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Evaluation of *in vitro* immunomodulatory effect of fractions of *Ficus septica* Burm. f. and their total flavonoid and phenolic contents

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Abstract

The study aimed to evaluate the *in vitro* effects of some fractions of ethanolic extract of *F. septica* Burm. f. on Balb/c mice macrophage phagocytosis and lymphocyte proliferation. In addition, we also determined the total flavonoid and phenolic contents in the fractions. These fractions were *n*-hexane soluble fraction, ethyl acetate soluble fraction and ethyl acetate insoluble fraction. These fractions were then tested on lymphocytes proliferation by a MTT reduction method and on macrophage phagocytosis activity based on the number of latex beads uptake by the cells. In the study, the total phenolics content of ethyl acetate-soluble, ethyl acetate-insoluble and *n*-hexane soluble fractions were 35.53; 7.59 and 0.33%, respectively. Whereas their total flavonoids content were 3.27; 0.81 and 0.05%, respectively. All fractions exhibited to increase the lymphocytes proliferation and macrophage phagocytosis activity. Among them, ethyl acetate fraction had highest immunomodulatory effects. In conclusion, ethyl acetate fraction of ethanolic extract of *Ficus septica* Burm. f is potential to develop as an immunomodulatory agent.

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Introduction

Reportedly, cancer is a main cause of death in the world. WHO (2011) reported that about 13% of cases of death due to cancer in 2008, and more than 70% of all death due to cancer occured in developing countries. There are several approaches used for treating cancer i.e surgery, radiotherapy and chemotherapy. One of the most popular chemotherapeutics is doxorubicin (Rang *et al.*, 2003; Tan *et al.*, 2009). To date, the use of doxorubicin is limited due to its side effects in high- and repeated-doses. The drug could induce cardiotoxicity and affect immune functions (Santos *et al*., 2010; Bowles *et al*., 2012). Zhang *et al.* (2005a) reported that doxorubicin influenced the immune systems by significantly decreasing the production of IL-2 and INF-gamma, and also mildly decreasing NK cell cytotoxicity, proliferation of lymphocyte and CD4+/CD8+ ratio in tumour-bearing mice. Besides, doxorubicin could stimulate the reduction of plasmatic IL-1, IL-10, and TNF-α levels in advanced breast cancer patients (Panis *et al*., 2012). To overcome these side effects, an immunomodulatory agent can be combined with doxorubicin in order to protect and enhance the immune functions during chemotherapeutics administration.

Biodiversity of Indonesia is the second largest after that of Brazil including medicinal plants. One of Indonesian medicinal plant is awar-awar or *Ficus septica* Burm. f. (Moraceae). The ethanolic extract of this plant exhibited cytotoxic effect on MCF-7 and T47D cells with IC_{50} value of 13 and 6 µg/mL, respectively (Mubarok *et al*., 2008; Pratama *et al.*, 2011; Nugroho *et al.*, 2011). The extract showed a synergistic effect in combination with doxorubicin (3.75 nM). The extract also induced apoptosis and downregulated the expression of Bcl-2 protein in MCF-7 breast cancer cells (Sekti *et al*., 2010). The extract (750 mg/kg BW) succeeded to induce apoptosis throught p53-independent pathway in liver cancer of 7,12-dimethylbenz[a]nthracene-induced rat (Septhea *et al.*, 2011).

In our previous study, both ethyl acetate-soluble fraction and hexane-insoluble fraction of ethanolic extract of *F. septica* leaves synergically increased the cytotoxic effect of doxorubicin through apoptosis, decrease of PARP expression and modulation of cell cyce arrest in T47D breast cancer cells (Nugroho *et al*.,

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2012; Nugroho *et al*., 2013a). Single administration of both fractions also exhibited potent cytotoxic effects in T47D breast cancer cells by IC_{50} value of 8 and 9 µg/mL, respectively. Based on these facts, active fractions of *F. septica* leaves is potential to develop as co-chemotherapeutic agents for doxorubicin in breast cancer therapy. In the study, the fraction were evaluated for its *in vitro* immunomodulatory activities.

Materials and Methods

Materials

F. septica Burm. f. was collected during June 2012 from area around Sumber Arum Moyudan, Yogyakarta, Indonesia. *F. septica* Burm. f. was authenticated by a botanist at Department of Pharmaceutical Biology, Universitas Gadjah Mada, Indonesia. The voucher specimen was deposited in herbarium of the department. Materials for assay were [3 - (4,5-dimetilthiazol-2-yl) -2.5-diphenyl tetrazolium bromide] (MTT), Roswell Park Memorial Institute (RPMI) medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), latex beads, lipopolysaccharide, penicillin-streptomycin (Pen-Strep), phytohemagglutinin (PHA) purchased from Sigma Chemical, St Loius, MO. Other materials were fungizone (Gibco, USA), Giemsa (Merck, Jerman).

Preparation of fractions of F. septica

Firstly, fresh leaves of *F. septica* were washed entirely with distilled water. The leaves were dried, ground and weighed. Subsequently, the dried powder was extracted with 70% ethanol with a ratio of 1:7. The filtrate was evaporated to provide a viscous ethanolic extract. The extract was diluted in 10% ethanol and fractionated with *n*-hexane (1:1) using Liquid-Liquid Continuous Extraction (LLCE) yielding *n*-hexane soluble and insoluble fractions. The *n*-hexane soluble fraction was acidified with 0.1 N HCl until pH 5. After addition with distilled water, the extract was then neutralized with 0.1 N NaOH to pH 7, and evaporated to provide a viscous fraction. On the other side, the *n*-hexane insoluble fraction was then fractionated with ethyl acetate (1:1) to yield non-ethyl acetate insoluble and ethyl acetate soluble fractions. Both fractions were then evaporated to provide viscous fractions. The fractions were dried using a Freeze dryer ALPHA 1-2 LD plus (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) to eliminate the existence of the remaining traces of water.

Determination of total phenolic content

The determination of total phenolic content was performed regarding to Chun *et al*. (2003). Samples (0.1 mL) were reacted with 0.1 mL reagent of Folin-Ciocalteau and incubated for 5-8 minutes. The solutions were added with 1.0 mL of 7% Na_2CO_3, and then added with distilled water. Blank solution contained only distilled water and Folin-Ciocalteau reagent. The mixture was allowed to stand for 2 h, and its absorbance was measured at 725 nm. Total phenolic content was expressed in mg gallic acid equivalent (GAE) of each 100 mg sample of dry weight.

Determination of total flavonoid content

Total flavonoid contents were based on modified colorimetric method of Chang *et al.* (2002). Either 0.2 mL of sample or catechin (reference standard) was added with 3.7 mL of 95% ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1M potassium acetate and 1.0 mL distilled water, and then incubated for 30 minutes. The absorbance was measured in 510 nm wavelength, and distilled water with 10% $AlCl_3$ and 1M potassium acetate were used as a blank. Total flavonoid content was expressed in mg catechin equivalent (CE) of each 100 mg of sample dry weight.

Animals

BALB/c mice (8-10 weeks old, male, 20-30 g) were obtained from Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The mice were housed under constant temperature (23-27°C) with constant relative humidity (50-70%) on an automatically controlled light (12 h light/dark). All animals were fed with standard rodent chow and water ad libitum. The mice were acclimatized and quarantined for at least one week before the experiment. All animal handling protocols were performed in accordance with the guidelines of laboratory animals care of the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia.

DPPH free radical-scavenging activity

The antioxidant activity was based on the free radical scavenging activity. The activity were evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. In the assay, 50 µL of a series of concentrations of the fractions was added to 1.0 mL of 0.4 mM DPPH solution in methanol, and then added with methanol to 5.0 mL. The mixture

was shaken vigorously, and incubated for 30 min at room temperature in the dark room. The absorbance was read at 517 nm using a spectrophotometer.

Phagocytic activity of macrophages

The mice were sacrificed by decapitation and exsanguination. Macrophages were isolated by injection of 10 mL of cold RPMI. The peritoneal cavity was opened gently, and peritoneal fluid was collected. The fluid was centrifuged at 1,200 rpm 4°C for 10 min. After the upper layer containing other components was aspirated and discarded, the remaining cell pellet was added with 3 mL of RPMI 1640 complete media containing FBS 10% (v/v). After counted by a Neubaeur hemocytometer, the cell pellet was resuspended in a complete medium to provide a cell suspension with 2.5×10^6 cells mL^{-1} density. The cell suspension was then seeded into 24-well plates covered by round cover slips. Each well contains 200 µL suspension (5×10^5 cells).

The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 h. Subsequently, the medium was removed, and the cells were washed twice with 250 µL RPMI 1640, and then incubated with 100 µL of fraction (dissolved with 1% DMSO in complete medium) for 24 h. Two hundred µL latex bead suspension (2.5×10^7 particles mL^{-1}) were added into each well and incubated in a 5% CO_2 incubator at 37°C for 60 min. The cells were then washed with PBS three time to eliminate unfagocyted latex beads. After dried at room temperature, the cells were fixed with methanol for 30 sec. This methanol was removed, and the coverslips were dried at room temperature, followed by 20% Giemsa (v/v) staining for 30 min. Subsequently, the cells were washed with distilled water, and dried at room temperature. Macrophage – phagocyted latex beads were observed under inverted microscope (Olympus, Germany).

Lymphocyte proliferation activity

Briefly, mice were sacrificed by decapitation and exsanguination. Spleen of mice were isolated aseptically, transferred to a petri dish containing 5 mL of RPMI and minced with a sterile forcep gently to provide lymphocytes suspension. Lymphocytes were isolated by centrifugation at 1,500 rpm 4°C for 10 min. Erythrocytes were lysed by resuspending the pellet with 2 mL Tris ammonium chloride buffer at room temperature for 2 minute. FBS (1 mL) was added by locating at bottom of centrifuge tubes. The suspension was then centrifuged at 1,500 rpm 4°C for 5 min. Supernatant was discarded, the remaining cell pellet was washed twice with RPMI and then diluted with complete medium. Lymphocytes were counted by hemocytometer and resuspended in RPMI to obtain cell suspension with 1.5×10^6 cells mL^{-1}. The lymphocytes were then seeded into 96-well plates, and incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 h. After incubation, 100 µL sample were added into each well and incubated again for 24 h. Subsequently, 100 µL MTT (5 $mg.mL^{-1}$) was added into each wells, and inducated at 37°C for 4 h. Viable cells will react with MTT to result in purple formazan. Finally, each well was added with stopper reagent (10% SDS) in 100 µL of HCl 0.01 N. Optical densities were read at ELISA reader at 550 nm.

Statistical analysis

All data were presented as mean ± the standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test was used for statistical analysis to compare more than two groups. P-values of less than 0.05 were considered significant.

Results and Discussion

Total phenolic content

The total phenolic content was estimated by using Folin-Ciocalteu reagent. The absorbances of a concentration series of gallic acid were plotted to their concentratios to yield a linear calibration curve of gallic acid ($y = 1.212x + 0.040$) with coefficient of determination (r^2) value of 0.994 (Fig. 1). The total phenolic content of *n*-hexane soluble, non-ethyl acetate and ethyl acetate soluble fractions (HSF, NESF, ESF) were 0.33±0.05%, 7.59±1.27% and 35.53±1.79%, respectively (Table 1). These results mean that each 100 mg dry weight of HSF, NESF, ESF containing total phenolics equivalent to gallic acid were 0.33±0.05 mg, 7.59±1.27 mg and 35.53±1.79 mg, respectively. Based on these data, the total phenolics content in these fractions varied widely. The phenolics content depended on the polarity of solvent. Highest total phenolic content was found in ESF, followed by NESF and HSF. High solubility of phenols in polar solvents resulted in high phenolic content in ethyl acetate soluble fraction.

Total flavonoid content

Total flavonoid content was estimated using a modified method based on the procedure of Chang *et al.* (2002). The method is based on complex formation between flavonoid and $AlCl_3$ producing a yellow coloured solution. The absorbance was measured spectrophotometrically at maximum wavelength of 510 nm (Nugroho *et al.*, 2013b). The total flavonoid content is represented in terms of catechin equivalents

Table 1. Total phenolic and total flavonoid contents in fractions of ethanoic extract of *F. septica* expressed in term of gallic acid andcatechin equivalent (Data represent mean±SEM, n=3).

Fracttion	mg of GA/100 mg of extract	mg of Catechin/g of extract
n-hexane soluble (HSF)	0.33±0.55	0.0486 ± 0.0005
non-ethyl acetate (NESF)	7.59 ± 1.27	0.81 ± 0.012
ethyl acetate soluble (ESF)	35.53 ± 1.79	3.27 ± 0.05

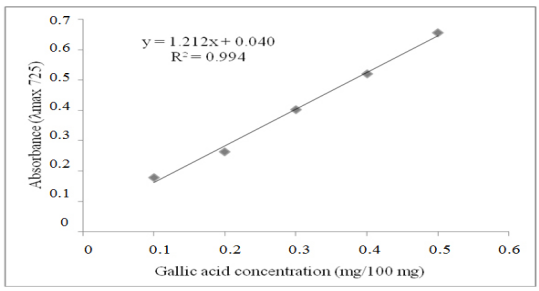

Figure 1. Linear curve of gallic acid concentration (mg/100 mg) vs. absorbance for determination of total phenolic content

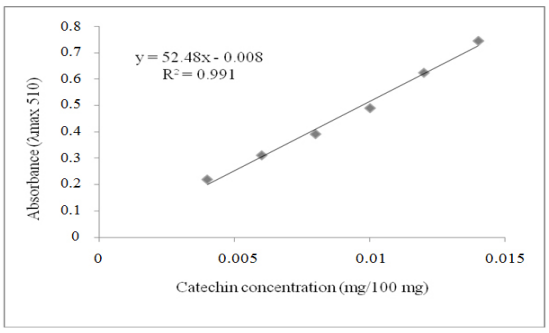

Figure 2. Linear curve of catechin concentration (mg/ 100 mg) vs. absorbance for determination of total flavonoid content

per gram of dry material of sample. The absorbances of a concentration series of catechin were plotted to their concentrations to yield a linear calibration curve of catechin (y = 52.48x - 0.008) with coefficient of determination (r^2) value of 0.991 (Fig. 2). The result showed that the total flavonoid contents of HSF, NESF, ESF were 0.05±0.001%, 0.81±0.01% and 3.27±0.05%, respectively (Table 1). These results mean that each 100 mg dry weight of HSF, NESF, ESF containing total phenolic equivalent to catechin were 0.05±0.001 mg, 0.81±0.01 mg and 3.27±0.05 mg, respectively. Based on this fact, ESF has the highest total flavonoid content followed by NESF and HSF.

DPPH free radical-scavenging activity

The antioxidant activity was performed using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals scavenging assay. The method is based on the reducion of DPPH solution due to the presence of antioxidant substance possessing hydrogen donating moieties such as phenolic and flavonoids that cause

Table 2. Antioxidant activity of ethanolic extract of *F. septica* (n=3).

No	Concentration (mg/mL)	% scavenging activity	IC_{50} value
1.	HSF		
	2.0 mg/ml	53.33	1.8 mg/ml
	1.6 mg/ml	46.54	
	1.4 mg/ml	41.04	
	1.2 mg/ml	36.08	
	1.0 mg/ml	34.38	
	0.8 mg/ml	28.76	
2.	ESF		
	1.6 mg/ml	68.75	0.4 mg/ml
	1.2 mg/ml	64.54	
	0.8 mg/ml	58.93	
	0.6 mg/ml	53.06	
	0.4 mg/ml	49.36	
	0.2 mg/ml	46.30	
3.	NESF		
	2.4 mg/ml	67.26	1.5 mg/ml
	2.0 mg/ml	62.44	
	1.8 mg/ml	58.25	
	1.6 mg/ml	52.16	
	1.4 mg/ml	48.48	
	1.2 mg/ml	43.65	
	1.0 mg/ml	37.94	

Table 3. Effect of fractions of ethanolic extract of *F. septica* in macrophage phagocytosis. (Data represent mean±SEM, n=3).

Treatment	Macrophage phagocyted-latex	% control
Control	322.67±2.33	
HSF 25 µg/mL	348.33±3.76	7.95
HSF 50 µg/mL	350.67±1.76	8.68
HSF 100 µg/mL	396.33±2.33	22.83
ESF 25 µg/mL	398.00±2.65	23.34
ESF 50 µg/mL	481.67±5.78	49.28
ESF 100 µg/mL	530.67±2.03	64.46
NESF 25 µg/mL	381.00±6.08	18.08
NESF 50 µg/mL	409.00±3.79	26.76
NESF 100 µg/mL	481.00±6.08	49.07

Table 4. Effect of fractions of ethanoic extract of *F. septica* in on mice lymphocytes proliferation. (Data represent mean±SEM, n=3).

Treatment	*Optical Density*	% control
Control	0.0192±0.0013	-
HSF 25 µg/mL	0.0128±0.0064	2,26
HSF 50 µg/mL	0.0322±0.0034	4,89
HSF 100 µg/mL	0.0288±0.0044	3,76
ESF 25 µg/mL	0.0348±0.0054	6,02
ESF 50 µg/mL	0.0442±0.0012	9,4
ESF 100 µg/mL	0.0938±0.0084	28,2
NESF 25 µg/mL	0.0222±0.0035	1,13
NESF 50 µg/mL	0.0425±0.0023	9,02
NESF 100 µg/mL	0.0532±0.0059	12,78

the formation of non-radical DPPH-H form. In the study, ESF revealed the highest activity with IC_{50} of 0.4 mg/mL, followed by that of NESF and HSF (IC_{50} 1.5 and 1.8 mg/mL, respectively). The result was shown in Table 2.

Effect on macrophage phagocytosis

In vitro macrophage phagocytosis assay was conducted using non-specific phagocytosis activity on latex beads (Pratten and Lloyd, 1984). ESF exhibited the most potent immunomodulatory activity regarding to macrophage phagocytosis. Its effect was concentration-dependent. The EC_{50} value of ESF was 67 µg/mL. ESF at concentration of 50 and 100 µg/mL stimulated the activity of macrophage phagocytosis by 49 and 65%, respectively (Table 3). In addition, NESF 100 µg/mL exhibited the increase of macrophage phagocytosis by 49%. However, HSF showed a mild activity.

Effect on lymphocytes proliferation

In the study, treatment of phytohaemagglutinin markedly stimulated proliferation of lymphocytes (data not shown). The substance is a lection responsible for induction of proliferation of human T lymphocytes (Hutchins and Steel, 1983). Based on the result, ESF exbihited highest stimulatory effect on lymphocytes proliferation in concentration-dependent manner (Table 4). ESF at concentration of 100 µg/mL stimulated the lymphocytes proliferation by 30%. However, mild stimulatory effects on lymphocytes proliferation were observed after NESF and HSF treatments.

Our previous studies, ethanolic extract of *F. septica* and its fractions could potentiate the cytotoxic effect of doxorubicin on T47D breast cancer cells (Pratama *et al*., 2011; Nugroho *et al*., 2011). Its fractions in combination with doxorubicin increased the incidence of cells undergoing apoptosis. Its fractions also improved the cytotoxic effect of doxorubicin by changing the inhibition of cell cycle phase G2/M to G1 phase, and stimulating the cleaved-PARP expression in T47D cells (Nugroho *et al*., 2012; Nugroho *et al.*, 2013a). The agent combined with chemotherapeutic agents to produce better effects is named co-chemotherapeutic agent. Since the use of doxorubicin could induce cardiotoxicity and suppress the immune functions (Zhang *et al.*, 2005a; Bowles *et al*., 2012), investigation of possible immunomodulatory effect of co-chemotherapeutic agent is interesting.

In the study, ethanolic extract of *F. septica* was fractionated to yield *n*-hexane soluble, non-ethyl acetate and ethyl acetate soluble fractions (HSF, NESF, ESF). Among them, ESF contained highest total phenolic content. In line with this result, qualitative phytochemical analyses with TLC showed the presence of phenolics and flavonoids in ESF and NESF, however not found in HSF (data not shown). The content of phenolic depends on the polarity of solvent used in fractination. Phenolic compound is highly soluble in polar solvent such as ethyl acetate resulting in high concentration of phenolic compounds in ESF. Besides, ESF also contained highest total flavonoid content. Some flavonoids contain one or more hydroxyl moieties which can be soluble in polar solvent.

ESF exhibited highest immunomodulatory effects regarding to macrophage phagocytosis and lymphocytes proliferation. Its high immunomodulatory effect is related to high content of phenolic compounds. The phenolic compounds are potential antioxidant (Rice-Evans *et al.*, 1997). Phenolic compounds are characterized by aromatic rings possessing one or more hydroxyl moieties responsible for their antioxidative effects. Antioxidative activity is closely related to the immunomodulatory activity. Changes in cellular oxidant status provides a stress to immune system cells (Krifa *et al*., 2012). The antioxidative action promotes redox-sensitive pathways responsible to control immune cell function (Ramiro-Puig and Castle, 2009).

Macrophage has a main role in non-spesific defence (innate immunity) and spesific defence (adaptive immunity) in vertebrate animals. The cells mainly function in immune system as phagocytic cells. The cell have a main role to phagocytose, to digest the celluler debris and dead cells as well as pathogens. Besides, the cells can stimulate other immune components including cytokines and lymphocytes to respond to antigens or pathogens (Rang *et al*., 2003; Shibata and Glass, 2009; Shalhoub *et al.*, 2011). In the study, the fractions especially ESF stimulated the activity of macrophage phagocytosis. Its stimulatory effect on macrophage phagocytosis was more potent than this of HSF and NESF. Lymphocyte is a key leucocyte cell responsible in adaptive immune response. The cell can be divided into three main groups : B cells (responsible for antibody production), T cells (induction of immune response and cell-mediated immune response) and NK cells (active in non-immunological, innate response) (Rang *et al*., 2003). In addition, ESF also stimulated the lymphocytes proliferation. Its stimulatory effect on lymphocytes proliferation was also more potent than this of HSF and NESF.

In line with our findings, Wu *et al.* (2002) have identified some flavonoids including genistin, kaempferitrin, and coumarines from leaves of *F. septica.* In addition, Yang *et al.* (2005) reported anti-inflammatory effect of *F. septica* leaves extract through inhibition of inducible nitric oxide synthase (iNOS) and cyclooxigenase-2 (COX-2). Induction of iNOS occurs in an oxidative condition. The iNOS is responsible to produce NO, a highly reactive free radical, in inflammation and multiple stages of carcinogenesis (Wong *et al*., 2001; Lo *et al.*, 2002). High level of NO can cause cell toxicity (Rang *et al*., 2003).

Reportedly, some flavonoids and polyphenols exhibited anticancer and immunomodulatory activities (Zhang *et al*., 2005b; Ghiringhelli *et al.*, 2012). Previous *in vivo* study, the combination of doxorubicin and proanthocyanidin potently improved the anti-tumour effect of doxorubicin and the immune responses. In addition, this combination suppressed myocardial oxidative

stress on doxorubicin-induced rats (Zhang *et al*., 2005a). *In vivo* study, apigenin exhibited to decrease doxorubicin-induced genotoxicity, and decrease the genotoxicity on cyclophosphamide-induced rats to inhibition of chemotherapeutics metabolic activation (Bokulic *et al.*, 2011). Quercetin is reported to induce lymphocyte proliferation and regulate imbalance of Th1/Th2. Its combination with doxorubicin induced persistent T-cell tumor-specific responses in breast cancer mouse model (Du *et al*., 2010).

Conclusion

Based on the results, it can be concluded that ethyl acetate soluble fraction of ethanolic extract of *F. septica* that contains phenolics and flavonoids exhibited immunomodulatory effects regarding to macrophage phagocytosis and lymphocytes proliferation. This fraction will be a good candidate as a co-chemotherapeutic agent acting to stimulate the immune function.

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