required for RNA isolation namely TRI reagent (Cat: T9424), chloroform (Cat: V800116), isopropanol (Cat: V800228), formaldehyde (Cat: V800189), 3-(N-morpholino) propanesulfonic acid, (MOPS, Cat: M1254), Diethyl (DEPC, Cat: D5758), thymol (Cat: T0501) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent (MTT, Cat: M2128) were purchased from Sigma Aldrich. Lactate Dehydrogenase cytotoxicity assay kit (LDH, BioVision, Cat: K311-400), acridine orange (Cat: A6014), propidium iodide (Cat: P4864) and all quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) primers were purchased from Sigma Aldrich. Fetal bovine serum (Gibco, Cat: 10270), Dulbecco's Modified Eagle's medium (DMEM, Cat: SH30243.01), antibiotic anti-mycotic solution (Cat: A002) and trypsin-Ethylene-diamine tetra acetic acid (EDTA, Himedia Labs, Cat: TCL007).

Plant extraction and phytochemical evaluation

Seeds of *T. ammi* were collected from local market in Madurai, identified by a botanist and voucher specimen was kept for future studies. The seeds were rendered free from all impurities by washing with distilled water, shade dried and ground into fine powder. About 100 g of seed powder was subjected to Soxhlet apparatus for extraction using 500 ml methanol. The process of extraction was carried out for 48 h. The extracts were concentrated by vacuum evaporator. Dried extract were used for further studies. The crude *T. ammi* extract was qualitatively screened to determine the presence of bioactive compounds as per the standard tests described by Kaur and co-workers [15], Trease and Evans [16].

Isolation and characterization

The crude methanolic extract was subjected to silica gel column chromatography (SGCC) for purification of thymol as previously described [17]. 1 g of the residue was subjected to silica gel column with toluene: ethyl acetate as eluent in the ratio of 9.7:0.3. The eluted fractions were collected in different test tubes based on their colouration into 9 fractions (fractions 1-9). The residues in the column were washed out with methanol. All the collected fractions from the silica gel column were spotted on pre-coated thin layer chromatography (TLC) plates with silica gel G as explained by Skoog *et al*. [18]. It was then kept in previously saturated developing chamber containing mobile phase (Toluene: Ethyl acetate), and allowed to run 3/4th height of the plate. The developed plate was removed and air dried. Plate was observed under UV light in a chamber and the spots were identified. Retention factor (Rf) values were determined by using the following formula:

Retention Factor (Rf) = Distance traveled by solute / Distance traveled by solvent

Gas chromatography-Mass spectrometry (GC-MS)

The samples were subjected for analysis using GC-MS-QP2010-P (Shimadzu) equipped with Quadra pole detector and split injection system. The GC was fitted with a ZP-624 capillary column (30 mm x 1.4 mm, film thickness 0.25 µm). The temperature programmed were as follows: injector temperature 220 °C, initial oven temperature of 50 °C for 2 min, then rise to 250 °C at the rate of 10 °C/min for 25 min, and transfer line temperature 220 °C. Helium was used as the carrier gas at 35.6 kPa pressure with flow 2.5 ml/min and electronic pressure control being kept on. The EM voltage was 952.9 V with lower and upper mass limits set at 30 and 350 *m*/*z*. Samples were dissolved in *n*-hexane and injected automatically. MS spectra of separated compounds were compared with Wiley7 Nist 05 mass spectral database.

High performance liquid chromatographic (HPLC) analysis

An isocratic HPLC method was employed to determine the major active phytoconstituents in *T. ammi* extract. The reversed phase (C8) analytical column (Phenomenex) (Dimension: 150 x 4.6 mm and Pore size: 5 µm) at the flow rate of 1.5 mL/min at 50 °C was used for the analysis. Both crude extract of *T. ammi* and the fractions were collected from silica gel column were dissolved in methanol and analyzed by HPLC fitted with PDA detector at 272 nm.

Screening for anti-bacterial activity

The anti-bacterial activity of the crude extract and the isolated thymol was carried out by disc diffusion and agar well diffusion methods, respectively, against both Gram positive bacteria namely *Bacillus subtilis*, *Streptococcus pyogenes* and *Methicillin Resistance Staphylococcus aureus* (*MRSA*) and Gram negative bacteria namely *Pseudomonas sp*., *Klebsiella sp*. and *Escherichia coli*. The bacterial strains were purchased from MTCC, IMTECH, Chandigarh. Ampicillin (10 mg/ml) was used as the standard control as demonstrated by Bauer and Kirby [19]. The stock culture of each bacteria used were sub-cultured at 37 ºC for 24 h.

Evaluation of anti-oxidant activity

The evaluation of the anti-oxidant potential of the isolated thymol to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was assessed by the standard colorimetric method as described by Mensor [20]. DPPH is a stable free radical, which changes its colour from violet to yellow upon reduction by the process of electron donation. Thymol converts it to 1,1-diphenyl-2-(2, 4, 6-trinitophenyl) hydrazine. The scavenging potential of the thymol can thus be determined by their degree of discolouration to yellow.

Additionally we evaluated the anti-oxidant ability of thymol in scavenging the hydrogen peroxid*e* (H_2O_2) free radicals. A total of 4 ml of (1.25-10 µl/ml) thymol was added to 0.6 ml of hydrogen peroxide solution (4 mM) in phosphate buffer (0.1 M and pH 7.4). After incubating for 10 min at 37 ºC, the absorbance at 230 nm was measured. Corresponding blanks were taken. The experiment was performed in triplicate. The absorbance of phosphate buffer as control was measured at 230 nm. Hydrogen peroxide produces hydroxyl radicals in the cells. Scavenging of these

radicals is seen by the decrease in absorbance at 230 nm with increasing concentration of the test sample. The scavenging effect (%) was measured using the formula:

H_2O_2 radical scavenging activity (%) =
$$[(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

In-vitro hepatoprotective activity

Cell culture

Normal WRL-68 liver cell line (ATCC. No: CL-48) was purchased from the National Centre for Cell Sciences (NCCS), Pune. These cells were grown in Dulbecco's modified eagles medium (DMEM) with high glucose and were cultured with 10% fetal bovine serum (FBS) and 1X antibiotic anti-mycotic solution (containing penicillin, streptomycin and amphotercin-B). The cultures were maintained at 37 ºC, 5% CO_2 and sub-cultured periodically using 0.25% trypsin-EDTA solution.

MTT assay

The MTT assay is a simple non-radioactive colorimetric assay used to directly measure cell proliferation or viability and indirectly measure cell toxicity. The MTT assay relies on the mitochondrial activity of cells and thus serves as a biomarker of cell metabolism. To determine cell viability and proliferation, the WRL-68 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate at 37 ºC in 5% CO_2 and grown for 24 h. At 75-80% confluency, cells were treated at four different conditions for 24 h at 37 ºC. 1). Cells treated only with paracetamol in various concentrations (25, 50, 100, 250 and 500 µg/ml), in order to determine the half lethal concentration (LC_{50}); 2). Cells treated only with thymol in the same concentrations as paracetamol to determine the half maximum effective concentration (EC_{50}); 3). Cells treated in combination, paracetamol followed by thymol and 4). Cells without any treatments that act as negative control. After 24 h treatment, culture media were removed and collected accordingly. Fresh medium (100 µl) was added along with MTT dye (10 µl) (5 mg/ml in phosphate buffer pH 7.4) and incubated for 4 h at 37 ºC in 5% CO_2. The medium was removed and the formazan crystals were solubilized with Dimethyl Sulphoxide (DMSO) (200 µl). The absorbance of each well was read on a micro plate reader at 575 nm. The relative cell viability (%) related to control wells containing cell culture medium without any treatment was calculated using the formula $[A]_{test} / [A]_{control} \times 100$.

Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase is an oxido-reductase enzyme present in almost all organisms. Cells release LDH into the blood circulation (*in-vivo*) or into the culture supernatant (*in-vitro* settings) when exposed to any injurious stimuli or cells undergoing death. In this colorimetric LDH quantification assay, LDH reduces Nicotinamide Adenine Dinucleotide (NAD) to (Nicotinamide Adenine Dinucleotide-Hydrogen (NADH), which then interacts with a specific probe to produce a colour which can be read at λmax = 450 nm.

Trypan blue exclusion assay

Trypan Blue assay was employed to further confirm the cell viability of WRL-68 cells with and without various treatments. Live and intact cells exclude the uptake of the dye whereas, cells with damaged cell membrane receives the dye and appears blue in colour.

RT- PCR studies

Semi-quantitative PCR was performed in Veriti Thermal Cycler (Applied Biosystem) using Taq DNA Polymerase. Total RNA was isolated using the TRI reagent. Reverse transcription was performed with 1 µg DNAse-treated RNA for 3 hours at 37 °C. The expression levels of IL-6 and IL-8 were quantified. Relative amount of target mRNAs were normalized to β-actin mRNA as internal control.

Statistical analysis

Data analyses were performed using Windows-based Graph Pad PRISM (GraphPad Software, Version 6) and are expressed as mean ± SEM. We employed Kolmogorov Smirnov (KS) test to determine the normality of distribution. Comparisons between two groups were performed by the unpaired parametric Student t-test or one way analysis of variance, followed by Tukey's multiple comparison post test. Statistical significance was accepted if the null hypothesis was rejected at $p \leq 0.05$.

RESULTS

Extraction yield and phytoconstituents

The yields of methanol-extracted crude residue from seeds of *T. ammi* and the active fraction after removal of solvent in rotary evaporator were around 15% w/w and 5% w/w, respectively. The phytochemical screening of the methanolic seed extract of *T. ammi* showed that the seeds are rich in alkaloids, flavonoids, terpenoids, tannins and phenol. The presence of these various phytoconstituents was confirmed by standard phytochemical tests which were evident by the colour formation (Figure 1A).

Purification and characterization

The SGCC was used to isolate and identify the active components in the plant extract and separate out the active fraction components based on the colour. In this study, 1 g of the crude extract was fractionated into fractions 1-9 (Figure 1B & 1C). Thin layer chromatography was employed to identify the specific component in the fractions based on the refractive index (Rf) value. TLC was performed to confirm the presence of thymol. Based on the Rf value, fractions 1-5 and 6-9 were pooled together. The results showed a spot with a Rf value of 0.64 in the TLC plate which was identified as

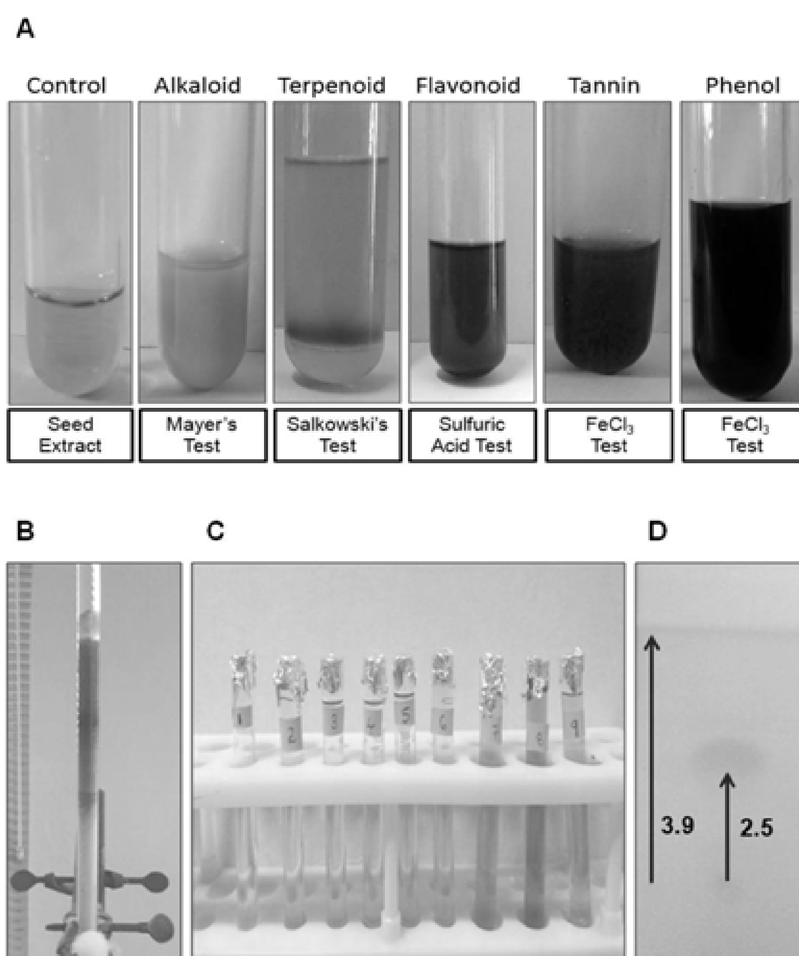

Figure 1. Phytochemical screening and separation. (A) Test tubes showing the results of confirmatory tests for various phytoconstituents present in the seed extract of *Trachyspermum ammi* which were identified based on the colour formations. **(B)** Separation of thymol using silica gel column, **(C)** Test tubes showing separated active fractions (1-9) and **(D)** Thin layer chromatographic plate showing the fraction migrated along the mobile phase Toluene: Ethyl acetate (9.7:0.3).

thymol (Figure 1D) and confirmed by comparing the standard thymol Rf value.

GC-MS analysis reported that eight active compounds were identified in the crude methanolic seed extract of *T. ammi*. The identified constituents are listed in table 1. GC-MS chromatogram showing the peak at the retention time (RT) 7.474 min^{-1} (Figure 2A) indicates the presence of thymol (m/z-135.10) which was compared by the standard thymol peak (Figure 2B). The reverse phase HPLC analysis confirmed that the isolated compound was thymol by the chromatogram peak with a retention time of nearly 6.18 min (Figure 2C) as previously reported.

Table 1. Major phytoconstituents in the crude extract of *T. ammi* seeds.

Peak	Chemical compounds	Retention time (RT)
1	Thymol	7.474
2	Apocynin	9.50
3	Cadecadienoic acid	17
4	2,4-Dioxaspiro	19.50
5	Octadecanoic acid	20
6	9-octadecanoic acid	21.50
7	Durohydroquinone	21.50
8	Benzo quinoline	23.50

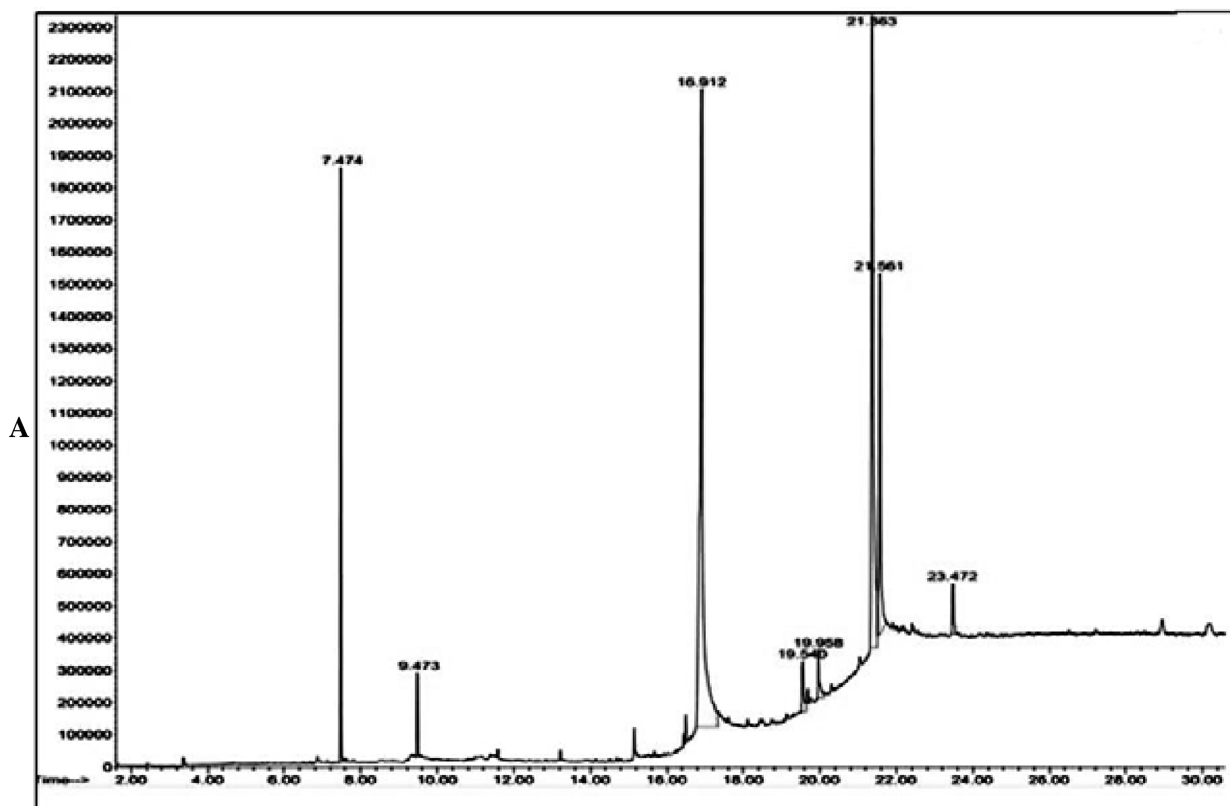

Figure 2

Figure 2 continued..

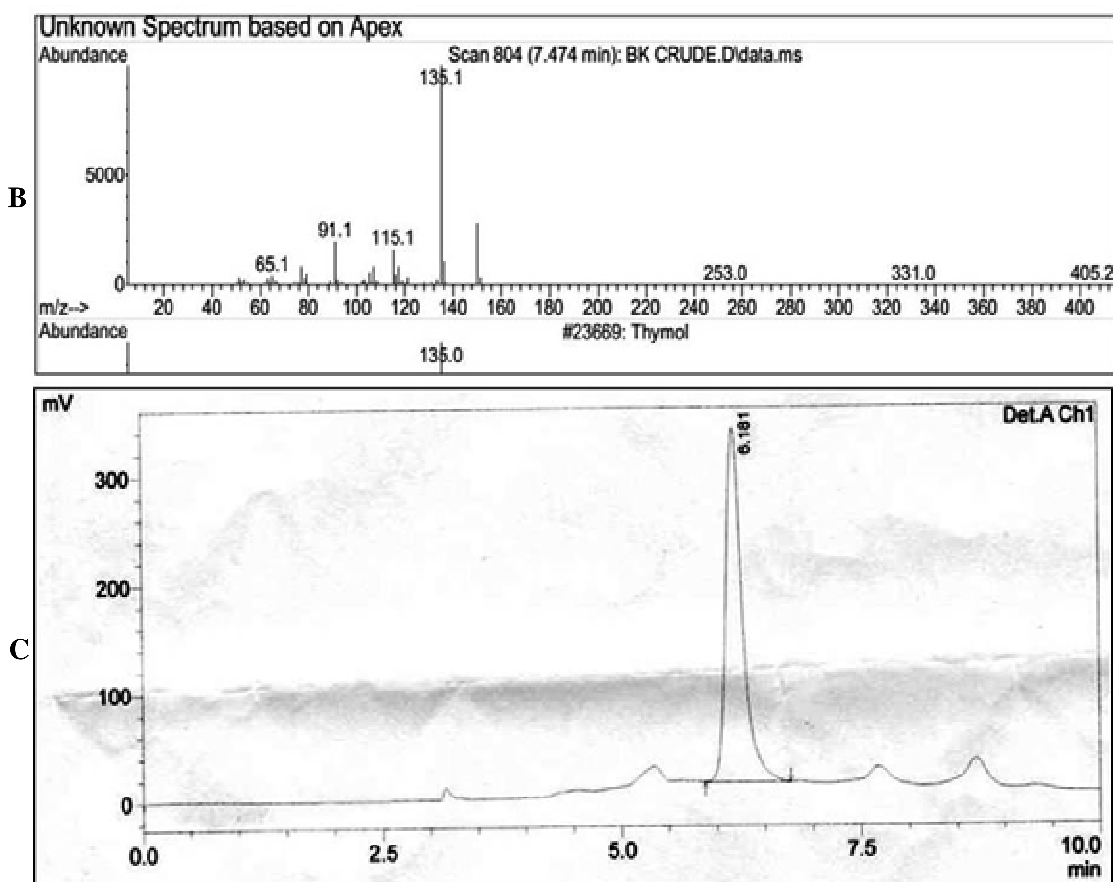

Figure 2. Characterization of phytoconstituents using analytical techniques. GC-MS chromatogram profile showing various phytoconstituents present in the seed extract of *Trachyspermum ammi* **(A)** and standard thymol chromatogram **(B)**. **(C)** Reverse phase HPLC chromatogram showing thymol peak with a retention time at 6.18 min.

Anti-bacterial activity

The anti-bacterial activity of the crude seed extract (Figure 3A) and the isolated thymol (Figure 3B) against different bacterial strains shows zones of inhibition. A clear inhibition zone was formed around the disc containing the crude extract (disc diffusion) and the well containing different concentrations of thymol (agar diffusion) with the standard antibiotic ampicillin as the positive control. The diameter of the zones formed as a result of the inhibited bacterial growth was measured using the vernier calliper.

Anti-oxidant activity

Our data showed that thymol scavenges the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical in a dose-dependent manner. Increasing the concentration of thymol (10-50 µl/ml), increases the percentage of the scavenging activity ($p = 0.004$), which is indicated by the intensities of the colour change. The H_2O_2 (Hydrogen peroxide) radical scavenging ability of the thymol was higher than ascorbic acid at the same concentration ($p = 0.008$). The results were represented using line graphs as shown in figure 4A & B.

In-vitro hepatoprotective activity of thymol

Cell viability of thymol and paracetamol was evaluated at different concentrations (25, 50, 100, 250 and 500 µg/ml) in WRL-68 liver cell line at 24 h, to determine the half maximum effective concentration (EC_{50}) and half lethal concentration

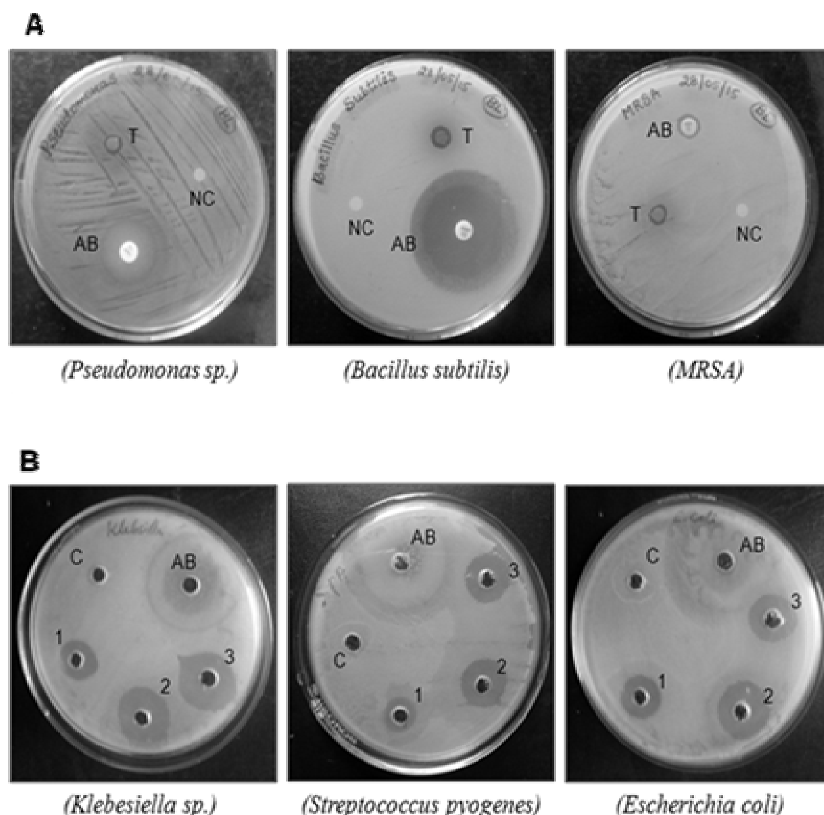

Figure 3. Screening of anti-bacterial activity of thymol. Representative images showing Mueller Hinton Agar plates to screen anti-bacterial activity of crude extract by disc diffusion method **(A)** and isolated thymol by agar well diffusion method **(B)**. Zone of Inhibition was observed against Gram positive bacterial strains in response to crude extract (T) and no zone was found around the disc without any treatment (NC). Similarly zone of inhibition was observed against Gram negative bacterial strains in response to thymol at three different concentrations (1, 2 & 3) and no zone the found around the control well **(C)**. Ampicillin (10 mg/ml) was used as the standard (AB) control.

(LC_{50}) values, respectively using the MTT assay. The percentage cell viability of WRL-68 cells was significantly decreased (from 80 to 20%) with increasing concentrations of paracetamol as shown in Figure 5A. At the same time, the cytotoxicity of WRL-68 cells significantly increased (from 50 to 100%) with increasing concentrations (25, 50, 100, 250 and 500 µg/ml) of paracetamol quantified in the cell culture supernatant using LDH cytotoxicity assay (Figure 5B). The LC_{50} value of paracetamol in WRL-68 cells was found to be 175 µg/ml (between 100 and 250 µg/ml), whereas thymol does not show any toxicity in WRL-68 cells (Figure 5C). Based on these observations we further evaluated the cell viability against paracetamol and thymol separately and in combination at two different concentrations (100 and 250 µg/ml). We observed that percentage viability of WRL-68 cells was significantly decreased with increase in paracetamol concentrations ($p = 0.041$ and $p = 0.011$). Interestingly, thymol at the same concentration recovers the decreased cellular viability induced by paracetamol within 24 h ($p = 0.129$ and $p = 0.031$). No significant differences were observed in the viability of WRL-68 cells with thymol treatments (Figure 5D).

Thymol inhibits the expression of IL-6 and IL-8 in WRL-68 cells

We quantified the expression of inflammatory cytokines namely IL-6 and IL-8 in WRL-68 liver cells in response to paracetamol treatment (250 µg/ml) after 24 h. Parallely cells were incubated

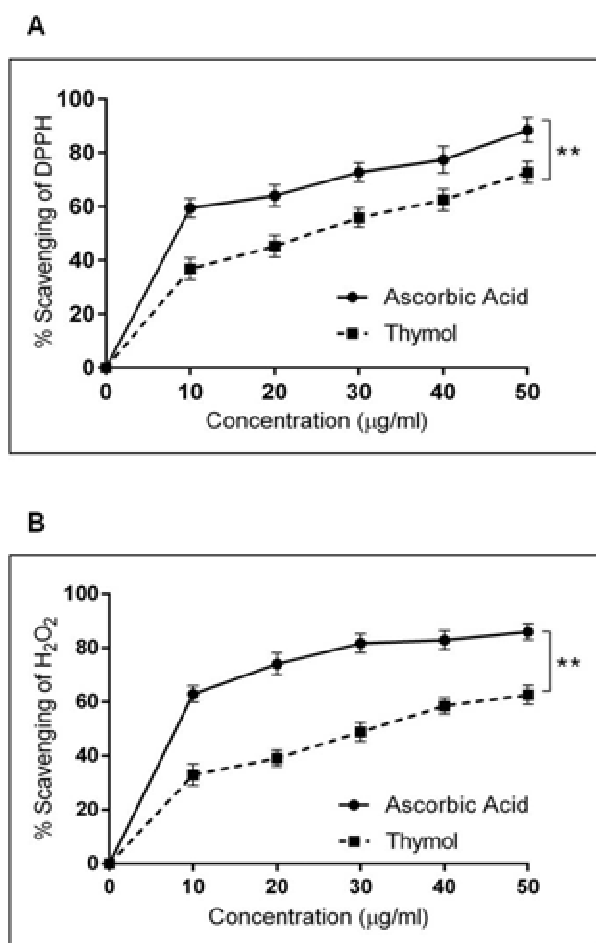

Figure 4. Evaluation of anti-oxidant activity of thymol. Line graphs showing cumulative calorimetric data of diphenyl picryl hydrazyl (DPPH) radical **(A)** and hydrogen peroxide (H_2O_2) radical **(B)** and the scavenging potential of thymol using ascorbic acid as the reference standard. The absorbance were read out at 230 nm. Data were represented as mean ± SEM of three separate experiments. **indicates $p < 0.01$ with the reference standard.

with the same concentrations of thymol alone and in combination with paracetamol in separate wells for 24 h at 37 ºC. Cells without any treatments act as negative control. All the primers used for semi-quantitative RT-PCR are given in table 2. We observed a significant increase in the expression of IL-6 ($p = 0.046$) in response to paracetamol treatments compared to the control group. Whereas cells treated with both paracetamol and thymol show inhibited expression of IL-6 ($p = 0.043$) and IL-8 ($p = 0.048$) which is as similar to cells without any treatments (Figure 6A and B).

DISCUSSION

Lymphatic filariasis is a painful and profoundly disfiguring disease caused by parasitic worms. Adult filarial worms dwell in the lymphatic vessels and eventually lead to chronic inflammation. Inflammatory damage induced by filarial parasites appears to be multifactorial and asymptomatic with endogenous parasite products, endosymbiotic bacteria, and host immune responses. Also stagnation of lymph fluid nourishes the growth of bacteria predominantly in the dermal cells and tissues which further fuels the complications. Current treatments using diethylcarbamazine citrate (DEC) and albendazole (ALZ) are promising and effective against microfilaria. Patients and physicians are in need for an appropriate and efficient therapeutic agent which can combat the complications of chronic lymphedema in a multifunctional way.

Pharmacognostic studies pertaining to the therapeutics of dietary agents have shown that consumption of naturally available fruits, vegetables and spices lowers the incidence of inflammatory diseases as they possess biologically active compounds [21]. Thymol has been reported as one of the prime phytoconstituent in *Trachyspermum ammi* having macrofilaricidal activity against the adult bovine filarial worm *Setaria digitata* [22]. *T. ammi* is commonly known as 'Ajwain' or Bishop's weed, known for its good medicinal value in India. Hence in the present study we investigated the invaluable and incredible prowess of thymol extracted from the methanolic fraction of *T. ammi* for its possible application in the treatment and management of LF. We studied the hepatoprotective role of thymol against paracetamol-induced toxicity in normal WRL-68 liver cell and free radical and inflammatory cytokine-mediated oxidative stress and its inhibitory potential against infectious bacterial growth.

Thymol (2-isopropyl-5-methylphenol) is a natural monoterpene and a phenolic derivative of cymene. It has been reported as one of the major essential oil in *T. ammi*. The natural ability of this phenolic-rich thymol has high oxidative and bacterial aggression. Extensive studies proclaim the potential of thymol for both internal and external applications. Nieddu *et al*. described that

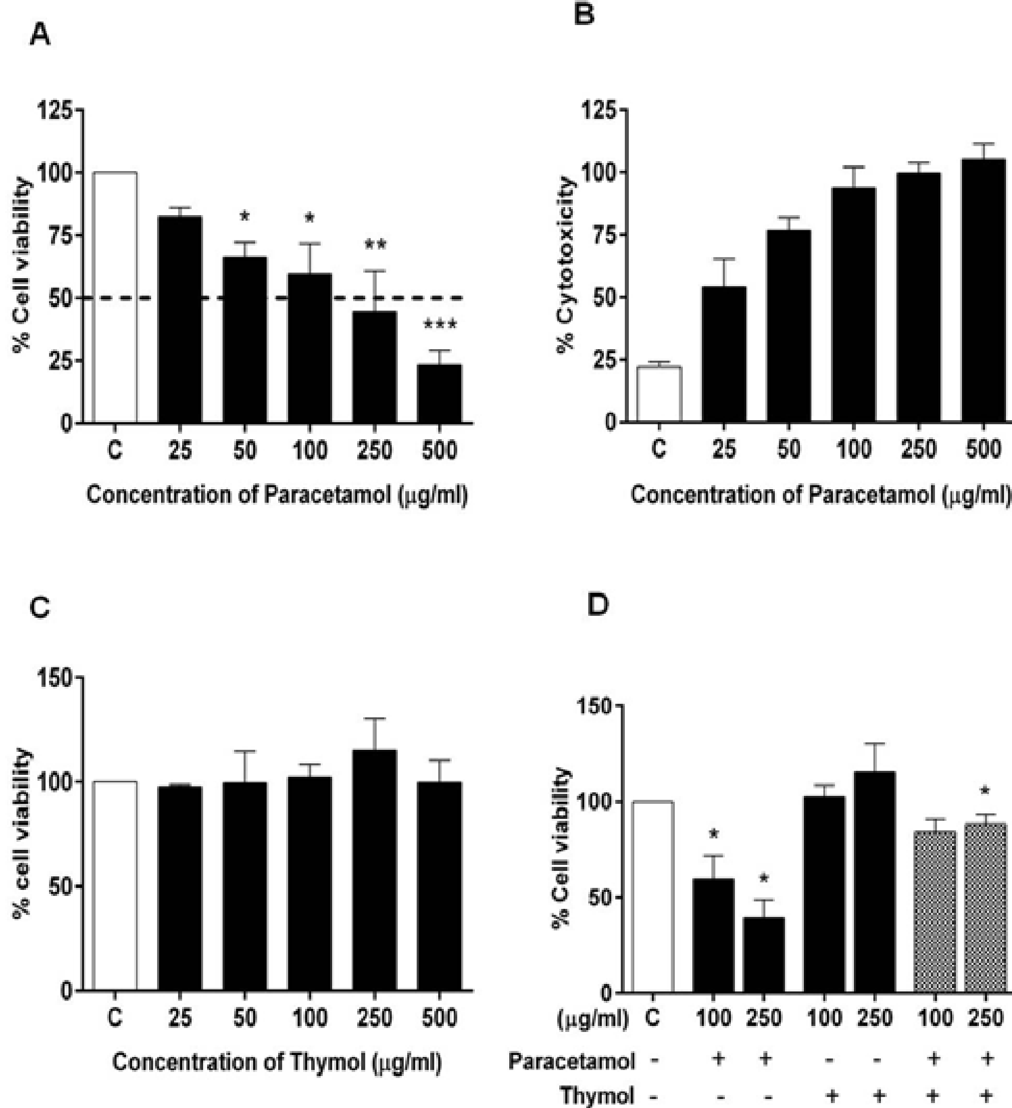

Figure 5. *In-vitro* hepatoprotective potential of thymol. Cell viability and cytotoxicity of thymol were evaluated using WRL-68 liver cells. Bar graph shows % cell viability **(A)** and **(C)** measured by MTT assay and % cytotoxicity **(B)** measured by LDH assay with various concentrations of paracetamol and thymol treatments after 24 h. Paracetamol reduced % cell viability significantly, whereas thymol restored the cell viability as shown in **(D)**. Data were represented as mean ± SEM of three separate experiments. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ vs control (without any treatment).

Table 2. List of RT-PCR primers used in the study.

Oligo	Forward primers sequence (5'- 3')	Reverse primers sequence (5'- 3')
IL-6	ATGAACTC CTTCTCCACAAGC	GTTTTCTGCCAGTGC CTCTTTG
IL-8	ATAAAGACATACTCCAAACCTTTCCAC	AAGCTTTACAATAATTTCTGTGTTGGC
β-actin	CCTTGCACATGCCGGAG	GCACAGAGCCTCGCCTT

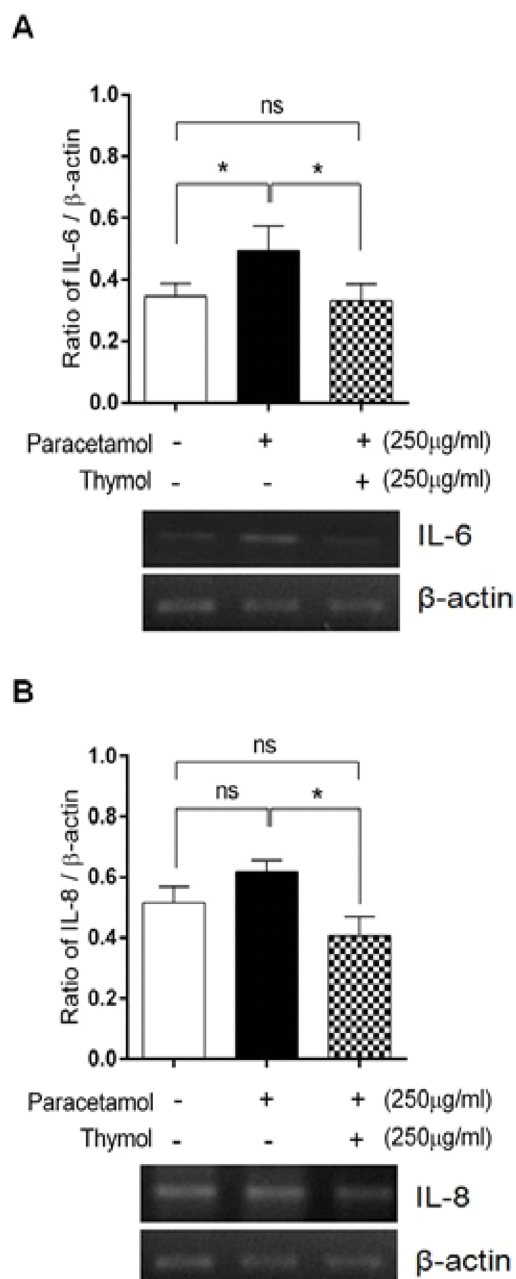

Figure 6. Semi-quantitative RT-PCR analysis. Bar graph shows the expression levels of IL-6 **(A)** and IL-8 **(B)** in the WRL-68 liver cells in response to paracetamol (250 µg/ml) and thymol (250 µg/ml) after 24 h. Expression of IL-6 was increased significantly in paracetamol treatment confirming the induction of toxicity. At the same time, treatment with thymol inhibits the expression of IL-6 and IL-8, similar to the expression in cells without any treatment. Ratio of expression was normalized with β-actin. Data were represented as mean ± SEM of three separate experiments. *$p < 0.05$ vs control (without any treatment).

oral administration of thymol was rapidly absorbed and slowly eliminated within 24 h [23]. Unabsorbed thymol was found in intestinal walls when taken in excess leaving no adverse effects. Santurio and co-workers demonstrated the antimicrobial activity of thymol against *E. coli* strains of intestinal origin [24]. Gilani *et al.* evaluated the antimicrobial cream formulated with the essential oil of *T. ammi* for dermal applications [25]. Recently Moon and Rhee examined the synergistic effect of thymol for its antimicrobial potential against major food-borne pathogens [26]. We observed a similar potential for the crude extract of *T. ammi* and for thymol against Gram positive and Gram negative bacterial strains associated with filarial lymphedema. It was very evident that the zone of inhibition increased with corresponding increase in concentrations of thymol, indicating the anti-bacterial activity of thymol against infectious pathogens.

Over dose of paracetamol leading to liver toxicity was first observed and reported by Davidson and Co-workers in two individuals. They observed eosinophilic degeneration along with chromatin condensation in the necrotic hepatocytes histopathologically [27]. Several other studies have reported that increased oxidative stress induced by reactive oxygen species (ROS) and reactive nitrogen (NO) species plays a major role in the recruitment of inflammatory cells and elevated levels of pro-inflammatory cytokines in individuals with paracetamol toxicity [28, 29]. Consequent reports showed the ROS scavenging activity of *T. ammi* crude extract. Sreemoyee and colleagues [30] demonstrate that the essential oil of ajwain has good DPPH and moderate H_2O_2 radical scavenging activity. In the present study we attempted to determine the anti-oxidant potential of thymol in scavenging DPPH and H_2O_2 free radicals *in-vitro*. Our calorimetric assay result clearly indicates that the scavenging potential of thymol is directly proportional to its concentration. Increasing the concentrations of thymol reduces the levels of DPPH and H_2O_2, which was further confirmed by the scavenging potential of ascorbic acid. In general, free radicals like hydroxyl (OH) and peroxynitrite (OONO) can easily cross the cell membranes at specific sites, and cause tissue damage and cell death.

Thus, removing OH and OONO are very important for the protection of living systems. The ability of thymol to quench hydroxyl radical seems to be directly related to the process of preventing lipid peroxidation. Thus thymol proved to be good anti-oxidant in suppressing oxidative stress by scavenging the reactive oxygen species.

The property of a drug or chemical or agent which protects liver cells from toxicity, injury or damage has been described as hepatotonic. Alam *et al.* studied the *in-vivo* hepatoprotective activity of thymol against carbon tetra chloride (CCL_4)-induced toxicity in rodent model [31]. Gilani *et al.* investigated the *in-vivo* hepatoprotective action of aqueous-methanolic extract of *Carum copticum* in mice model against CCL_4 and paracetamol-induced liver damages [32]. We investigated the hepatoprotective potential of thymol against paracetamol induced-toxicity in normal WRL-68 liver cell line which shows identical features as those of primary hepatocytes. We examined cell viability, proliferation, cell damage and expression of cytokines (IL-6 and IL-8) in response to varying concentrations of paracetamol and the counter action by thymol in WRL-68 liver cells. The results of our study were convincing and well correlated with the previously published *in-vitro* and *in-vivo* reports. We observed that the percentage viability of WRL-68 cells was significantly decreased with increase in paracetamol concentrations (100 and 250 µg/ml). Interestingly, thymol at the same concentration recovers the decreased cellular viability by paracetamol within 24 h. No differences in the viability were observed in different thymol treatments, indicating that thymol promotes cell survival and proliferation in normal liver cells but may exert cytotoxicity in cancerous cells. Moreover thymol supports the process of hepatic regeneration during liver injury. Our RT-PCR data reconfirm the same findings that the expression levels of IL-6 and IL-8 were significantly elevated in cells treated with paracetamol, whereas cells treated with both paracetamol and thymol show inhibited expression of IL-6 and IL-8 similar to cells without any treatments. This suggests that thymol has the ability to attenuate the expression of these inflammatory cytokines which were in excess during acute liver injury. At the same time thymol protects liver from certain inflammatory insult, and preserves liver function for its self renewal. The results showed that thymol offered maximum protection against hepatotoxicity caused by paracetamol.

CONCLUSION

In conclusion, our data suggests that *Trachyspermum ammi* (a good dietary agent) may be a promising natural source for thymol and its inherent, multifunctional therapeutic potential can effectively harnessed for the treatment and management of human lymphatic filariasis along with the drug of choice.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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