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# Thymus vulgaris Extract Effect on Blood TNF- $\alpha$ and IL-10 Level and Bacterial Colonies in Escherichia coli Infected Mice Urinary Bladder

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**Abstract** : The *Thymus vulgaris* extracts are effective as anti-inflammatory, immunomodulatory, antioxidant, antibacterial and antifungal. *E. coli* is a major pathogen involved in nosocomial infections and one of multi-drug resistance organisms. UTI is the most common extraintestinal *E. coli* infections and is caused by uropathogenic *E. coli* (UPEC). This study aimed to prove the *Thymus vulgaris* ethanol extract effect in increasing IL-10 and decreasing TNF- $\alpha$  and bacterial colonies in the urinary bladder. All *Thymus vulgaris* parts, which bought from Gharian-Libya, were dried, then macerated 3 times, and its ethanol was evaporated. Final extraction results were stored in the freezer. This study use 20 female mice divided into 5 groups; (1) positive control (infected with *E. coli*); (2) negative control (without infection); and treatment groups T1, T2, T3 (infected mice administrated with ethanol extract of *Thymus vulgaris* (ETV) 250, 500, 750 mg/kg B.wt). The bacterial colonies in urinary bladder were analyzed; the blood levels of TNF- $\alpha$ , IL-10 were analyzed by ELISA method. There was no visible *E. coli* colonies infection in urinary bladder in all treated groups. ETV increased the IL-10 and decrease TNF- $\alpha$  level in blood.

**Keywords:** Thymus vulgaris, TNF- $\alpha$ , IL-10, Escherichia coli, in vivo..

## Introduction:

*Thymus vulgaris* is aromatic herbs and subshrubs, predominantly found in Mediterranean region, Asia, Southern Europe and North Africa <sup>1</sup>. Thyme is a species of flowering plant in the mint family Lamiaceae, native to southern Europe from the western Mediterranean to southern Italy. Growing to 15–30 cm (6–12 in) tall and 40 cm (16 in) wide, it is a bushy, woody-based evergreen subshrub with small, highly aromatic, grey-green leaves, and clusters of purple or pink flowers in early summer <sup>2</sup>. *T. vulgaris* contains about 2.5% but not less than 1.0% of volatile oil (essential oil) which contains terpenes, terpenoids, and phenylpropenes <sup>3</sup>. Thymol (2-isopropyl-5 methyl phenol) is the main monoterpene phenol, isomeric with carvacrol, found in thyme extract. Ashnagar et al. Explain the component of phenols tymol (40%) and carvacrol (15%) <sup>4</sup>. These compounds have shown anti-inflammatory, immunomodulatory, antioxidant, antibacterial and antifungal properties <sup>4,5</sup>. The activity of carvacrol is extended to drug-resistant microorganisms strains with a particular significance for pathogenesis, which is difficult to treat <sup>6</sup>. We used *Thymus vulgaris* extract against *Escherichia coli* in mice model of UTI to see the antimicrobial effect.

The mechanisms of thyme to destroy the bacteria through the release of LPS and also acts on the cytoplasmic membrane to alter the transport of ions <sup>6</sup>. The activity of carvacrol seems to be linked to the presence of a hydroxyl group that may function as a trans-membrane carrier of monovalent cations by carrying

H<sup>+</sup> into the cell cytoplasm and transporting K<sup>+</sup> back out<sup>6</sup>. Carvacrol can affect the folding or insertion of outer membrane (OM) proteins, and inhibit the synthesis of another microbial protein<sup>7</sup>. The important cytokines in immune-pathogenesis of *Escherichia coli* are TNF- $\alpha$  (pro-inflammatory) and IL 10 (anti-inflammatory). The role of IL-10 during infection has emerged as a key immune regulator during infection with bacteria, ameliorating the excessive Th1 and CD8 T cell responses (by overproduction of TNF- $\alpha$ ) that are responsible the immunopathology associated with infections<sup>8</sup>.

*Escherichia coli* is an extracellular Gram-negative, a rod-shaped bacterium belonging to the family Enterobacteriaceae. It causes a wide spectrum of diseases to the humans, ranging from self-limiting to life-threatening intestinal and extra-intestinal illnesses<sup>9,10</sup>. *Escherichia coli* is one of the most predominant pathogens, causing 80–90% of all episodes of UTIs<sup>11</sup>. Bacteria attach to the urothelium through receptors that exist on the surface of the urothelium. Based on the pile type, there are two types of bacteria that have different virulence: (1) bacteria Pili type 1, which may cause infection in cystitis; and (2) P-type pile, which often cause severe infections such as acute pyelonephritis<sup>12</sup>. Some bacteria have the property to form antigen, produces a toxin (hemolysis), and produces the enzyme urease that can change the atmosphere of the urine to alkaline<sup>12</sup>. Adhesin protein is a virulence factor of the fimbria, afimbria (OMP), capsules, siderophores, bacterial motility, changes in surface antigens, toxic proteins that can kill phagocytic cells. Another factor that can damage host cells includes hydrolytic enzymes, or lipopolysaccharide endotoxin (LPS), and exotoxin<sup>13</sup>.

*Escherichia coli* can adhere and invade host cells, leading to stimulation of the pro-inflammatory immune response which is initiated by TLR4 recognition of LPS that activates MAPK and NF- $\kappa$ B pathways. After this transcription activation and secretion of pro-inflammatory mediators such as TNF- $\alpha$  and KC/IL-8 and MIP-2. These chemokines recruit granulocytes and lymphocytes that are required for controlling infection<sup>14</sup>. *Escherichia coli* expresses several virulence factors implicated in pathogenesis. The bacteria is captured by macrophage which presented the bacterial antigen to CD4 (Th1) which activate the macrophage to secrete the cytokines like TNF- $\alpha$ , N.O, R.O.I, and proteolytic enzymes; There is another cytokine secreted by CD4(Th2) like IL-10 which inhibit the macrophage activation as well<sup>15</sup>. The thymol and carvacrol also led to decreasing Th1, Th2 (IL-4) and Th17 levels in the sera of mice but increased levels of IL-10<sup>15</sup>. We want to prove that the extract of *Thymus vulgaris* decreases the secretion activities of TNF- $\alpha$ ; increases the secretion activities of IL-10, and decreases the number of the bacterial colony in the urinary bladder of *E. coli* infected mice.

## Materials and Methods

### Extraction of plant material

The *Thymus vulgaris* sample was bought from a local herbalist in Gharian-Libya. The preparation of ethanol extract of *Thymus Vulgaris* carried out in Pharmacology Laboratory, Medical Faculty, Brawijaya University. 200 grams of every plant parts were macerated in one liter of 70% ethanol. The container was sealed with paper foil to prevent loss of volatile solvent and left at room temperature for 24 hours. At the end of this period, the contents were filtered using filter paper (No.1) into a beaker. The filtered solution then concentrated by evaporating the solvent in a hot air oven at 40°C for 24 hr. The extract was then weighed, kept in sterile bottles, labeled accordingly, and stored in the refrigerator.

### Bacterial preparation:

Isolation of *Escherichia coli* bacteria obtained from patients with urinary tract infection (UTI) who were hospitalized in dr. Saiful Anwar Hospital. Patients were selected based on these criteria: (1) Urine of patients obtained significant bacteriuria  $\geq 10^5$  CFU (colony forming units) / ml; and (2) *Escherichia coli* singly emerges from urine culture of patients with UTI.

### *E. coli* Culture

The bacteria used in this research was *E. coli* derived from the Clinical Microbiology Laboratory in Saiful Anwar General Hospital at Malang, Indonesia. The *E. coli* was made an inoculum in  $10^9$  CFU/mouse. It was metallic green on EMBA and identified by using MICROBACT™ GNB 12/A/B/E, 24E.

## Experimental animals

Healthy female mice (6 - 8 weeks; 25 - 30 g) were obtained from the Pharmacological Laboratory of Brawijaya University, Malang, Indonesia. Animals were placed on Animal Research Care. Adaptation time was done in one week to prepare the mice. Animals were housed in polypropylene cages and maintained at  $25 \pm 2^\circ\text{C}$  and constant humidity degree according to the alteration of day and night.

## Experimental design

The mice are adapted for 7 days in the laboratory with standard feed, then grouped randomly. 20 mice were divided into the 5 groups. The group of T1, T2, T3 were given an extract of *Thymus vulgaris* (ETV) administered orally for 7 days with concentration 250, 500, 750 mg/kg, respectively, and were infected with *Escherichia coli* ( $10^9$  CFU / mice) at the same time. The Group C2 positive control was infected with *Escherichia coli* ( $10^9/50 \mu\text{L}$  / mice) for 7 days. The Group C1 were not treated nor infected for 7 days. On day 14, every mouse was sacrificed by Ether inhalation. The entire ventral surface of mice was sprayed with alcohol 70%. A small incision was made in the skin using scissors on the medial abdomen, and then the skin was torn using tweezers in the direction of the head and tail so that peritoneum was exposed. Peritoneal moistened with alcohol 70% to remove the hairs. The peritoneum was opened, and then the urinary bladder and whole the blood were taken with aseptic technique.

## Bacterial Colonies Calculation in urinary bladder

Calculation of bacterial colonies was done in Microbiology Laboratory in Medical Faculty Brawijaya University, Malang, Indonesia.

## TNF- $\alpha$ and IL 10 Blood Level Determination

Blood was collected by cardiac puncture immediately after sacrifice. Levels of TNF- $\alpha$  and IL-10 in the plasma were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kit was purchased from PlatesBioLegend, Inc.

## Ethical issue

The study was approved by Health Research Ethics Committee (Medical Faculty, Brawijaya University), serial No.381/EC/KEPK/10/2016.

## Result and Discussion

ETV showed the decreasing effect to TNF- $\alpha$  blood level in all treatment groups after 14 days of treatment. The decrease is proportional with the given doses, with T1 (250 mg/kg ETV) exhibited lower TNF- $\alpha$  compared to control. T2 (500 mg/kg ETV) exhibited lower TNF- $\alpha$  level compared to T1; and T3 treatment group (750 mg/kg ETV), which is the highest ETV concentration in this study, exhibited the lowest blood TNF- $\alpha$  level of all (figure 1).

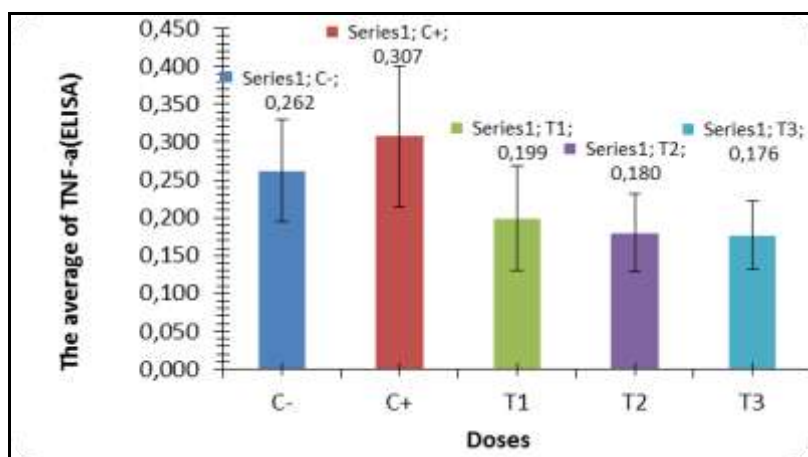


Figure 1: Graph of TNF- $\alpha$  at each dose *Thymus vulgaris* extract

TNF- $\alpha$  implies a potent inflammatory response involving macrophages and neutrophils. In accordance with our results, Olszyna *et al.*, (1998) reported that concentrations of TNF in serum and urine were below the limit of detection in the vast majority of the positive control and negative control groups<sup>16</sup>. The production of pro-inflammatory cytokines can be inhibited by anti-inflammatory cytokines, of which IL-10 is the most potent. IL-10 is primarily produced by monocytes early in UTIs, induced by recognition of LPS.

ETV increased IL-10 concentration in T1 compared to control. The higher dose of TV, the higher concentration of IL-10 in plasma. T2 mice had higher IL-10 concentration compared to T1. The highest recorded IL-10 concentration was in T3 mice, which was given the highest concentration of ETV (figure 2).

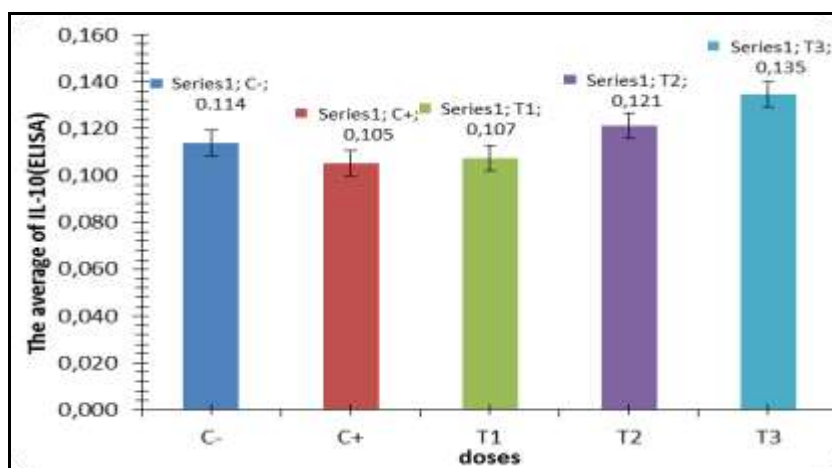


Figure 2: Graph of IL-10 at each dose *Thymus vulgaris* extract

Anti-inflammatory sIL-1RA was significantly higher in females than in males<sup>17</sup>. Our study showed that the anti-inflammatory cytokine (IL-10) had a higher concentration in the plasma than the pro-inflammatory cytokine (TNF- $\alpha$ ). Bacterial colony in the urinary bladder were significantly decreased by ETV administration ( $p < 0.05$ ). The higher ETV concentration administered, the lower *E. coli* bacterial colony count (figure 3).

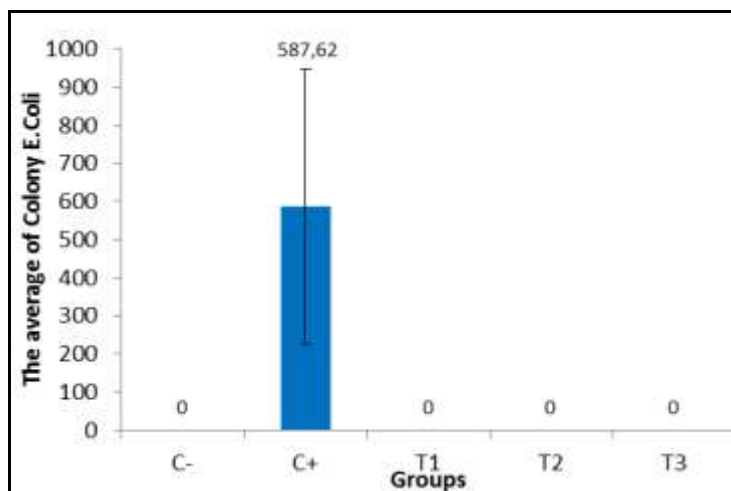


Figure 3. Graph of the colony of *E. coli* at each dose *Thymus vulgaris* extract

## Conclusion

*Thymus vulgaris* extract in *E. coli* infected female mice will provide a reduction of the TNF- $\alpha$  and increase IL10 serum level and reduction of bacterial colonies in the urinary bladder.

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