

## ANTIMICROBIAL ACTIVITIES OF ANDROGRAPHOLIDE AND PROPOLIS AGAINST INTRACELLULAR *MYCOBACTERIUM TUBERCULOSIS* PHAGOCYTOSED BY MONOCYTES DERIVED MACROPHAGES

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### ABSTRACT

*One of the major health problems worldwide is Tuberculosis (TB). According to WHO report, Indonesia has the third highest TB prevalence. Due to the fact of multidrug resistance, there is an urgent need for more potent antibiotics and other drugs. Natural products have been regarded as one of the most successful strategies for creating new medicines. The plant andrographolide and propolis is a superb immune system enhancer in TB, and might be more effective when combined to achieve an higher effect. Macrophages are central effector cells of host defence mechanisms against mycobacteria. However, M. tuberculosis uses macrophages as its preferred habitat. It is not much known how macrophages either kill M. tuberculosis or become its breeding ground. In the present study we investigated the co-stimulant action of Andrographis paniculata and propolis associated with bactericidal activity on macrophages. Incubation of monocytes derived macrophages of healthy participants with the extract of A. paniculata and propolis activated bactericidal activity against intracellular Mycobacterium tuberculosis, but no significant effect on the macrophages of tuberculosis patient. These findings suggest that andrographolide and propolis may have a limited effect on bactericidal activity in tuberculosis patient.*

**Keywords :** andrographolide, propolis, macrophages, tuberculosis

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### INTRODUCTION

Tuberculosis (TB) remains a major worldwide health problem. The WHO report noted that Indonesia has the third highest prevalence of TB, after China and India. The estimated numbers of new TB cases in China, India and Indonesia were 1,828,000, 1,414,000 and 591,000 cases, respectively in 1998 (Subronto 2002).

Macrophages are central effector cells of host defence mechanisms against mycobacteria, but *M. tuberculosis* uses macrophages as its preferred habitat. Activated macrophages can control the growth of the microbe, although sterile eradication is seldom achieved. Little is known regarding how macrophages either kill *M. tuberculosis* or become its breeding ground. Macrophages are capable of destroying microorganisms, including oxygen ( $H_2O_2$ ) and nitrogen (NO) intermediate metabolite mechanisms. Patients with active TB had strongly depressed IFN- $\gamma$  production, and may cause the failure of macrophages to produce  $H_2O_2$  and NO (Kaufmann 2001).

TB has become a reemerging disease. Major factors that have aggravated the spread of TB are co-infection with

HIV, and development of multi drug resistant bacilli. The multidrug-resistance is typically a result of inadequate drug therapy. There seems to be an urgent need for more potent antibiotics and other drugs to possibly reduce the duration of treatment. Immunomodulating agents may have potential in some treatment regimens. The use of natural products has been one of the most successful strategies for the discovery of new medicines (Yildirim 2004).

Propolis is a resinous hive product collected by honeybees *Apis mellifera* from various plant sources. Propolis exhibits several biological activities, such as antimicrobial, antiinflammatory and hepatoprotective. In tuberculosis, granuloma formation was more prominent in the group treated with propolis than the isoniazid group. Propolis inhibited Mycobacterium activity in the samples and did not inhibit the effectiveness of tuberculosis drugs. Propolis has a moderate growth inhibitory activity against *M. tuberculosis* and has a synergistic effect with streptomycin, rifamycin, isoniazid and ethambutol. Preliminary results show that 10  $\mu$ g/ml propolis induced an elevation in  $H_2O_2$  production, but no significant

alterations in NO production (Duarte 2003; Valcic 1999; Yildirim 2004).

*A. paniculata* (sambiloto) also known commonly as "King of Bitters," is one of herbs reputed to be effective to be treatment of cold, fever and inflammation. It grows abundantly in Indonesia. Andrographolide, an active component of this plant, has immunostimulative activity, anti-inflammatory, antibacterial, antipyretic, expectorant and hepatoprotective. Its role on proliferation of human lymphocytes and on production of Th1 cytokines (INF- $\gamma$  and TNF- $\alpha$ ) were determined in vitro. Nitric oxide synthase is induced by Th1 cytokines, while Th1 cytokines induced by andrographolide. Thus, andrographolide appears to be a promising source that may induces NO release in macrophages (Batkhoo 2002).

Taken together, these data suggest that both andrographolide and propolis is a superb immune system enhancer in TB, and might be more effective when combined to achieve an higher effect. First line drugs of TB consists of a combination of drugs designed to achieve maximum result. Dangerous side effects, such as hepatotoxic, can develop or become worse in patients. Earlier work showed that andrographolide and propolis has anti-hepatotoxic activity and might be helpful when combine with antituberculosis drugs. Many compounds do not penetrate the blood-brain barrier. However, andrographolide does so and concentrates in the brain and particularly in the spinal cord. Its benefit for meningitis TB (Panossian 2002).

In this study we investigated the effect of andrographolide and propolis that might have complementary and synergic effects on TB therapy especially on bactericidal activity of macrophages.

## MATERIALS AND METHODS

### Extraction of *A. paniculata*

The dried leaves (300 g) were macerated in methanol and kept at room temperature for 3 d. After filtration, the methanol solution was evaporated under reduced pressure to give the methanol extract (20.5 g). The water soluble was extracted with *n*-butanol. Filtration of the precipitate formed in the *n*-butanol gave crude andrographolide (1.96 g) and chromatographed on a silica gel column using chloroform/methanol as a solvent to yield pure andrographolide (6.19 mg). 10  $\mu$ g andrographolide dissolved in 1ml RPMI before use (Batkhoo 2002).

### Propolis

Commercial product 20% Standardised Purified Propolis Resin were obtained from High Desert - USA.

### Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors and tuberculosis patient. Allow the tube of anticoagulated blood (10 ml) to stand in a vertical position for 20 min at room temperature until most of the RBCs have settled but the WBCs are contained in the plasma. Layer the WBC-rich plasma over the 7.5 ml of Ficoll Hypaque. Centrifuge at 900 x g for 10 min at room temperature. Collect the mononuclear WBCs.

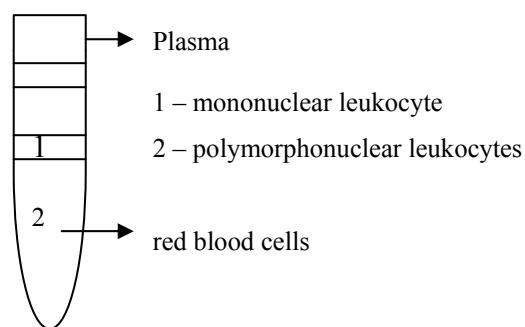


Figure 1. Separation of mononuclear WBCs and PMNs into two fractions after centrifugation

Prepare 0.1 ml of the cell suspension in a 0.4% solution of trypan blue 0.9 ml for viable cell counts (unstained cells). Cell counting with a hemacytometer. PBMC were seeded at a density of  $4 \times 10^5$  cells/well on 24 well tissue culture plates and allowed to adhere (incubated for 2 h) in humidified CO<sub>2</sub> incubator. Non-adherent cells were gently removed twice with fresh medium. Remaining adherent cells (>95% macrophages) were culture for 6 days to enable differentiation into macrophages with 1000  $\mu$ l RPMI 1640 medium containing 5% heat-inactivated fresh pooled human serum, 25mM HEPES, 2 mM L-glutamine and 100 U/ml penicillin. For treated cells, macrophages were treated with 10  $\mu$ g/ml andrographolide and 10  $\mu$ g/ml propolis. After overnight treatment, cells were washed twice and fresh medium was added.

### Organisms

*Mycobacterium tuberculosis* H37Rv ATCC 27294 and *M. tuberculosis* clinical isolates were cultured in Lowenstein Jensen medium. Pick 3-5 colonies, and emulsify them in 4 ml of PBS in a glass tube to which 6

sterile glass beads have been added. Vortex, allow the tube to stand, so the larger particles can settle. Transfer supernatant to sterile glass tube, add 4500 µl of PBS. Vortex and centrifuge at 3500 rpm, 4°C for 10 min. Bacteria were opsonized as follows: resuspend the cell pellet in 1 ml of RPMI containing 10% human serum and rocked for 20 min at 37°C. Following incubation, organisms were washed twice and fresh RPMI was added Adjust the turbidity to no. 1 McFarland standard (contains approximately  $10^7$  CFU/ml).

### Antimicrobial activity

Adherent macrophages were infected with viable *M. tuberculosis*. Remove 100 µl medium and replace with RPMI 1640/10% human serum containing  $2 \times 10^6$  CFU of *M. tuberculosis*. After 2 h incubation at 37°C, extracellular bacteria were removed by washing three times with RPMI. The infected macrophages were

cultured for up to 7 days in the presence or absence andrographolide and propolis. Evaluations for efficacy were performed day 1, 3, and 7 of the treatment. 200 µl of the macrophages suspension were lysed (washed with PBS by centrifugation 1000 rpm at 4°C. Ad pellet with 10 ml aquabidest, incubated for 30 min at 4°C and then vortex). 50 µl of suspension were cultured and the numbers of residual bacterial colony forming unit (cfu) in the resultant samples were counted after 2–6 week cultivation (Sato 1998).

### RESULTS

All data are expressed as the mean  $\pm$  the standard deviation (SD). Statistical analysis was performed using Student's t test. and the differences were considered significant if  $p < 0.05$ .

Table 1. The effects of the andrographolide and propolis on the bactericidal activity

Incubation time (days)	Means (x $\pm$ SD) phagocytosed intracellularly growing <i>M. tuberculosis</i> (CFU/ml)							
	None added				+ 10 µg/ml andrographolide and 10 µg/ml propolis			
	Healthy Person		Tuberculosis Px		Healthy Person		Tuberculosis Px	
	ATCC 27294	Clinical isolate	ATCC 27294	Clinical isolate	ATCC 27294	Clinical isolates	ATCC 27294	Clinical isolates
1	4050.0 $\pm$ 210.0	5003.3 $\pm$ 802.3	4000.0 $\pm$ 511.6	4008.3 $\pm$ 897.8	2823.3 $\pm$ 367.3	3466.6 $\pm$ 847.7	3806.6 $\pm$ 685.3	3603.3 $\pm$ 302.8
3	2516.6 $\pm$ 401.2	3950.0 $\pm$ 378.1	3090.0 $\pm$ 547.9	3028.3 $\pm$ 618.4	1940.0 $\pm$ 35.7	2210.0 $\pm$ 739.2	2908.3 $\pm$ 593.2	2816.6 $\pm$ 651.3
7	2420.0 $\pm$ 384.2	3003.3 $\pm$ 493.0	3013.3 $\pm$ 510.0	2950.0 $\pm$ 956.0	1093.3 $\pm$ 109.3	2080.0 $\pm$ 114.5	2241.6 $\pm$ 74.5	1983.3 $\pm$ 87.1

*Mycobacterium tuberculosis* H37Rv ATCC 27294 and *M. tuberculosis* clinical isolates, were used for antibacterial assay. The present study shows that, the clinical isolates was more resistant to andrographolide and propolis than the laboratory strain (figure 2). This observation is important because the laboratory strain, which has been commonly used to determine susceptibility to antimicrobial, may not express the same virulence or resistance level compared with strain recently isolated from clinic.

Andrographolide and propolis caused significant killing of intracellularly growing *M. tuberculosis* (IG-Mtb) in healthy person ( $p < 0.05$ ) (figure 3 and 4). However, its efficacy in killing IG-Mtb was less than that in TB patients (figure 5 and 6). Andrographolide and propolis had a weak bactericidal effect in TB patients but failed to reach statistical significance by the t-test ( $p > 0.05$ ).

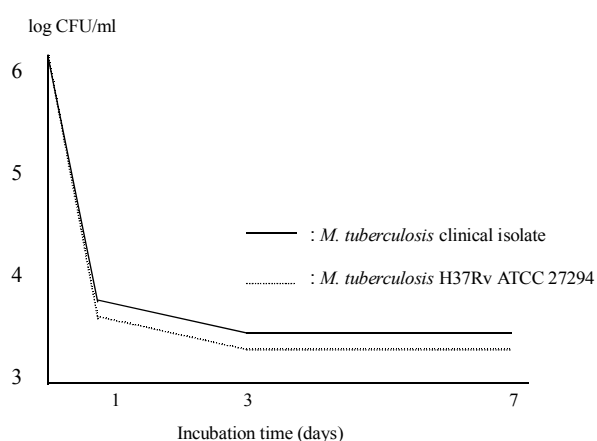


Figure 2. Antimicrobial activity of andrographolide and propolis against phagocytosed intracellularly growing *M. tuberculosis*

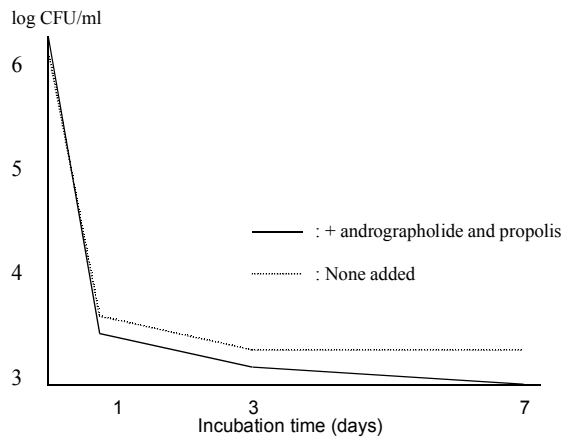


Figure 3. Andrographolide and propolis accelerate degradation of *M. tuberculosis* H37Rv ATCC 27294 in healthy person

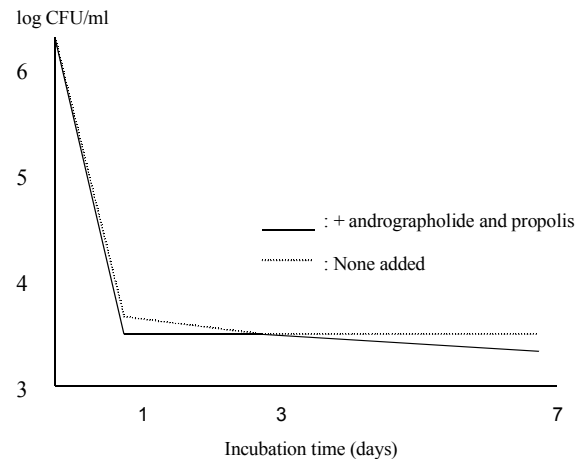


Figure 6. Effect of andrographolide and propolis against intracellular *M. tuberculosis* clinical isolates in tuberculosis patient

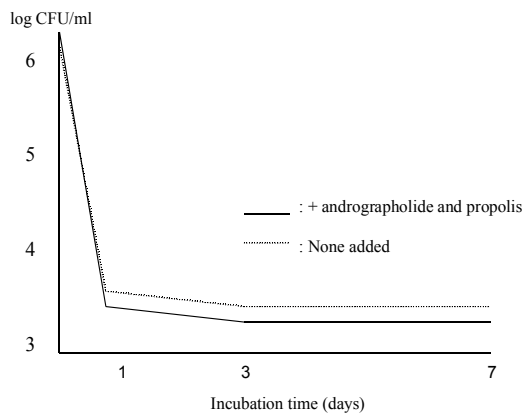


Figure 4. Andrographolide and propolis accelerate degradation of *M. tuberculosis* clinical isolates in healthy person

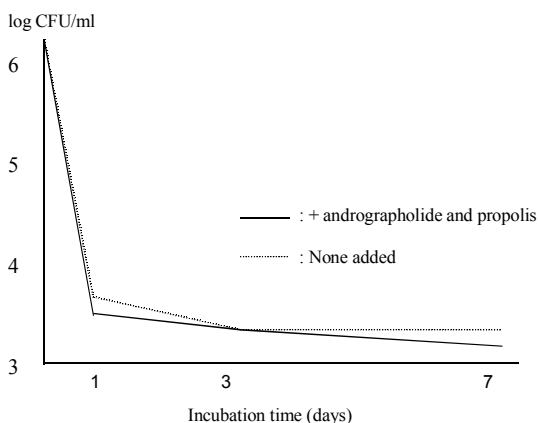


Figure 5. Effect of andrographolide and propolis against intracellular *M. tuberculosis* H37Rv 27294 in tuberculosis patient

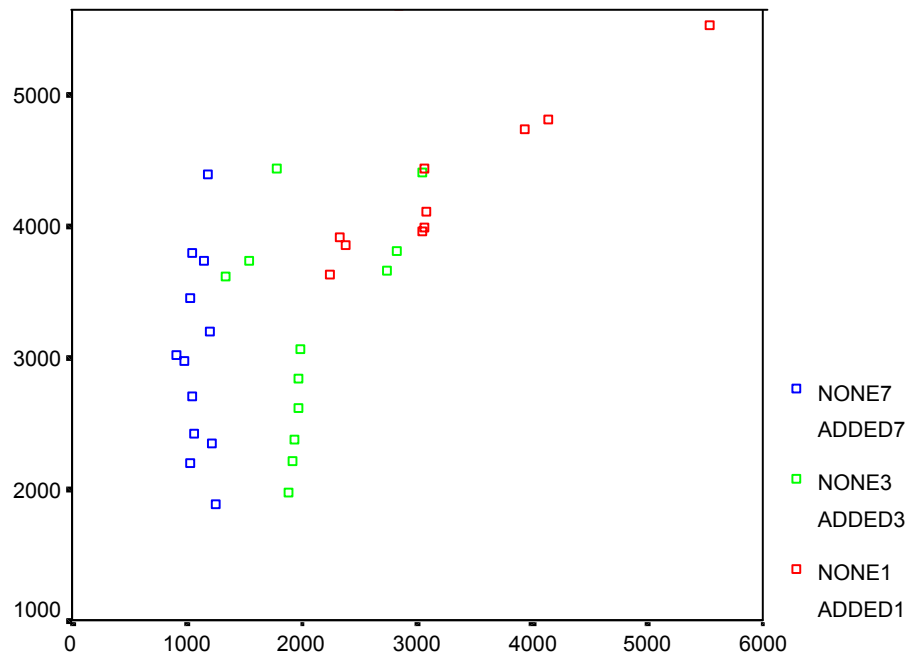
Evaluations for efficacy were performed day 1, 3, and 7 of the treatment. As shown in figure 7, at day 1 of treatment a significant decrease of bacteria as compared with day 3 and 7. Pearson Correlation at day 1 is 0,370, day 3 is 0,336 and day 7 is 0,061. This finding confirms a previous observation that andrographolide and propolis action on the immune system has a short-term effect. Its immune-boosting activity achieved when gave continuous.

## DISCUSSION

When mycobacteria exposed to macrophages, within minutes, mycobacteria adhere to the macrophage surface. Initiate signal transduction, are internalized, and take up residence. Following infection, the macrophages undergo morphological changes, often assuming giant cells and demonstrating highly interactive membranes (figure 8a), compared with uninfected cells (figure 8b).

The data obtained in the present study showed that andrographolide and propolis activated bactericidal effect of macrophages. Interestingly, only macrophages of healthy person activity, not macrophages of TB patients. The reasons for failure to predict outcome in TB patients are unclear. The mechanism that may contribute to this, might be an interesting secreted Ag85.

Among various *M. tuberculosis* products, proteins that are actively secreted into the culture medium are currently of particular interest. Ag85 is a protein family involved in cell wall synthesis. The Ag85 induces strong T-cell proliferation and IFN- $\gamma$  production in most healthy individuals infected with *M. tuberculosis*, but not in TB patient (Subronto 2002).



## CONCLUSION

This study indicated that andrographolide and propolis are used mainly in the prevention and treatment and may have great promise for close contact TB. Andrographolide and propolis is less effective than antibiotics but less toxic, microbes do not develop resistance to it, and it doesn't suppress the normal flora. The natural elements tend to work slowly but harmonise with the body's own immune defences and are more decisive in the end.

## REFERENCES

- Batkhuu, J, Koichi, H, Takano, F, Fushiya, S, Oshiman, K & Fujimiya, J 2002, 'Suppression of NO production in activated macrophages in vitro and ex vivo by neoandrographolide isolated from *Andrographis paniculata*', *Biol. Pharm. Bull.*, vol. 25, no. 9, pp. 1169-1174.
- Duarte, S, Koo, H, Bowen, WH, Hayacibara, MF, Cury, JA, Ikegaki, M & Rosalen, PL 2003, 'Effect of a novel type of propolis and its chemical fractions on glucosyltransferases and on growth and adherence of mutants *Streptococci*', *Biol. Pharm. Bull.*, vol. 26, no. 4, pp. 527-531.
- Kaufmann, SHE 2001, *How can immunology contribute to the control of tuberculosis?* [www.nature.com/reviews/immunol](http://www.nature.com/reviews/immunol) 1 : 20-30.
- Keane, JHG, Remold, H & Kornfeld 2000, 'Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages', *J. Immuno.*, vol. 164, p. 2016.
- Mehta, PK, King, CH, White, EH, Murtagh, JJ & Quinn, FD 1996, 'Comparison of in vitro models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication', *Infection and Immunity*, vol. 64, pp. 2673-9.
- Nagaoka, T, Banskota, AH, Tezuka, Y, Midorikawa, K, Matsushige, K & Kadota, S 2003, 'Caffeic Acid Phenethyl Ester (CAPE) Analogues: Potent Nitric Oxide Inhibitors from the Netherlands Propolis', *Biol. Pharm. Bull.*, vol. 26, no. 4, pp. 487-491.
- Panossian, A, Davtyan, T, Gukassyan, N, Gukasova, G, Mamikonyan, G, Gabrielian, E & Wikman, G 2002, 'Effect of andrographolide and Kan Jang-fixed combination of extract SHA-10 and extract SHE-3-on proliferation of human lymphocytes, production of cytokines and immune activation markers in the whole blood cells culture', *Phytomedicine*, vol. 9, no. 7, pp. 598-605.
- Sato, K, Akaki, T & Tomioka, H 1998, 'Antimicrobial activities of benzoxazinorifamycin KRM-1648, clarithromycin and levofloxacin against intracellular *Mycobacterium avium* complex phagocytosed by murine peritoneal macrophages', *Journal of Antimicrobial Chemotherapy*, vol. 41, pp. 77-83.
- Scheller, S, Dworniczak, S, Waldemar-Klimmek, K, Rajca, M, Tomczyk, A & Shani, J 1999, 'Synergism between ethanolic extract of propolis (EEP) and anti-tuberculosis drugs on growth of mycobacteria' *Z Naturforsch [C]*, vol. 54, no. 7-8, pp. 549-53.
- Subronto, YW 2002, *Antigen Recognition and Immunopathogenesis of Tuberculosis: Clinical Studies in Yogyakarta, Indonesia*, Proefschrift, KNAW.
- Yildirim, Z, Hacievliyagil, S, Kutlu, NO, Aydin, NE, Kurkcuglu, M, Iraz, M & Durmaz, R 2004, 'Effect of water extract of Turkish propolis on tuberculosis infection in guinea-pigs', *Pharmacol. Res.*, vol. 49, no. 3, pp. 287-92.