

## Potential Use of Pomegranate (*Punica granatum*) Extract as an Immune-Stimulant Based on *in vitro* and *in vivo* Models

Laily N<sup>1,4\*</sup>, Harahap AR<sup>2</sup>, Aji GK<sup>1</sup>, Sukarti I<sup>1</sup>, Ascobat P<sup>3</sup> & Wijayanti RDE<sup>1</sup>

<sup>1</sup> Center of Agroindustrial Technology-The Agency for the Assessment and Application of Technology (BPPT), Indonesia

<sup>2</sup> Department Clinical Pathology, Faculty of Medicine, Universitas Indonesia

<sup>3</sup> Department of Pharmacology, Faculty of Medicine, Universitas Indonesia

<sup>4</sup> Postgraduate Student at Faculty of Public Health, Universitas Indonesia

### ABSTRACT

**Introduction:** Emergency foods containing immune-stimulants may enhance the immunity of disaster victims suffering from low immune status. This experiment investigated the potential use of pomegranate as an immune-stimulant. **Methods:** *In vitro* lymphocyte proliferation analysis showed pomegranate extract as an immune-stimulant. An *in-vivo* study was conducted with mice fed pomegranate extract added to a high calorie biscuit preparation (Testing Group), and their IgG assays were compared with mice fed a biscuit added commercial immune-stimulant (Comparison) and without immune-stimulant (Control). These 3 groups of mice were starved before the experiment to mimic an emergency condition. Their IgG assays were also compared with two groups of non-starved mice fed a biscuit to which was added pomegranate extract (Non-hunger Testing Group) and without immune-stimulant (Non-hunger Control). Total serum IgG determinations were carried out in weeks 0, 2, 4 and 8 to determine the humoral immunity stimulation. **Results:** Up to 4<sup>th</sup> week, there was no significant difference in total IgG among the groups. However, at 8<sup>th</sup> week, there was a significant increase of total IgG in mice fed with the Test and Comparison foods compared to the Control. This finding indicates pomegranate extract performed as well as the commercial immune-stimulant in increasing IgG. **Conclusion:** Increase in serum IgG is a sensitive immune-marker that represents a specific humoral immune response due to infections. Pomegranate extract shows potential for use as a supplemental food in improving immunity status of subjects in an emergency situation.

**Key words:** Emergency-food, IgG, immune-stimulant, pomegranate-extract

### INTRODUCTION

Prevention of infectious diseases is a major public health concern after a natural disaster (Noji, 2005). Mitigation is done by maintaining environmental health such as sanitation, hygiene and vector management. Food aid for food security and the fulfilment of the immunisation programs are for prevention of infectious diseases (Noji, 2005).

The success of mitigation of infectious diseases after natural disasters depends on catastrophe management. It takes time to assess the needs of disaster victims. In an emergency situation, when the infrastructure and facilities to meet the needs of the victims have not been identified, for example, to meet the nutritional needs and to prevent the occurrence of infectious diseases,

development of a ready to eat emergency food containing immune-stimulatory agent is a good solution which could be adopted.

The body's immune system plays a role in fighting against infectious diseases. Disorders of the immune system can lead to vulnerability of the body against infectious diseases. One of the government programs to prevent infectious diseases during a natural disaster is mass immunisation (Noji, 2005). On the other hand, there are problems with mass immunisation programs given after a disaster because it is counter-productive. Mass immunisation only gives a false sense of security. What is important for optimal results in containing the spread of infectious diseases in emergency situations is adequacy of food intake rather than hygiene and sanitation. Mass vaccination is only recommended when recommended sanitary actions have no effect and there is evidence of an increasing number of cases with the risk of an epidemic (Noji, 2005).

Emergency food can be defined as a processed food product that is specially designed to be consumed during emergencies such as floods, landslides, earthquakes, hunger, fires, wars and other events. The forms of emergency food products that have been used in several countries are bar biscuits, canned food, and retort pouch food. These products are ready-to-eat foods with sufficient energy load. As disasters may occur at any time, emergency food therefore requires to be maintained as stock. To serve as emergency food stock, this product should provide a long shelf-life and be fairly easy to distribute. The Institute of Medicine (IOM) (2002) states that emergency food must have five critical characteristics which are: safe, palatable, easy to use, easy to eat and nutritionally complete. A dried product like the biscuit, is a type of food that meets the criteria of an emergency food.

Disaster victims in camps are very vulnerable to infectious diseases such as acute respiratory infection and diarrhoea

(Noji, 2005). In addition to the requirements mentioned earlier, the development of emergency food with the addition of an active substance like an immune-stimulatory agent is expected to be useful for maintaining health during evacuation. An immune-stimulant is an active ingredient that can enhance the body's immune system. Alkaloids, terpenoids, quinone and phenolic compounds are low to medium molecular weights compounds that show immune-stimulatory properties. High molecular weight compounds that show immune-stimulant properties are glycoproteins, nucleotides and polysaccharides (Das *et al.*, 2013; Oliveira *et al.*, 2010; Stier, Ebbeskotte & Gruenwald, 2014).

Many studies have highlighted that oral administration of antioxidants can stimulate the immune system and protect cells from oxidative damage. The addition of antioxidants such as beta-carotene might increase the activity of macrophages, protect macrophages from oxidative damage and enhance the T-lymphocyte proliferative response (Sadek, 2012). *In vitro* and animal studies also demonstrate that administration of plant extracts can improve and protect the immune systems (Benmebarek *et al.*, 2013; Devasagayam & Sainis, 2002). Polyphenols and lycopene are secondary metabolites possessing antioxidant activity. Administration of polyphenols in humans can also enhance macrophage phagocytosis (Mertens-Talcott *et al.*, 2006; Ratnaningsih, Asmara & Sismindari, 2004).

The health benefits of pomegranate are associated with active compounds in the fruit. Pomegranate contains carbohydrates, glycosides, phenol, tannins, flavonoids, anthocyanin and alkaloids. In traditional medicine, pomegranate fruit (*Punica granatum* L.) is consumed to treat dysentery, microbial infections, diarrhoea, respiratory pathologies and diseases such as herpes and influenza (Oliveira *et al.*, 2010; Vidal *et al.*, 2003). Pomegranate

appears to exhibit interesting anti-viral activity. Pomegranate extracts have been shown to be effective against the herpes virus, and hydro-alcoholic extracts of whole pomegranate fruits exhibit a high activity against the influenza virus (Vidal *et al.*, 2003). Lansky & Newman (2007) reported that antioxidants in pomegranate fruit have anticancer activity, while another study demonstrated *in vitro* immunostimulatory activity of pomegranate fruit extracts (Laily *et al.*, 2011). However, the use of pomegranate extracts in human studies particularly in enhancing immune responses have not been well studied.

This study explored the potential usage of pomegranate extract as an immune-stimulant to boost lymphocyte proliferation based on *in vitro* and *in vivo* models.

## METHODS

### Extraction of pomegranate

Pomegranate fruit pulp was washed and dried at 40°C and ground into powder. The powder was extracted with 80% ethanol, and evaporated to yield a concentrate of pomegranate (1.0 g/ml) in dimethyl sulfoxide.

### *In vitro* test : Lymphocyte isolation

Human lymphocyte cells were isolated from peripheral blood by centrifugation at 514 ×g for 10 min to separate the cellular components. Red blood cells being denser remained at the bottom, while blood lymphocytes were concentrated at the top of the solutions. The buffy coat layer (composed mainly of human lymphocytes) was pipetted out carefully using a syringe and dissolved in 3 ml of RPMI medium.

The lymphocyte suspension was added slowly on top of the ficoll-hypaque solution to form two layers. Centrifugation was done at 1430 ×g for 30 min to separate granulocyte and red blood cells from the lymphocyte, monocyte and platelet cells at the top layer. The former was washed two

times with basic media and centrifuged at 228 ×g for 30 min to obtain the lymphocytes in the precipitate. Lymphocyte cells were counted by the trypan blue method and dissolved with RPMI medium to acquire a 10<sup>6</sup> cells /ml concentration.

### Lymphocyte proliferation response analysis

Lymphocyte proliferation response analysis was conducted with MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (Zakaria *et al.*, 2011)

A volume of 80 µl lymphocyte suspension cells (10<sup>6</sup> cells/ml) grown in RPMI medium was dispensed into micro wells. Into each well, 20 µl pomegranate extract of various concentrations were added. The final concentration of the pomegranate extract in the microwells were 0.1, 0.25, and 0.5 µl/ml. The control consisted of only the RPMI 1640 solution without pomegranate extract. Incubation was performed for 72 h at 37°C in 5% CO<sub>2</sub> incubator. Following incubation, the cells were treated with MTT and incubated further for 4 h and its absorbance was measured at 570 nm using a micro plate reader (Thermo, Shanghai, China). Immune-stimulant activity was expressed as % stimulation index (% SI), based on the formula:

$$SI = \frac{OD_{control}}{OD_{treatment}} \times 100\%$$

where *SI* is stimulation index, *OD*<sub>treatment</sub> is optical density of treatment (with stimulation) and *OD*<sub>control</sub> is optical density of control (without stimulation, medium only), [%].

### Preparation of emergency food

A high calorie biscuit (463 kcals/100g) containing cream of pomegranate extract as an immune-stimulatory ingredient was prepared as an example of an emergency food. The biscuit consisted of wheat and corn flour, whey, sugar, eggs and vegetable fat, while the cream was made from palm oil, margarine, whey, corn starch, sugar

flour and 1% of pomegranate extract. The emergency food was dissolved in 0.5% of carboxyl methyl cellulose by stirring for 10 min.

Inbred BALB/c mice, 6-8 weeks of age were supplied by the University of Gadjah Mada, Indonesia. All the mice were provided with food and water *ad libitum*. This study obtained the approval of the Animal Ethics Committee (AEC), Faculty of Medicine, University of Indonesia.

### ***In vivo* experimental model**

**Step One.** After one week of adaptation process, the mice were separated into Hunger and Non-Hunger Groups. The Hunger Group comprised mice that were insufficiently fed fulfilling 50% of daily calorie requirement but given water *ad libitum*. The Non-Hunger mice were provided with sufficient calories and water. **Step Two.** The feeding experiment was conducted for one month with the Hunger Group divided into three groups, as follows:

- Group (1): 18 mice fed the biscuit with commercial immune-stimulant extract (Hunger Comparison Group);
- Group (2): 18 mice fed the biscuit with pomegranate extract (Hunger Testing Group);
- Group (3): 24 mice fed normal food (Hunger Control Group).

Meanwhile the Non-Hunger mice were divided into two groups:

- Group (4): 18 mice fed normal food (Non-Hunger Control Group),
- Group (5): 12 mice fed biscuit with pomegranate extract (Non-Hunger Testing Group).

The dose of biscuit with pomegranate extract administered to the experimental mice was equal to 400g/day based on estimation of food consumed by a 70-kg man.

**Step 3:** Sampling analysis was done at weeks 0, 2 and 4, in which six mice each time were sacrificed for immunological assay. The rest of the mice from Groups (1), (2) and (3) were challenged with tetanus toxin. Immunological assay was done at week 8, which was one month after vaccination. Six mice from each group were obtained for further immunological analysis.

### **Immunological analysis - determination of mouse serum IgG**

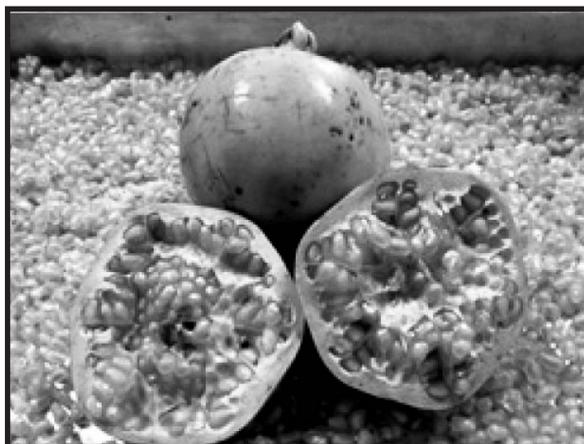
The mouse IgG ELISA kit is designed for measurement of IgG in mouse serum or plasma. Test samples were diluted and incubated in micro-titre wells for 45 min alongside prepared mouse IgG standards. The micro-titre wells were subsequently washed, and HRP conjugate was added and incubated for 45 min. IgG molecules were thus sandwiched between the immobilisation and detection antibodies. The wells were then washed to remove unbound HRP-labelled antibodies, and TMB reagent was added and incubated for 20 min at room temperature. This resulted in the development of a blue colour. The formation of blue colour was stopped by addition of a stop solution (acid-isopropanol) resulting in a change of colour from blue to yellow, and optical density was measured using a spectrophotometer uv-vis 2001 (Hitachi, Japan) at 450nm. The concentration of IgG was proportional to the optical density of the test sample and was derived from a standard curve.

### **Statistical analysis**

Analysis of total IgG was undertaken by ANOVA test. Body weight determination results were analysed using the student's *t*-test. In all cases, the level of significance was defined by  $p < 0.05$ .

## **RESULTS AND DISCUSSION**

A preliminary study on potency of pomegranate extract (Figure 1.) as an



**Figure 1.** Pomegranate fruit (*Punica granatum*).

**Table 1.** Lymphocyte proliferation test of pomegranate extract and other comparable agents

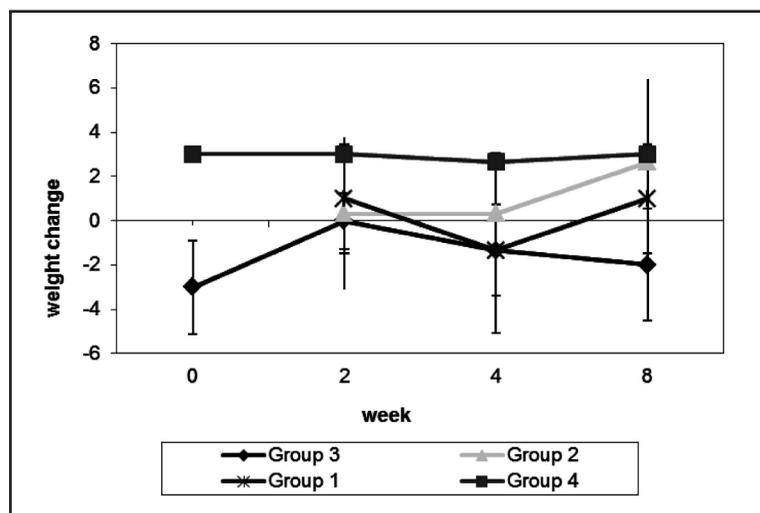
Label	Sample	Concentration (ppm)	% Stimulation index
1.	Pomegranate extract	100	1245
2	Commercial extract	100	1024
3	LPS	20	605
4	Concovalin A	20	645

immune-stimulant was tested *in vitro* based on lymphocyte proliferation using MTT assay. Potency of immune-stimulatory activity of pomegranate extract was compared with other mitogens (LPS and concovaline A) and other commercial immune-stimulants. The test results showed that pomegranate extract has potency as an immune-stimulant with its stimulation index being higher than the commercial immune-stimulants (Table 1). Lymphocyte proliferation activity of pomegranate was 1.2 times greater than the commercial immune-stimulant at the same concentration.

Furthermore, the ability of pomegranate extract as an immune-stimulant was tested using experimental animals in

the form of a high calorie emergency food biscuits capable of maintaining the body's immunity. Hunger treatment was intended to mimic the emergency situation in which the body might be more prone to infections during a disaster to test the effect of supplementing emergency food biscuits with pomegranate extract.

Administration of tested food to hungry mice showed an increase in body weight, which was almost similar to non-hunger control group (Group 4), by 2.67 and 3 respectively. Hunger testing group (Group 2) also had a higher body weight with the increase being 1.67 times higher than the mice in the hunger comparison group (Group 1) (Figure 2). The hunger testing group (group 2) was able to restore



**Figure 2.** Mean weight changes of groups of mice during 8 weeks of feeding experiment

*Note:* Group (1): Mice fed the biscuit added with commercial extract (Hunger Comparison Group);  
 Group (2): Mice fed the biscuit added with pomegranate extract (Hunger Testing Group);  
 Group (3) Mice fed the biscuit without immune-stimulant (Hunger Control Group).  
 Group (4) Mice fed the biscuit without immune-stimulant (Non-Hunger Control Group),

body weight under hunger conditions closer to the non-hunger control group (group 4) than other groups (Group 1 and 3).

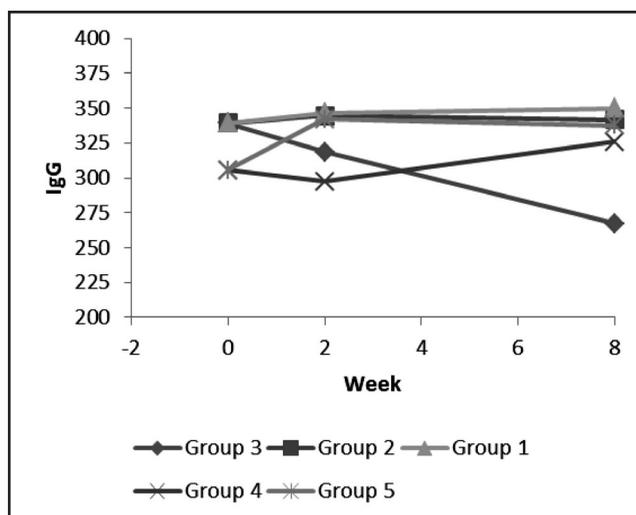
Tetanus toxin injection at week 4 caused body weight to decrease significantly in the hunger control group (Group 3). Similarly, reduced body weight after infection was also noticed in the hunger comparison group (Group 1) even though in this group, the increase occurred after week 8. However, this condition did not occur in the hunger testing group (Group 2), with the mice belonging to this group still being able to maintain their body weight at week 4 after infection, and then increase their body weight, similar to the weight of the normal treated mice, which were non-hunger treated and not infected with the toxin (Group 4, non-hunger control group) (Figure 2).

Humoral response test (total IgG) towards the emergency food formula fortified with pomegranate extract was done on hunger-treated mice, as well as

untreated mice (mice fed with normal diet). The tested food was given after week 2 and the observation was continued for up to week 8. On week 4, selected mice were challenged with tetanus toxin. These findings were compared with a commercial immune-stimulant.

The results showed that administration of the sample for 4 weeks either on hunger testing group (Group 2) or hunger comparison group (Group 1) did not raise total IgG. However at week 8, there was a significant increase in total IgG in either Group 1 or Group 2 (Figure 3.). There was no significant difference between hunger testing group and hunger comparison group. This is in contrast to the hunger control food group (Group 3) which experienced a 21% reduction in total IgG during treatment for up to 8 weeks.

The emergency food formula fortified with pomegranate extract could be used to combat tetanus toxin-mediated infection at a dose equivalent to 400g/days (human



**Figure 3.** Total IgG during treatment on hungry-mice groups

*Note:* Group (1): Mice fed the biscuit added with commercial extract (Hunger Comparison Group); Group (2): Mice fed the biscuit added with pomegranate extract (Hunger Testing Group); Group (3) Mice fed normal food (Hunger Control Group). Group (4) Mice fed normal food (Non-Hunger Control Group), Group (5) Mice fed the biscuit added pomegranate extract (Non-Hunger Testing Group).

dose) for 8 weeks of the experiment. The hunger comparison group with the commercial immune-stimulant, an agent that had passed the clinical test, also gave comparable results. However, mice in the hunger control group (Group 3) could not maintain their immunity and could not cope with the infection induced at week 4 (Figure 3).

For the non-hunger tested mice (Groups 4 and 5), the effect of tested food was also studied in the mice fed with food based on calorie requirements. After 2 weeks of feeding, total IgG of the mice in non hunger testing group (Group 5) increased significantly ( $p < 0.05$ ) compared to mice in the non-hunger control group (Group 4). On the other hand, Group 4 (non-hunger control group) showed a significant increase in total IgG at week 8. Mice in non-hunger testing group (Group 5) and non-hunger control group (Group 4) did not show any significant increase in

total IgG. The result indicates that under normal conditions, (e.g. no infections or hunger), feeding with emergency food formula for 2 weeks is sufficient to boost total IgG. However, mice fed control food needed at least 4 weeks for their total IgG to increase.

The immune system is enormously important for combating infectious diseases. A poor immune system will cause the body to be susceptible to infectious diseases. An immune-modulator is an active agent that is capable of controlling the desired immune response. Supplementation of emergency food biscuits with pomegranate extract can potentially boost the immune system of the animals by increasing the level of total IgG. Pomegranate extract is rich in polyphenols and antioxidants which are the active compounds and has immune-stimulatory potent. In a previous study, Laily *et al.*, (2011) mentioned that immune-stimulatory activity of pomegranate extract increased

by increasing the concentration of active compounds such as total phenols, antioxidant and ellagic acid. Immune-stimulatory properties of pomegranate extract were influenced by the levels of polyphenolic compounds in the extract. Punic acid is an active compound in fruit extracts and has strong ability against oxidation, is an anticancer and improves immune response (Bassaganya-Riera, 2005). Singh, Murthy & Jayaprakasa (2002) mentioned that pomegranate peel extract had higher antioxidant activity than pomegranate seed extract due to the different phenolic composition of these extracts. The statement was further supported by Kotamballin *et al.* (2002) that pomegranate peel is rich in phenolic compounds, and different activities of the pomegranate extract could be ascribed to their different phenolic composition. On the other hand, Norshazila *et al.* (2010) highlighted a negative correlation between antioxidant activity and total phenol compound. Various phenolic compounds respond differently in DPPH assay, depending on the number of hydroxyl groups in the phenolic compounds.

*Stachys mialhesi* extract has a powerful immune-stimulatory effect due to the flavonoids, terpenoids and phenolic components in the extract. Immune-stimulatory activity test was done based on an animal experimental model using carbon clearance method. The principle advantage of this method is the speed of removal of foreign material such as colloidal carbon (Benmebarek *et al.*, 2013). Pomegranate extract contains bioactive compounds similar to *S. mialhesi*, where both the extracts have immune-stimulatory activities tested *in vitro* and *in vivo*.

## CONCLUSION

An *in vitro* study revealed that pomegranate extract had potential as an immune-stimulatory agent. The supplementation of

emergency food formula with pomegranate extract and fed to mice boosted the body's immune system during an emergency condition, such as starvation. The mice were able to defend themselves from infection (tetanus toxin) by increasing the levels of total IgG and body weight during 8 weeks of observation. The provision of emergency food formula for 2 weeks in normal circumstances (no starvation) could also increase the total IgG levels more rapidly than control food.

The stimulation effect of increased levels of total IgG in mice occurred at week 8 after treatment in both groups (hunger testing group and hunger comparison group) and differed significantly from the hunger control group (mice fed with emergency food without immune-stimulant). Thus, to have a better understanding of the implications of the immune-emergency food on the human immune system, the logical next step is to investigate the ability of this immuno-emergency food to boost immunity in human during emergencies.

## Conflict of interest

The authors declare there are none.

## REFERENCES

- Bassaganya-Riera (2005) Method of using punic acid to enhance immune response and metabolic disorder US-Patent 2005/0159483 A1.
- Benmebarek A, Zerizer S, Laggoune S & Kabouche Z (2013) Immuno-stimulatory activity of *Stachys mialhesi* de Noé *Allergy, Asthma & Clinical Immunol* 9: 2.
- Das BK, Pattnaik P, Debnath C, Swain DK & Pradhan J (2013). Effect of  $\beta$ -glucan on the immune response of early stage of *Anabas testudineus* (Bloch) challenged with fungus *Saprolegnia parasitica*. *Springer Plus* 2: 197.
- Devasagayam TPA & Saini KB (2001). Immune systems and antioxidants, especially those derived from Indian medicinal plants *Indian Exp Biol* 40: 639-655.

- Institute of Medicine(IOM) (2002). Emergency Relief Food Products. National Academic Press, Washington, pp. 2-5.
- Kotamballin N, Murthy C, Guddadarangavahally K, Jayaprakasha & Singh RP (2002). Studies on antioxidant activity of pomegranate (*Punica granatum*) peel extract using *in vivo* models. *Agric Food Chem* 50: 4791-4795.
- Lansky EP, & Newman RA (2006). *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer *Ethno pharmacol* 109(2): 177-206.
- Laily N, Wijayanti RDE, Bachtiar A & Sukarti I (2011). Determination of immunostimulant activity of pomegranate fruit (*Punica granatum*) by *in vitro* test [Kajian Aktivitas Immunostimulan Buah Delima Putih (Pomegranate, *Punica granatum*) secara *in vitro*]. *Prosiding Seminar Nasional Perhimpunan Ahli Teknologi Pangan Indonesia (PATPI)*. pp. 474-480.
- Mertens-Talcott SU, Jilma-Stochlawetz P, Rios J, Hingotani L & Derendord H (2006). Absorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum* L.) polyphenols after ingestion of a standardized extract in healthy human volunteers *J Agric Food Chem* 54: 8956-8961.
- Noji EK (2005). Public health issues in disasters. *Crit Care Med* 33(1): S29-S33.
- Norshazila S, Syed Zahir I, Mustapha Suleiman K, Aisyah MR & Kamarul Rahim K (2010). Antioxidant levels and activities of selected seeds of Malaysian tropical fruits. *Mal J Nutr* 16(1): 149-159.
- Oliveira RA, Narciso CD, Bisinotto RS, Perdomo MC, Ballou MA, Dreher M, & Santos JEP (2010). Effects of feeding polyphenols from pomegranate extract on health, growth, nutrient digestion, and immune-competence of calves *Dairy Science* 93: 4280-4291.
- Ratnaningsih T, Asmara W & Sismindari (2004). Polyphenols extracted from green tea augments the protective immune responses in mice challenged with *Salmonella typhimurium*. *Med Indones* 13(1): 1-7.
- Sadek KM (2012). Antioxidant and immunostimulant effect of *Carica papaya* Linn. aqueous extract in acrylamide intoxicated rats *Acta Inform Med* 20(3): 180-185.
- Singh RP, Murthy C & Jayaprakasha K (2002). Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models. *Agric Food Chem* 50(10): 81-86.
- Stier H, Ebbeskotte V & Gruenwald J (2014). Immune-modulatory effects of dietary Yeast Beta-1,3/ 1, 6-D-glucan. *Nutrition* 13: 38.
- Vidal A, Fallarero A, Pena BR, Medina ME, Gra B, Rivera F, Gutierrez Y & Vuorela PM (2003). Studies on the toxicity of *Punica granatum* L. (Punicaceae) whole fruit extracts. *Ethno-pharmacol* 89: 295-300.
- Zakaria FR, Prangdimurti E, & Puspitawati GAKD, Thahir R, Suismono (2011). Diets based on sorghum (*Sorghum bicolor* L Moench) improve lymphocyte proliferation and antioxidant capacity of rat liver [*Diet berbasis sorgum (Sorghum bicolor L Moench) memperbaiki proliferasi limfosit dan kapasitas antioksidan hati tikus*]. *Majalah Pangan: M Med Kom* 3(20): 209-222. [Bahasa Indonesia]