Polyphenols extracted from the Green Tea (*Camellia sinensis*) augments the protective immune responses in mice challanged with *Salmonella typhimurium*

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Abstrak

Teh hijau diolah dari daun teh yang tidak difermentasi. Berbagai aktivitas biologis teh hijau telah dilaporkan. Bentuk infus dan kandungan polifenolnya telah diketahui mempunyai efek antimutagenik, antibakterial, menurunkan kadar kolesterol, antioksidan, dan mitogen limfosit B. Penelitian membuktikan bahwa polifenol teh hijau dapat meningkatkan produksi IL-12. Infeksi yang disebabkan oleh kuman salmonella spp sampai saat ini masih merupakan masalah kesehatan di berbagai negara di seluruh dunia. Peran imunitas tubuh, diantaranya imunitas seluler yang diperantarai sel T helper sangat diperlukan untuk mengatasi infeksi akibat kuman ini. Berbagai penelitian menunjukkan bahwa IL-12 berperan penting dalam mekanisme imunitas seluler. Penelitian ini bertujuan untuk mengetahui efek polifenol terhadap respon imun seluler mencit selama infeksi Salmonella typhimurium. Subjek penelitian adalah mencit Balb/C betina berumur 6-8 minggu yang dibagi menjadi 3 kelompok. Kelompok pertama mendapat polifenol dosis 10 mg/hari, kelompok kedua 5 mg/hari selama 1 bulan, dan kelompok ketiga tidak mendapat polifenol. Pada hari ke-31 semua kelompok diinfeksi dengan Salmonella typhimurium 10^8 CFU per oral. Pada hari 0, 3, 5, dan 7 setelah infeksi masing-masing kelompok dibunuh 3 ekor mencit untuk diekstraksi splenosit dan sel makrofag peritonealnya. Kadar IFN-γ supernatan kultur splenosit dan aktivitas fagosit oleh makrofag peritoneum diperiksa pada hari tersebut. Kadar IFN-γ pada supernatan kultur splenosit meningkat selama infeksi pada semua kelompok, tetapi kadarnya pada kelompok yang mendapat polifenol lebih tinggi daripada kelompok kontrol. Persentase aktivitas fagositosis makrofag peritoneal juga lebih tinggi pada kelompok yang mendapat polifenol daripada kelompok kontrol. Peningkatan aktivitas fagositosis makrofag ini berkorelasi positif dengan kadar IFN-γ pada supernatan kultur splenosit. ***(Med J Indones 2004; 13: 1-7)***

Abstract

Green tea is an aqueous infusion of dried unfermented leaves of Camellia sinensis. Numerous biological activities of green tea have been reported. The aqueous infusion and its polyphenolic substance are known for their activity as an antimutagenic, antibacterial, hypocholesterolemic, antioxidant, and mutagenic of B lymphocyte. Studies have demonstrated that green tea polyphenols increase IL-12 production. Salmonella spp infection is an important public health problem in many countries. Cell-mediated immunity (CMI), especially T-cell help is important for protection against this infection. Recent evidence indicates that IL-12 is one such factor that plays a crucial role in the development of CMI. These studies were carired out to investigate the effect of green tea polyphenols to the immune cellulare in mice responses of mice during Salmonella typhimurium infection. The subject consisted of 36 female mice (Balb/C), 6-8 weeks old, divided into 3 groups. The first group was given 10 mg polyphenols/mouse, the second group was given 5 mg polyphenols/mouse, and the third group as the control. In day 31, all mice were infected with 10^8 CFU Salmonella typhimurium orally. On day 0, 3, 5, and 7 postinfection, 3 mice from each groups were sacrificed, the splenocytes were extracted and cultured to measure the level of IFN-γ in the supernatan and. The peritoneal macrophages were also extracted and cultured to measure the phagocytic activity. The level of IFN-γ in splenocyte culture supernatant increased during infection in all groups, but the level of the experimental groups were higher than in control group. The percentage of phagocytic activity of peritoneal macrophages were higher in the experimental groups than in the control group. The increase of the phagocytic activities were seen corelate with the level of IFN-γ supernatan splenocyte culture. ***(Med J Indones 2004; 13: 1-7)***

Keywords: *polyphenols, green tea, macrophages, phagocytosis*

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Tea is one of the most widely consumed beverages in the world, only second to water. It contains a number of organic substances, some of which are useful to human health as firstly found by the Chinese. Besides protein and carbohydrate, tea also contains polyphenol. This compound has a unique biological activity, and is presumed to have some effects on human health. A

lot of research is carried out intensively regarding this matter.[1,2]

Polyphenol contained in the green tea consists of three sub-classes: flavanol, flavon and flavonol. Four main flavanol or cathecin, namely: epicathecin (EC), epicathecin gallat (ECG), epigallocathecin (EGC), and epigallocathecin gallat (EGCG), together make up one-third of the green tea's dry-weight.[1,3]

Cathecin can also be found, with lower concentrations, in other plants, such as red wine, apple and outer parts of particular trees.[4] Cathecin concentration in the green tea is higher than that in the black tea, since the green tea is made of fresh leaves. The cathecin content varies due to the age of the leaves. The young ones tend to have cathecin in higher concentration. Scientific researches have shown the effects of green tea on health. It can act as an anti-mutagenic, anti-bacterial, anti-fungi, mitogen lymphocyte-B, cholesterol, blood glucose and blood pressure reducer, anti-oxydant, and anti-tumor.[3-5]

Polyphenol extract can also inhibit the growth of in vitro *Staphylococcus aureus* and *Candida albicans.*[6] The green tea has long been used against diarrhea. Researches show that cathecin protects mice against infection from vibrio-cholera and prevents its toxic activities[7]. It has also been proved to prevent caries by inhibiting the growth and accumulation of bacteries in the mouth such as *Streptococcus mutans* and *Porphyromonas gingivalis.*[8]

Katiyar et al has shown that administration of topical EGCG prior to ultraviolet exposure on mice skin can increase the production of IL-12 in the Draining Lymph Node (DLN), which is predicted as a mediator and ajuvan for the contact sensitivity induction.[9]

Salmonella spp, as well as *Shigellae* and *Camphylobacter jejuni*, is a general cause of acute infectious diarrhea. Salmonellosis is an urgent health problem in developing countries including Indonesia. Annually, there are about 16 to 21 million of *Salmonella spp* infection cases worldwide, with a mortality rate of 600-700 thousands. In developing countries throughout Africa, West, South and Southeast Asia, and Latin America, there are about 12.5 to 16 million cases per year with more than 700 thousands mortality.[10]

Salmonella spp is an intra-cellular bacteria which enters the body via digestive tracts. The symptoms can be gastroenteritis, enteric fever (typhoid-like disease), bacteriemia with and without extra-intestinal infection and asymptomatic carrier.[11] The cause of non-typhoid salmonellosis are *S. typhimurium, S. dublin, S. cholerasuis and S. enteritidis*. They can cause various diseases on human, from gastrointestinal infection which can recover by itself to lethal systemic infections. Bacterial multiplication and host factor determine the outcome of *Salmonella spp* infection.[12]

Cytokines plays an important role in the host immunity response against salmonella, especially in the production of Interferon gamma (IFN-γ) induced by by Interleukin-12 (IL-12). The production of IFN-γ is substantially induced by IL-2 and therefore its neutralisation will decrease the production of IFN-γ and host's resistance against infection. IFN-γ is needed by the macrophage to encourage its activitites. Activated macrophage can eliminate intracellular bacteries by phagocytosic and microbicydal mechanisms, i.e. the production and secretion of intracellular ROI.[12,13]

CMI deficiency and clearance by reticuloendothelial cells are the cause for prolonged infection with recurrences in Salmonella infection.[14,15] Classically, the macrophage activating factor (MAF) is considered to be identical with IFN-γ. It stimulates the macrophage by producing free radicals such as super-oxyde anion (O_2^-) and nitric oxyde (NO), which is an anti-microbial action against *Salmonella spp* and other pathogens. The drop in O_2^- production by *invivo* super-oxyde dismutation (SOD) will increase the number of bacteries in liver.[16,17]

The researches above show that the green tea polyphenol can inhibit the development of infectious diseases and increase the production of IL-12.[9] This fact leads to the study to understand the effects of green tea polyphenol, which will be referred to as 'polyphenol', on IFN-γ production by mice splenocyte and phagocytic activity of mice peritoneal macrophage during *S. typhimurium* infection.

This work is desired to be useful when considering the administration of green tea or polyphenol as daily healthy drink as well as a supplement against infectious diseases.

METHODS

This research is an experimental one with *post-test only control group* scheme. The independent variables

is polyphenol, while the dependent variables are the IFN-γ concentration in supernatant of splenocyte culture and fagocytic activity of peritoneal macrophage.

Female Balb/C mice, aged 6-8 weeks were randomly selected and divided into three groups, each consisting of 12 mice. Group I was given with polyphenol 10 mg/day, group II with 5 mg/day, while group III was left without administration of polyphenol. Polyphenol was given orally using intragastricum canule every day in a month.

Green tea polyphenol extract was prepared as follows: 250 g of commercially available green tea was crushed into grains. Extract was collected using a mixture of methanol 95% and water (1:1 ratio). This crude polyphenol extract was then filtered and dried at room temperature, before it was redissolved with water and freeze-dried. The result was mixed with water again, and at its aqueous phase was extracted with ethyl acetic and evaporated. The result is polyphenol and checked with *thin layer chromatography.*

The probands were infected with S. typhimurium in 0.2 ml of PBS 0.01 M pH 7.4. It was given orally, each with a dosage of 10^8 CFU. In day 0, 3, 5 and 7 after bacterial inoculation, 3 mice from each group were killed to obtain the splenocyte and peritoneal macrophage. The IFN-γ production was determined using Quantikine^R M Mouse IFN-γ immunoassay, and the fagocytic activity of the peritoneal macrophage was observed.[18]

To determine the survival of mice during *S.typhimurium* infection, 36 mice were separated into 3 groups as mentioned. In day 31, all groups were infected with *S. typhimurium* at lethal dosage (10^6) intraperitoneally.

RESULTS

This work attemps to understand the effects of polyphenol on cellular immune response during *S. typhimurium* infection on Balb/C mice.

In the preliminary research, a 10^6 CFU dosage was injected intraperitoneally in line with the method by Umezawa et al to determine the survival of mice during S. typhimurium infection.[17] Figure 1 shows that polyphenol prolongs the life survival during infection.

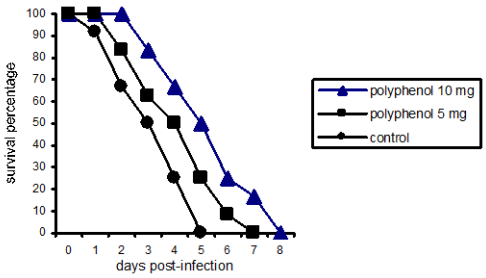

Figure 1. Survival of mice infected intraperitoneally with 10^6 CFU of S. typhimurium Each group consists of 12 mice and the tests were done twice with similar results.

Level of IFN-γ in the supernatant of splenocyte culture

The level IFN-γ was measured using ELISA (Quantikine^R M). In this work, the level of IFN-γ was found to increase from time to time, and the value was higher in mice treated with polyphenol (Figure 2).

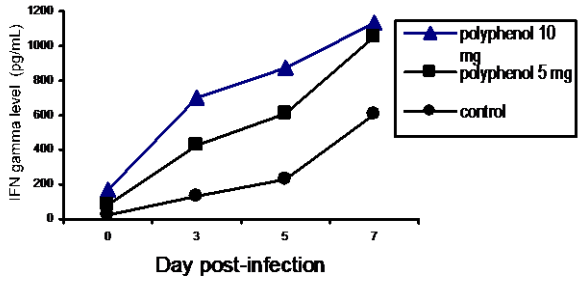

Figure 2. IFN-γ level in the supernatant of splenocyte culture of Balb/C mice during S. typhimurium infection.

In day 0, the IFN-γ level found in the supernatant of splenocyte culture of the group with 10 mg/day dosage was 7.5 times as high as that of the control group. In the group with 5 mg/day dosage, it was found 3.6 times as high. The difference of IFN-γ level in day 0 is probably due to the administration of polyphenol which inceases IL-12, which in turn will induce the Th1 and NKC cells to produce IFN-γ. In the control group, the absence of polyphenol means less number of IL-12 so that proliferation and differentiation of the T-type lymphocyte cells are not as active as those in the other groups. This agrees with

results by Mastroeni which show that IL-12 neutralisation *invivo* decreases the IFN-γ production by splenocyte *invitro*. It also explains why the level of IFN-γ in the supernatant of splenocyte is found higher in group with polyphenol.[19]

After the infection, the level of IFN-γ found in all groups increased from time to time. In day 7, the level of IFN-γ in 10 mg/day group increased to 1132.794 ± 33.22 pg/ml, (51.8 x the level in control group in day 0 / normal condition), the level in 5 mg/day group increased to 48.2 x normal condition, and the level in control group increased to 27.6 x normal condition. The increase of IFN-g level in all groups is caused by *S. typhimurium* which acts as an immunogen. This immunogen will be presented by macrophage to the T-type lymphocyte cells, which then secrete IFN-γ.

Macrophage phagocytic capability

The phagocytic capability of peritoneal macrophage cells was determined by counting the number of latex particles phagocytosed in 100 macrophages (Figure 3).

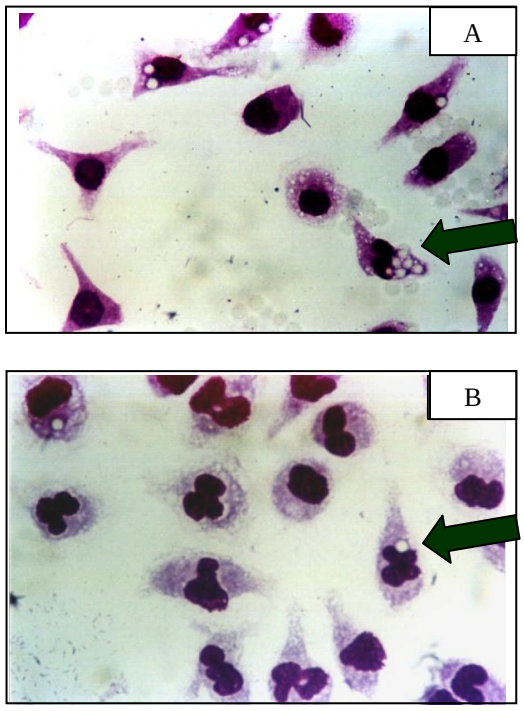

Figure 3. Illustration of peritoneal macrophage of Balb/C mice phagocytosing latex particles invitro, in day 0 (magnified at 400x). Arrows indicate latex particles being phagocytosed by the macrophages. In group with 10 mg/day polyphenol (A) it is more obvious than the control group (B).

Macrophage phagocytic capability increases at all time in all groups (Figure 4). Statistically, there is a very significant difference between Group I and the control group ($p<0.01$) at all times and a significant difference ($p<0.05$) amongst all groups from day 0 to day 7, except in day 5 where there is a very significant difference ($p<0.01$). When Group II and the control group is compared, there is a very significant difference in day 0 and day 5 post-infection ($p<0.01$), while there is a significant difference ($p<0.05$) in the other days of observation.

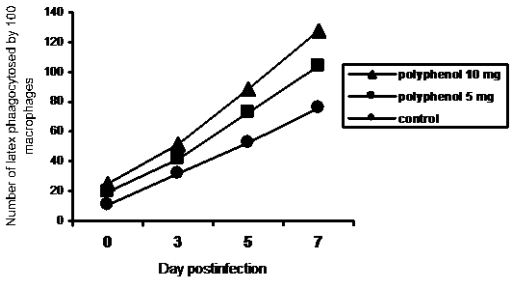

Figure 4. Average number of latex phagocytosed by 100 peritoneal macrophages of the Balb/C mice during S. typhimurium infection.

Percentage of phagocytic macrophage

The percentage of macrophage phagocytosing latex particles increases in all groups (Figure 5). The percentage of peritoneal macrophage cells phagocytosing the latex particles *invitro* in the polyphenol group is higher than that in the control group at all observation periods. The *Tuckey* test shows a very significant difference ($p<0.01$) between Group I and the control, in all observation days.

Differences can also be noted between Group II and the control in day 0 ($p<0.01$), and in day 3, 5 and 7 ($p<0.05$). Dosage variation shows significant difference ($p<0.05$) in day 0, 3 and 5.

Polyphenol increases macrophage activity in phagocytosing the latex by increasing the percentage of phagocytic macrophage and improving the capability of the macrophage to phagocytose the latex particles *invitro*. Higher phagocytic capability after treatment with polyphenol agrees with the results by De la Funte et al which found a better immune response in hospes consuming anti-oxydant, noted by the increase of adherence, chemotaxis and phagocytosis.

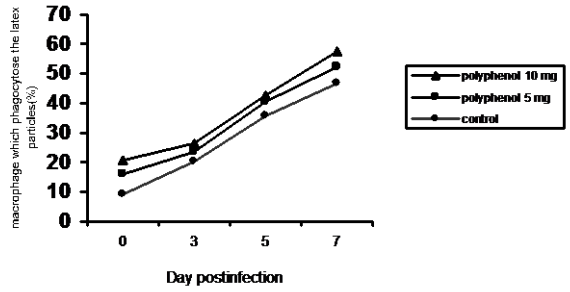

Figure 5. Percentage of peritoneal macrophage of Balb/C mice which phagocytose the latex particles during S. typhimurium infection.

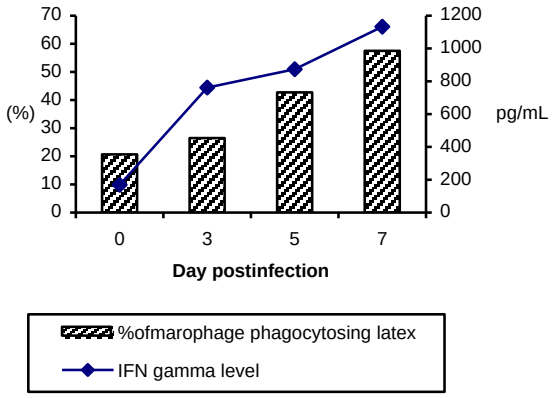

Figure 6. IFN-γ level and percentage of macrophage phagocytosing the latex in Balb/C mice treated with polyphenol at 10 mg/day.

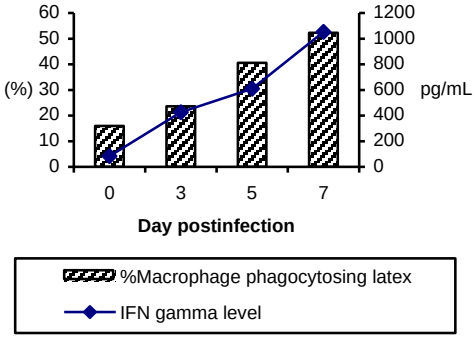

Figure 7. IFN-γ level and percentage of macrophage phagocytosing the latex and secreting ROI, and lymphocyte proliferation activity in Balb/C mice treated with polyphenol at 5 mg/day.

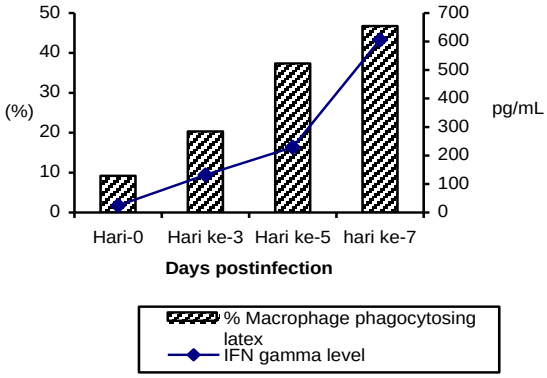

Figure 8. IFN-γ level and percentage of macrophage phagocytosing the latex and secreting ROI, and lymphocyte proliferation activity in Balb/C mice without polyphenol treatment

The IFN-γ level and percentage of macrophage phagocytosing the latex increases since day 0 after infection, and keeps on increasing with time (Figures 6-8). The increase in IFN-γ level is followed by peritoneal macrophage activities, which is shown by a strong correlation in every group: in Group 10 mg (r=0.93), Group 5 mg (r=0.97) and in the control group (r=0.93). There is not yet a definite reason why the IFN-γ level in Group 10 mg drastically increases in day 3 after infection, which is not seen in the other groups. This lack of explanation is owing to inadequate number of probands, the tests which were only carried out twice and other unknown *in vivo* mechanisms.

DISCUSSION

Measurement of IFN-γ level from the supernatant of splenocyte culture shows higher number in group with polyphenol than the control group. Previous research shows that polyphenol incerases the level of IL-12 [9]. This cytokine is known to help induce the production of IFN-γ by the T-helper 1 and NK cells [13,16]. The presence of IFN-γ activates the macrophage to produce IL-1 which is, due to its activities, called *Lymphocyte Activating Factor* (*LAF*). LAF affects the T-lymphocyte to produce IL-2, which stimulates itself to proliferate and produce IFN-γ which in turn will activate the macrophage. Interleukin 2 produced by the T-lymphocyte is also called as *B cell growth*

hormone (BCGF) which stimulates the proliferation of B-lymphocyte and produces antibody.[20]

In the control group, the absence of polyphenol means less number of IL-12 than that in the treated groups, hence proliferation and differentiation are not as active as those in the treated groups. Therefore, the level of IFN-γ in mice treated with polyphenol is higher than that in untreated ones.

In day 3, 5 and 7 after the infection, the level of IFN-γ increases in all groups due to the presence of immunogen which activates the immune system. This immunogen is presented by the *Antigen Presenting Cell* (APC), one of which is the macrophage which then interacts with T-lymphocyte cells. This interaction causes the T cells to produce cytokines, such as IFN-γ which activates the macrophage. This mechanism occurs in all groups, so that the T-lymphocyte of all probands undergoes proliferation and produce IFN-γ.

Polyphenol increases macrophage activities in phagocytosing the latex particles, by increasing the percentage of phagocytic macrophage and improving the capability of macrophage to phagocytose latex particles *invitro*. Higher phagocytic capability after administration of polyphenol is in line with the results shown by De la Funte et al.[21] He found an increase in immune response of hospes consuming anti-oxydant, as shown by the increase in adherence, chemotaxis and phagocytosis. In addition, there is still a possibility that polyphenol can increase phagocytic activity of macrophage by increasing the production of IL-12. As mentioned earlier, IL-12 is greatly needed in CMI immunity against *S. typhimurium.*[13,14] Interleukin-12 also helps induce IFN-γ which affects activation of microphage.[16] Neutralisation of these two cytokines causes the bacteria to grow more rapidly *in vivo*. This explain why administration of polyphenol improve mice survival infected in lethal dosage with *S. typhimurium*. Further, Yang et al proves that green tea polyphenol inhibits the production of TNF-α. This cytokine is predicted to be important in inflammation response causing lethal shock after administration of LPS.[22]

In an activated macrophage, there are some morphological changes and an increase in enzyme secretion. The macrophage becomes bigger and shows the formation of pseudopod and vacuole. IFN-γ increases the expression of Fc receptor at cell surfaces for more active phagocytic and ADCC processes. This cytokine also increases MHC II expression so that antigen presented by APC can be identified more easily by the T-lymphocyte. Based on these facts, it can be concluded that higher IFN-γ level in the treated groups causes the macrophage within these group to be more active compared to that in the control group. The results obtained here clearly show that there is a strong correlation between IFN-γ level and the percentage of macrophage phagocytosing the latex, and also between IFN-γ level and the number of latex particles phagocytosed by 100 macrophages in every group.

CONCLUSIONS AND RECOMMENDATIONS

The following remarks can be drawn from this research:

1. Polyphenol extends the life survival of mice during *S. typhimurium* infection.
2. During *S. typhimurium* infection, the level of IFN-γ in the supernatant of splenocyte culture of Balb/C mice treated with polyphenol is higher than that in the untreated ones.
3. Phagocytic capability of the peritoneal macrophage cells of Balb/C mice increases with the administration of polyphenol.
4. Higher percentage of peritoneal macrophage cells phagocytosing the latex particles is found in mice treated with polyphenol.

The effects of green tea polyphenol on the immune response of mice infected with *S. typhimurium* can be studied further by understanding its effects on cytokine secretion and the production of enzyme which has an influence on the microbicidal process against this bacteria. The effects on severity of infection should also be addressed.

Based on the results of this research, the consumption of green tea polyphenol extract or the green tea in general is recommended as the daily drink to optimize the immune system of the body. It is also encouraged for a supplement in the therapy of pasient diagnosed as well as suspected with salmonellosis along with treatment with antibiotics.

REFERENCES

1. Beecher GR, Warden BA, Merken H. Analysis of Tea Polyphenols. P.S.E.B.M 1999; 220.

2. Gunawijaya FA. LD-50 of Green Tea Extract in Strain C3H Mice. Maj Ilmiah Fakultas Kedokteran USAKTI 1996;15:4.
3. Merken HM, Beecher GR. Measurement of Food Flavonoids by High-Performance Liquid Chromatography: A Review. J Agric Food Chem 2000; 48(3): 577-99.
4. Dreosti IE. Bioactive Ingredients: Antioxidants and Polyphenols in Tea. Nutrition Reviews 1996; 54(11): S51-S8.
5. Vallcic S, Timmermann BN, Alberts DS, Wachter GA, Krutzsch M, WymerJ, et al. Inhibitory Effect of Six Green Tea Catechins and Caffeine on The Growth of Four Selected Human Tumor Cell Lines. Anticancer Drugs 1996;7:461-8.
6. Nakayama M, Suzuki K, Toda M, Okubo S, Hara Y, Shimamura T. Inhibition of the Infectivity of Influenza Virus by Tea Polyphenols. Antiviral Res 1993; 21:289-99.
7. Toda M, Okubo S, Ikigai H. The Protective Activity of Tea Catechin Againts Experimental Infection by Vibrio cholerae 01. Microbiol Immunol 1992;36: 999-1001.
8. Ooshima T, Minami T, Matsumoto M, Fujiwara T, Sobue S, Hamada S. Comparison of the Cariostatic Effects between Regimens to Administer Oolong Tea Polyphenols in SPF Rats. Caries 1998; 32: 75-80.
9. Katiyar SK, Challa A, McCormick TS, Cooper KD, Muchtar H. Prevention of UVB-induced Immuno-suppression in mice by the green tea polyphenol. Carcinogenesis 1999;20(11): 2117-24
10. Margawani KR, Sri P. Epidemiology of Salmonellosis. Jurnal JEN 1996; 2: 1-7.
11. Ryan K, Falkow S. Enterobactericeae. In Kenneth JR, editors. Sherris Medical Microbiology, 3th ed. London: Prentice-Hall International Inc. ;1994. p. 336-37.
12. Weintraub BC, Eckmann L, Okamoto S, Hense M, Hedrick SM, Fierer J. Infection Immun 1997; 65(6): 2306-12.
13. Elhovy A, Bost KL. Limited Interleukin-18 Response in Salmonella- Infected Murine Macrophages and in Salmonella-Infected Mice. Infection Immun 1999; 67(10): 5021-6.
14. Chong C, Bost, Clements JD. Differential Production of Interleukin-12 mRNA by Murine Macrophages in Response to Viable or Killed Salmonella spp. Infection Immun 1996; 64 (4): 1154-60.
15. Keush GT. Salmonellosis. In Wilson JD, Braunwald E, Isselbacher KJ, Peterdorf RG, Martin JB, Fauci AS, et al, editors. Harrison's Principles of Internal Medicine, 2th ed. New York: Mc Graw-Hill Co.;1991.p. 609-13.
16. Gulig PA, Doyle TJ, Salzler CMJ, Maise R, Matsui H. Systemic Infection of Mice by Wild-Type but Not Spv-Salmonella Typhimurium Is Enhanced by Neutralization of Gamma Interferon and Tumor Necrosis Factor Alpha. Infection Immun 1997 ; 65(12): 5191-6.
17. Umezawa K, Akaike T, Fujii S, Suga M, Setoguchi K, Ozawa A, et al. Induction of Nitric Oxide Synthesis and Xanthine Oxidase and Their Roles in the Antimicrobial Mechanism againts Salmonella Typhimurium Infection in Mice. Infection Immun 1997; 65(7): 2932-40.
18. Lewis CE, McGee JOD. The Natural Immune System : The Macrophage. New York Oxford University Press; 1992.
19. Mastroeni P, Harrison JA, Chabalgoity JA, Hormaeche CE. Infection Immun 1996; 64(1): 189-96.
20. Elgert KD. Immunology: Understanding the Immune System. New York: Wiley-Liss.Inc, 1996.
21. De La Fuente M, Fernandez MD, Burgos MS, Soler A, Praicito A, Miquel J. Immune Function in Aged Women is improved by Ingestion of Vitamin C and E. J Phisiol Pharmacol 1998; 76: 373-80.
22. Yang F, Villiers WJS, Mc Clain CJ, Varilek GW. Green Tea Polyphenols Block Endotoxin-Induced Tumor Necrosing Factor Production and Lethality in a Murine Model. Biochem Molecular Roles Nutrients 1998: 2334-40.