PHYTOTHERAPY RESEARCH
Phytother. Res. (2016)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5571

Immunomodulation in Middle-Aged Humans Via the Ingestion of LJ100/Physta® Standardized Root Water Extract of *Eurycoma longifolia* Jack —A Randomized, Double-Blind, Placebo-Controlled, Parallel Study

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This study was aimed to investigate the capacity of a standardized root water extract of *Eurycoma longifolia* (Tongkat Ali, TA), LJ100/Physta® to modulate human immunity in a middle-aged Japanese population. This random-ized, double-blind, placebo-controlled, parallel study was conducted for 4 weeks. Eighty-four of 126 subjects had relatively lower scores according to Scoring of Immunological Vigor (SIV) screening. Subjects were instructed to ingest either 200 mg/day of TA or rice powder as a placebo for 4 weeks [TA and Placebo (P) groups] and to visit a clinic in Tokyo twice (weeks 0 and 4). SIV, immunological grade, immunological age, and other immune param-eters were measured. Eighty-three subjects completed the study; 40 in the TA group and 41 in the P group were statistically analyzed, whereas two were excluded from the analyses. At week 4, the SIV and immunological grade were significantly higher in the TA group than those in P group ($p < 0.05$). The numbers of total, naïve, and $CD4^+$ T cells were also higher in the TA group than those in P group ($p < 0.05$). No severe adverse events were observed. The results suggest that ingestion of the root water extract of TA (LJ100/Physta®) enhances compre-hensive immunity in both middle-aged men and women. This study is registered in UMIN-CTR (UMIN000011753).

Keywords: Tongkat Ali; eurycomanone; comprehensive immunity; Scoring of Immunological Vigor.

INTRODUCTION

The tree *Eurycoma longifolia* Jack, classified as Simaroubaceae of Sapindales and also known as Tongkat Ali (TA), originally comes from Southeast Asia, such as Malaysia, Vietnam, Java, and Thailand (Bhat and Karim, 2010). TA includes a large number of compounds such as eurycomanone, glycosaponin, and polysaccharides, etc. (Kuo *et al.,* 2003). The roots of TA have traditionally been used as a tonic, energy enhancer, and aphrodisiac (Gimlette and Thomson, 1977; Ismail *et al.,* 1999; Jagananth and Ng, 2000; Bhat and Karim, 2010). Subsequent scientific researches into TA supplementation revealed testosterone hormone modulation, especially in hypogonadic men, increased muscle strength and size, recovery from fatigue, and improved moods (Tambi *et al.,* 2011; Hamzah and Yusof, 2003; Talbott *et al.,* 2006; Talbott *et al.,* 2013). Previous *in vitro* studies have revealed the anticancer effects of TA-derived quassinoids, including eurycomanone, against colon, breast, lung, and skin cancers (Tada *et al.,* 1991; Zakaria *et al*., 2009; Wong *et al.,* 2012). In addition, extracted TA has antioxidative effects (Varghese *et al.,* 2013). These cancer-suppressing and antioxidative effects of TA suggest that its ingestion enhances immune functions in humans.

The immune system protects the host from infections by pathogenic organisms (e.g. bacteria and viruses) and cancer growth. Humans possess both innate and adaptive immunity (Murphy, 2014). The innate immunity serves as a defense system against pathogens and cancers, beginning at the first infection or occurrence in the human body. In contrast, adaptive or acquired immunity results in the formation and storage of immune memory of a pathogen following infection for subsequent possible occurrence of the same infection (Murphy, 2014). Immunological functions

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Received 8 May 2015
Revised 15 December 2015
Accepted 16 December 2015

decrease with factors such as aging, stress, and infection, rendering the host vulnerable to infectious diseases. However, a comprehensive evaluation of human immunity has remained difficult, because many immune determinants are associated with the defense system depending on the situation and are evaluated as a whole, rather than individually (Hirokawa *et al.,* 2009).

A patented immune evaluation method, the Scoring of Immunological Vigor (SIV), was proposed by Utsuyama *et al.* (2009) and Hirokawa *et al.* (2009). The use of SIV can objectively evaluate human's immunity with eight immune parameters which are easily affected by aging, stress and illness. SIV is the sum of eight immunological functional scores with three-point grades: numbers of total T cells, naïve T cells and $CD8^+CD28^+$ T cells, NK cells, and B cells; ratios of $CD4^+/CD8^+$ T cells ($CD4^{+/}CD8^+$) and naïve/memory T cells; and the T cell proliferative index (TCPI). SIV was derived from a database of 300 Japanese people (Utsuyama *et al.,* 2009) and currently from a larger database. The values of these immune parameters were standardized by assigning scores of 3 (high), 2 (moderate), or 1 (low) (Utsuyama *et al.,* 2009). SIV has already been used to evaluate the immunomodulating influences of supplements and fruits in a Japanese population (Fujii *et al.,* 2011; Suzuki *et al.,* 2012). As SIV is calculated using multiple factors, it can describe 'comprehensive immunity' by considering eight parameters and not interpreting each immune function separately. In addition, it can assess the extent of age-related decreases in immunological functions. SIV was classified to five immunological grades according to the total score; (Grade V: sufficiently high zone; Grade IV: safety zone; Grade III: observation zone; Grade II: warning zone; Grade I: critical zone). The observation zone shows mean level of the SIV, but should be required to move up into the higher zone. The warning zone indicates that the level of SIV is not enough to keep health and significant effort is needed to improve SIV. The critical zone indicates that the susceptibility to infections is so high that the individual might easily get illness (Utsuyama *et al.,* 2009). Immunological age is another easily understood immune parameter. This parameter is calculated based on the relationship between age and the TCPI, which is derived from the T cell proliferation activity and number (Utsuyama *et al.,* 2009).

The objective of this study was to evaluate whether the ingestion of a propriety water extract of TA root would comprehensively enhance immune functions. This trial was designed as a randomized, double-blind, placebo-controlled, parallel study of Japanese adults aged 40–59 years. Participants were instructed to ingest either 200 mg of the water extract of TA or rice powder as a placebo for 4 weeks, and they visited the clinic twice (weeks 0 and 4) where their SIV and other immunological factors were measured.

MATERIALS AND METHODS

Subject recruitment. This study was approved by the ethics committee of Seishinkai Medical Association Inc., Takara Medical Clinic (Tokyo, Japan) prior to initiation and took place at Takara Medical Clinic from August to December 2013. Subjects were recruited using the recruitment site Go106 (http://www.monitor-touroku.jp/). All subjects provided and signed the informed consent before undergoing screening procedures.

Subjects were between 40 and 59 years old and were required to fulfill the following inclusion criteria: good health and answered 'Yes' to the screening question of 'Do you usually feel fatigued?' The exclusion criteria for the study were as follows: (i) any previous medical history of heart failure and cardiac infarction; (ii) treatment for one or more of the following diseases: atrial fibrillation, cardiac arrhythmia, hepatic disorder, renal disorder, cerebrovascular disorder, rheumatism, dyslipidemia, hypertension, or other chronic disease; (iii) use of medicines, herbal medicines, or dietary supplements within 30 days before providing informed consent; (iv) any allergies; (v) pregnancy, lactation, or plans to become pregnant during the trial period; (vi) pollinosis; (vii) current smoking history; (viii) enrollment in any other clinical trials within 3 months before providing informed consent; and (ix) investigator's determination of unsuitability for trial participation. The clinical investigation was conducted in accordance with the Declaration of Helsinki. The study has been registered on UMIN-CTR (http://www.umin.ac.jp/, UMIN000011753).

Study design. A randomized, placebo-controlled, double-blind, parallel group, single-centered study was conducted for 4 weeks. Subjects visited the clinic twice: once before starting treatment (week 0) and once at 4 weeks after ingestion (week 4). After fulfilling all inclusion and exclusion criteria, subjects with comparatively lower SIVs (see details in 'Outcome Measurements') at week 0 were recruited from among the 126 screened individuals. Recruited subjects were randomized to either the TA group (42 subjects) or Placebo (P) group (42 subjects).

Intervention. TA capsules included 200 mg of a standardized *E. longifolia* water-soluble TA root extract (LJ100/Physta®) and 30 mg of fatty acid sucrose esters in each hard gelatin capsule. P capsules each included 200 mg of rice powder instead of LJ100/Physta®. The LJ100/Physta® TA extract, which was standardized to contain 0.8%–1.5% eurycomanone, >40% glycosaponin, >30% polysaccharide, and >22% protein, was supplied by Biotropics Malaysia Berhad (Shah Alam, Selangor, Malaysia). Both TA and P capsules contained polysaccharides and were manufactured under Good Manufacturing Practices (GMPs) in Japan. Subjects were instructed to take one capsule of TA or P each day. Subjects were instructed to maintain their regular lifestyle until week-4 visit, including dietary and drinking habits.

Randomization. Stratified randomization sequences were created with computer-generated random numbers using the Statlight #11 program (Yukms Co., Ltd., Tokyo, Japan) on Microsoft Excel 2007 (Microsoft Japan Co., Ltd., Tokyo, Japan). The program allocated subjects based on age (40s/50s) and sex (male/female) into four groups; 40s male, 50s male, 40s female, and 50s female. Sex and age demographic stratifications were set and crossed. Finally,

the results of these four randomizations were combined and assigned as the final randomization sequence for this trial. Allocation and enrollment were performed by the staff of ORTHOMEDICO Inc., who did not perform any analyses or clinical procedures. The allocation information was disclosed to the investigator, subjects, and a statistician after all measurements were completed.

Outcome measurements. The primary endpoints of this study were the following immune parameters: numbers of neutrophil, lymphocyte, total T cells ($CD3^+$ cells), $CD4^+$ T cells, $CD8^+$ T cells, $CD8^+CD28^+$ T cells, naïve T cells ($CD4^+CD45^+$ cells), memory T cells ($CD45^+CDRO^+$ cells), B cells ($CD20^+$ cells), and NK cells ($CD16^+CD56^+$ cells); ratios of $CD4^+/CD8^+$ T cells and naïve/memory T cells; T cell proliferative activity, TCPI, immunological age, T lymphocyte age, immunological grade and SIV. TCPI was calculated by the following equation:

$$\text{TCPI} = \text{T cell proliferative activity} \times \left(\text{T cell number per mm}^3/1000\right).$$

Immunological age was determined from the average and standard deviation of the pre-immunological age. The pre-immunological age was calculated by the following regression equation of the TCPI and age:

$$\text{Immunological age} = (2.535 - \text{TCPI})/0.017.$$

T lymphocyte age was calculated by the equation between chronological age and $CD8^+CD28^+$ T cell count. SIV, immunological grade, immunological age, and T lymphocyte age comprised the comprehensive immune functions. All measurements and calculations of the above immunological parameters were outsourced to the Institute of Health and Life Science (Tokyo, Japan) (Hirokawa *et al.,* 2009; Utsuyama *et al.,* 2009).

An analysis of moods was performed as a secondary endpoint by evaluating the Profile of Mood States (POMSs, Japanese, brief version) (Yokoyama *et al.,* 1990), which measures six factors including tension/anxiety, depression, anger/hostility, vigor, fatigue, and confusion. Another secondary endpoint was safety (items for safety are described in Table 3 and Supplementary Tables 1 and 2). Blood measurements of 18 hematologic and 31 biochemical parameters were outsourced to Mitsubishi Chemical Medience Co., Ltd. (currently LSI Medience Co., Ltd., Tokyo, Japan). Ten urine and seven somatometric and sphygmomanometric parameters were examined at Takara Medical Clinic.

Sample size estimation and statistical analysis. A sufficient sample size of 42 subjects per group was determined through a power analysis of previous data used to evaluate SIVs (Fujii *et al.,* 2011; Suzuki *et al.,* 2012; unpublished data), with the following settings: statistical power of 80%, significance level of $p < 0.05$, and mean difference in the change in SIV between the two groups of two-thirds the standard deviation (SD), with an allocation ratio of 1:1 and the assumption of an approximate dropout rate of 20% per group. This estimation was performed using the EZR package ver. 1.11 (Kanda, 2013) on R 2.13.0 (2005; R Development Core Team, Vienna, Austria).

Baseline demographics were summarized as means and SDs; age, SIV, immunological age, immunological grade, and T-lymphocyte age were analyzed using Student's *t*-test, whereas the proportions of male subjects and those in their 40s were analyzed using the χ^2 test. The treatment effect (TA versus P) was evaluated for each endpoint using an analysis of covariance model adjusted for each week 0 value, sex, and age. A paired *t*-test was used to compare the weeks 0 and 4 values within groups. All statistical analyses were performed using PASW Statistics 18 (IBM Japan, Ltd., Tokyo, Japan).

RESULTS

Demographics

Subjects were recruited from August 21 through October 18, 2013; initial ingestion ranged from November 4 through 12, 2013 (depending on the subject visits), to week 4 visits from December 2 through 10, 2013. Fig. 1 shows the flow chart of subjects in this study. Eighty-four subjects completed the trial. One subject in the TA group was considered as a dropout by the investigator because this individual had begun vigorous exercise regimen after the week 0 visit. Two participants were excluded from the statistical analyses because one caught a cold during the trial period, which might have caused immune bias, and the other ingested less than 90% of the capsules to be consumed. All of the other 81 participants ingested 90% or more capsules during the study period and did not eat or drink much more than they were already used to, during the study as they were instructed during the informed consent that they were required to continue with the regular lifestyle and not to make severe changes to diet and lifestyle.

The final numbers of subjects in the TA and P groups included in the analyses were 40 and 41, respectively (Table 1). The ratios of male subjects in the TA and P groups were 0.50 and 0.51, and those of subjects in the 40s were 0.58 and 0.61, respectively. The mean ages in the TA and P groups were 48.7 ± 5.3 and 48.1 ± 5.5 years old, respectively. The SIVs in the TA and P groups were 17.9 ± 1.8 and 18.0 ± 1.8 points, respectively. There were no statistically significant differences between the TA and P groups at week 0 (Table 1).

Immunological functions

Immunological outcomes are described in Table 2. A significant difference in SIV was observed between the TA and P groups at week 4 (Fig. 2a, 18.80 ± 2.41 in TA versus 17.95 ± 2.40 in P, $p < 0.05$). In the TA group, SIV significantly increased over the baseline ($p < 0.01$), whereas no significant change in this parameter was observed in the P group after 4 weeks of ingestion. Of the eight subscores in the SIV, the total T cells, TCPI, and naïve/memory T cell ratio increased at week 4 in the TA group, whereas all parameters remained nearly unchanged after 4 weeks of ingestion in the P group (Fig. 3). Moreover, the immunological grade differed significantly between the TA and P groups at week 4 (Fig. 2b, 3.05 ± 0.78 in the TA group versus 2.83 ± 0.54 in the P group, $p < 0.05$). As with SIV, the

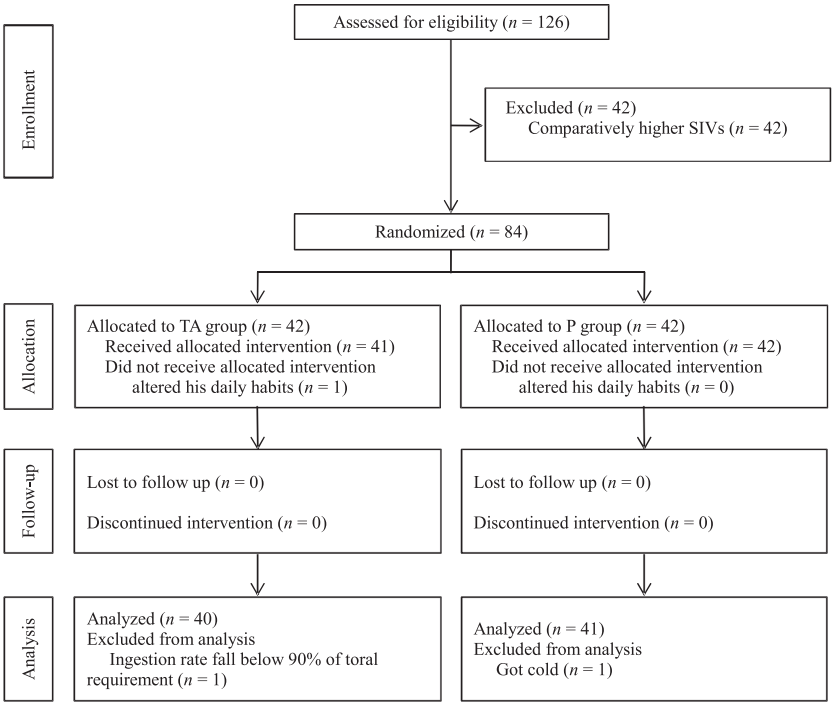

Figure 1. Subject flowchart. Eighty-four of the 126 subjects who participated in the week 0 examination were randomized; 40 in the TA group and 41 in the P group were included as the intention-to-treat population in the final analysis. TA, Tongkat Ali.

Table 1. Demographic information at week 0

	TA	*P*	Significance
Total number	40	41	
Sex ratio (men)	0.5	0.51	1
Ratio aged in 40s	0.58	0.61	0.93
Age	48.7 ± 5.3	48.1 ± 5.5	0.66
SIV	17.9 ± 1.8	18.0 ± 1.8	0.85
Immunological age	54.1 ± 6.8	54.4 ± 7.2	0.83
Immunological grade	2.7 ± 0.5	2.8 ± 0.4	0.42
T-lymphocyte age	51.3 ± 8.2	49.8 ± 8.0	0.41

Age, SIV, immunological age, immunological grade, and T lymphocyte age are shown as means and standard deviations.

immunological grade only increased significantly in the TA group after 4 weeks of ingestion ($p < 0.01$). The immunological grade changed from the warning (Grade II) to the observation zone (Grade III) in the TA group; on the other hand, the one was maintained as the warning zone (Grade II) in the P group. No significant differences in immunological age and T lymphocyte age were observed between the TA and P groups at week 4, although the immunological age significantly decreased in the TA group after 4 weeks of ingestion ($p < 0.001$). A between-group comparison of the week 4 values indicated four other parameters with significance differences: lymphocytes ($p < 0.05$), total T cells ($p < 0.05$), $CD4^+$ T cells ($p < 0.01$), and naïve T cells ($p < 0.05$) (Table 2; Fig. 2c–e).

Analysis of mood by POMS

There was no significant difference between the TA and P groups at week 4, although the tension/anxiety domain score of the TA group was nearly significantly lower than that of the P group ($p = 0.054$). All POMS items changed significantly between weeks 0 and 4 in both the TA and P groups.

Safety and adverse events

Blood and biochemical analyses (Table 3 and Supplementary Table 1), urinalysis, somatometry, and sphygmomanometry (Supplementary Table 2) were performed to evaluate the influence of TA on safety. Although some blood, biochemical, and physical analysis items indicated significant changes between weeks 0 and 4 in both the TA and P groups, all changes were clinically insignificant.

Eight subjects (1 male and 3 females in each group) reported a total of 29 adverse events (AEs) during the ingestion period (Supplementary Table 3), all of which were judged as mild by the investigator. Ten and 19 AEs were reported during 1136 total days in the TA group and 1151 total days in the P group, respectively, and the frequency of reported AEs did not statistically differ between the TA and P groups (odds ratio, 0.529; 95% confidence interval, 0.245–1.14).

DISCUSSION

The present study demonstrated that the ingestion of a standardized *E. longifolia* root water extract, LJ100/Physta®, improved various immunological parameters such as the total T cells, $CD4^+$ T cells, and naïve T cell numbers, as shown in the results, and improved comprehensive immune functions as demonstrated by the SIV and im-munological grade (Table 2; Figs. 2 and 3). The inges-tion of a water extract of TA did not lead to a significant difference in POMS, which measures param-eters related to subjects' moods, when compared with the ingestion of placebo, although improvements in the

Table 2. Outcomes of immunological parameters at weeks 0 and 4

Item		Group	Week 0	Week 4		
Neutrophil		TA	3188.33 ± 1307.64	3154.85 ± 1166.19		
		P	3147.46 ± 1025.83	3317.32 ± 1152.76		
Lymphocyte		TA	1497.40 ± 319.53	1511.98 ± 402.08		#
		P	1517.37 ± 379.79	1403.07 ± 404.20	*	
T cell	/μl	TA	1075.03 ± 227.69	1144.58 ± 319.72	*	#
		P	1082.71 ± 275.54	1031.22 ± 285.61		
$CD4^+$ T cell	/μl	TA	661.68 ± 154.63	741.65 ± 245.46	**	##
		P	658.29 ± 170.63	644.32 ± 187.90		
$CD8^+$ T cell	/μl	TA	395.75 ± 136.77	368.58 ± 132.02	*	
		P	429.59 ± 167.67	362.59 ± 147.77	***	
CD4/CD8 ratio	—	TA	1.85 ± 0.73	2.21 ± 1.01	***	
		P	1.73 ± 0.74	2.07 ± 1.00	***	
Naïve T cell	/μl	TA	231.80 ± 89.96	296.53 ± 142.07	***	#
		P	226.10 ± 71.96	244.29 ± 94.88		
Memory T cell	/μl	TA	429.88 ± 120.82	445.15 ± 158.25		
		P	432.20 ± 151.14	400.02 ± 153.05		
Naive/memory ratio	—	TA	0.58 ± 0.25	0.70 ± 0.32	***	
		P	0.59 ± 0.27	0.70 ± 0.38	**	
$CD8^+CD28^+$ T cell	/μl	TA	247.03 ± 94.41	239.88 ± 87.16		
		P	264.41 ± 102.49	240.59 ± 93.95	*	
B cell	/μl	TA	200.08 ± 91.59	150.85 ± 75.27	***	
		P	213.95 ± 112.89	161.54 ± 100.93	***	
NK cell	/μl	TA	153.15 ± 78.10	155.53 ± 71.54		
		P	156.22 ± 81.79	144.34 ± 78.22		
T cell proliferative index	—	TA	1.52 ± 0.23	1.72 ± 0.22	***	
		P	1.45 ± 0.26	1.70 ± 0.21	***	
T cell proliferative activity	—	TA	1.64 ± 0.45	1.96 ± 0.58	***	
		P	1.57 ± 0.49	1.75 ± 0.52	*	
Immunological age	Years	TA	54.09 ± 6.79	50.43 ± 8.54	***	
		P	54.43 ± 7.22	52.45 ± 7.80	*	
T lymphocyte age	Years	TA	51.30 ± 8.19	51.90 ± 7.50		
		P	49.82 ± 8.01	51.28 ± 7.56	*	
SIV	—	TA	17.93 ± 1.80	18.80 ± 2.41	**	#
		P	18.00 ± 1.76	17.95 ± 2.40		
Immunological grade	—	TA	2.70 ± 0.46	3.05 ± 0.78	**	#
		P	2.78 ± 0.42	2.83 ± 0.54		

*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$ between weeks 0 and 4 within the TA or P group.
#$p < 0.05$;
##$p < 0.001$ between the TA and P groups at week 4. P, placebo; TA, Tongkat Ali; SIV, Scoring of Immunological Vigor.

anxiety/tension domain within the TA group reached near significance ($p < 0.054$).

Immunomodulating effects of TA

This study targeted the middle-aged (40–59 years old) population. It is well known that immune responses decrease in their activity with age (Salam *et al.,* 2013). For instance, Hirokawa *et al.* (2009) clearly reported age-dependent immune dysfunction using the same immune parameters used in this study. Our target population of 40s and 50s are then the population which begin to experience the decline in immune functions and would therefore be potential candidates to investigate for immune improving and restorative properties of intervention.

The numbers of total and naïve T cells were significantly greater in the TA group than those in the P group at week 4 (Table 2; Fig. 2). An increase in T cell numbers represents improved adaptive, or cell-mediated, immunity. Naïve T cells normally undergo thymic involution as a result of aging, causing a decrease T cell repertoire (Salam *et al.,* 2013). In fact, immune system efficiency is said to decline with age through a process known as immunosenescence. A contributing factor to this process is a reduction in the number of naïve T cells (Hawkley and Cacioppo, 2004). However, the treatment with the TA water extract in this study was able to increase the numbers of both total and naïve T cells and lymphocytes. The TA extract might affect immunity through one of two processes. First, the standardized TA water extract, LJ100/Physta® contains a 4.3-kDa peptide (Sambandan *et al.*, 2006; Asiah *et al.,* 2007) (which might also contribute to T cell activation), as small peptides are known to stimu-late T cells (Watts *et al.,* 1985). Second, glucocorticoids such as cortisol, which is also known as a stress hormone, promote immunosenescence by inducing a shift from a $CD4^+$ to $CD8^+$ dominant pattern of immunity (Hawkley

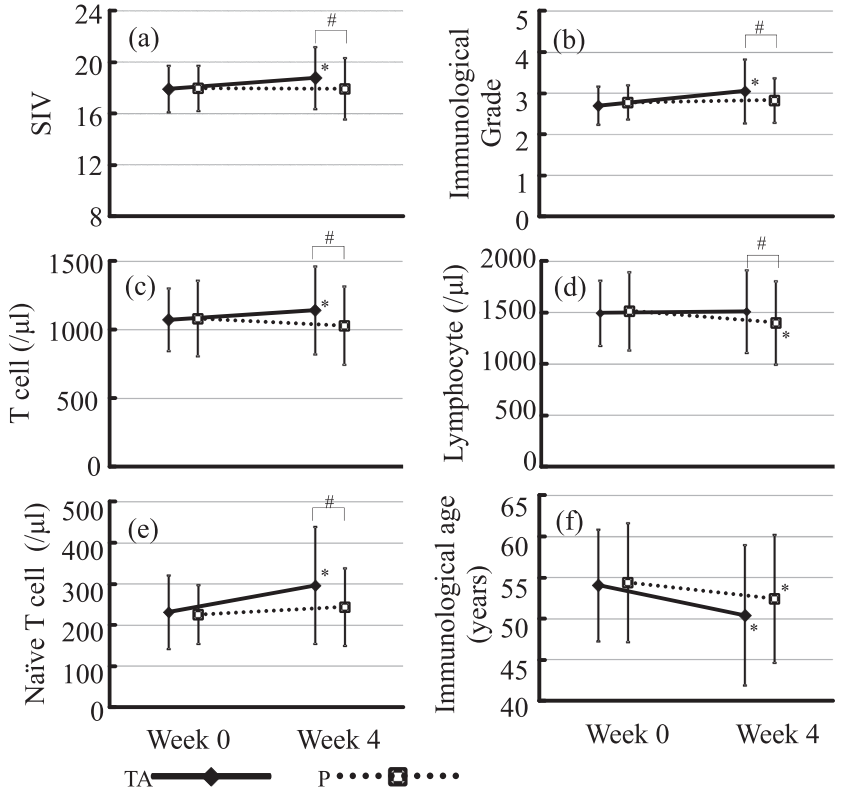

Figure 2. Immunological parameters with differences between and within groups. Six parameters [(a) SIV, (b) immunological age, (c) number of total T cells, (d) number of lymphocytes, (e) number of naive T cells, and (f) immunological grade] showed significant differences. $^{\#}p < 0.05$ between TA and P groups at week 4. $^{*}p < 0.05$ between weeks 0 and 4 within the TA or P group. Solid lines and rhombuses (♦) indicate the TA group and dashed lines and open squares (□) indicate the P group. SIV, Scoring of Immunological Vigor.

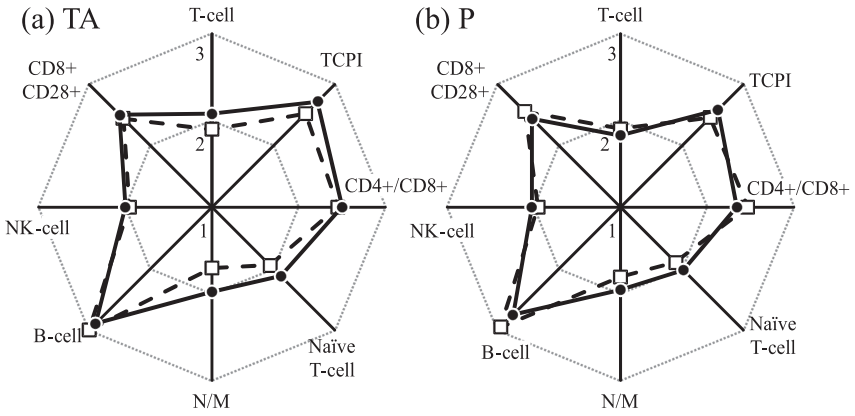

Figure 3. Eight immunological subscores in (a) TA and (b) P groups. Eight immunological subscores in the (a) TA and (b) P groups. Subscores ranged from 1 to 3. Open squares (□) with dashed lines express the mean subscores at week 0, and solid circles (•) with solid lines indicate the mean subscores at week 4. N/M, naive/memory T-cell ratio; TA, TA, Tongkat Ali; TCPI, T-cell proliferative index.

and Cacioppo, 2004). However, with TA supplementation, this dominance may have been reversed. The number of $CD4^+$ T cells in the TA group was significantly higher than that in the P group at week-4. In a report by Talbott *et al.* (2013), with a 4-week supplementation of a TA water extract, the stressed population had reduced cortisol levels and reported fewer symptoms of stress. As a result, the higher number of T cells in this study may have occurred as an indirect effect from possibly lowered cortisol levels. As with Talbott *et al.* (2013) study, an almost significant improvement in the tension/anxiety domain with TA supplementation was also observed.

According to Buford and Willoughby's review (2008), dehydroepiandrosterone (DHEA) opposes the action of the glucocorticoid cortisol, and acts in an immunomodulatory manner by shifting from a $CD8^+$ dominant pattern to $CD4^+$ dominance. This is in alignment with another study where an increase in $CD4^+$ T cells was observed with DHEA supplementation in a mouse model of *Mycobacterium tuberculosis* (Hernandez-Pando *et al.,* 1998). Water extract of TA has been found to increase DHEA levels in humans (Tambi, 2007). DHEA production gradually declines after adolescence while cortisol levels remain unaltered, creating an imbalance between DHEA and cortisol caused during aging. It is suggested that TA supplementation may have led to an increase in the levels of DHEA, thus affecting $CD4^+$ T and naïve T cell levels.

Table 3. Biochemical analyses of safety parameters

Item	Reference value	Group	Week 0	Week 4	
AST	10–40 IU/L/37 °C	TA	19.18 ± 4.48	19.85 ± 4.50	
		P	19.12 ± 5.25	23.15 ± 13.22	**
ALT	5–45 IU/L/37 °C	TA	16.28 ± 6.64	16.93 ± 6.97	
		P	17.80 ± 11.24	22.39 ± 25.02	
y-GTP	M: −80 IU/L/37 °C F: −30 IU/L/37 °C	TA	22.93 ± 15.69	22.60 ± 13.32	
		P	27.78 ± 27.27	28.59 ± 28.30	
ALP	100–325 IU/L/37 °C	TA	183.48 ± 55.67	185.00 ± 54.25	
		P	187.12 ± 69.29	193.41 ± 71.22	*
LDH	120–240 IU/L/37 °C	TA	193.75 ± 24.76	187.20 ± 27.90	*
		P	194.37 ± 26.28	188.37 ± 26.27	*
LAP	37–61 IU/L/37 °C	TA	50.80 ± 7.86	49.00 ± 7.74	***
		P	53.56 ± 10.36	52.32 ± 11.65	**
Total bilirubin	0.2–1.2 mg/dL	TA	0.82 ± 0.24	0.76 ± 0.20	
		P	0.81 ± 0.23	0.82 ± 0.24	
Direct bilirubin	0.0–0.2 mg/dL	TA	0.10 ± 0.05	0.09 ± 0.05	
		P	0.09 ± 0.04	0.09 ± 0.04	
Indirect bilirubin	0.2–1.0 mg/dL	TA	0.72 ± 0.21	0.68 ± 0.16	
		P	0.72 ± 0.20	0.73 ± 0.23	
Cholinesterase	200–452 IU/L/37 °C	TA	315.18 ± 63.73	325.73 ± 69.01	**
		P	305.27 ± 55.72	315.15 ± 63.59	*
ZTT	2.0–12.0 U	TA	7.24 ± 2.76	7.42 ± 2.78	
		P	6.52 ± 2.59	6.96 ± 3.10	*
Total protein	6.7–8.3 g/dL	TA	7.03 ± 0.27	7.07 ± 0.35	
		P	7.11 ± 0.33	7.14 ± 0.33	
Urea nitrogen	8.0–20.0 mg/dL	TA	12.43 ± 3.12	12.13 ± 3.01	
		P	12.36 ± 3.23	12.40 ± 3.46	
Creatinine	0.47–0.79 mg/dL	TA	0.74 ± 0.16	0.70 ± 0.14	***
		P	0.73 ± 0.13	0.72 ± 0.15	
Uric acid	M: 3.8–7.0 mg/dL F: 2.5–7.0 mg/dL	TA	4.91 ± 1.22	4.87 ± 1.29	
		P	4.81 ± 1.34	4.79 ± 1.34	
CK	40–150 IU/L/37 °C	TA	121.50 ± 59.18	108.93 ± 34.18	***
		P	93.98 ± 36.46	117.61 ± 60.12	
K	3.5–5.0 mEq/L	TA	3.86 ± 0.30	4.07 ± 0.38	***
		P	3.84 ± 0.26	4.09 ± 0.37	***
Cl	98–108 mEq/L	TA	102.75 ± 1.71	103.23 ± 1.61	*** #
		P	101.95 ± 2.00	102.44 ± 1.90	
Na	137–147 mEq/L	TA	141.48 ± 1.77	141.00 ± 1.52	
		P	141.44 ± 2.15	140.88 ± 1.44	
Ca	8.4–10.4 mg/dL	TA	9.45 ± 0.44	9.45 ± 0.36	
		P	9.52 ± 0.29	9.49 ± 0.29	
Inorganic P	2.5–4.5 mg/dL	TA	4.05 ± 0.75	3.25 ± 0.46	***
		P	4.10 ± 0.82	3.19 ± 0.43	***
Fe	M: 50–200 mg/dL F: 40–180 mg/dL	TA	95.28 ± 39.99	98.13 ± 41.91	
		P	98.39 ± 32.23	97.00 ± 34.64	
Amylase	40–122 IU/L/37 °C	TA	67.35 ± 21.45	70.33 ± 20.75	
		P	69.83 ± 17.83	79.88 ± 49.34	
Total cholesterol	120–219 mg/dL	TA	201.28 ± 29.99	211.23 ± 32.56	**
		P	201.29 ± 31.51	206.17 ± 33.15	
HDL cholesterol	40–95 mg/dL	TA	68.50 ± 17.52	72.88 ± 20.55	***
		P	68.66 ± 16.88	69.02 ± 17.77	
LDL cholesterol	65–139 mg/dL	TA	114.00 ± 24.42	119.43 ± 30.34	
		P	113.27 ± 27.40	117.22 ± 31.04	
Triglyceride	30–149 mg/dL	TA	86.90 ± 60.97	80.28 ± 42.63	
		P	88.88 ± 46.82	87.29 ± 42.86	
Free fatty acid	0.10–0.90 mEq/L	TA	0.78 ± 0.22	0.60 ± 0.24	***
		P	0.76 ± 0.23	0.68 ± 0.29	
Glucose	70–109 mg/dL	TA	82.68 ± 5.79	82.60 ± 10.27	
		P	84.90 ± 8.00	83.93 ± 8.59	
HbA1c	4.6%–6.2%	TA	5.39 ± 0.30	5.38 ± 0.29	
		P	5.33 ± 0.23	5.35 ± 0.26	
Glycoalbumin	12.3%–16.5%	TA	14.30 ± 1.24	14.16 ± 1.24	*
		P	14.19 ± 1.37	14.04 ± 1.22	*

*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$ between weeks 0 and 4 within the TA or P group;
#$p < 0.05$ between the TA and P groups at week 4. P, placebo; TA, Tongkat Ali.

In this study, TA supplementation caused significant increases in immunological age in the TA group (Table 2). Specifically, the TA group had a younger immunological age (by 4 years) after a 4-week TA supplementation. Other anti-aging properties of TA, such as improvements in hormonal balance, strength, quality of life, and sexual health, have also been previously reported (Tambi *et al.,* 2011; Henkel *et al.,* 2014). The TA group in the present study also had significantly higher numbers of lymphocytes relative to the P group after the 4-week supplementation period. In the *in vitro* DHEA treatment on peripheral blood leukocytes obtained from older donors (≥65 years old), increased leukocytic RACK-1 expression and lymphocyte proliferation were observed. This suggests the further role of this hormone in the modulation of RACK-1 expression and immune functions (Corsini *et al.,* 2005).

In a recent study by Muhamad *et al*. (2015), the TA water extract was evaluated in subjects for endurance running and immune functions. Subjects on TA had improved immune functions characterized by the increase in NK cells. Natural killer cell is an immune cell that is very important in defense against viral infection. It is one of the lymphocytes and a component of the innate immune system [31]. In this study, there was an increasing trend in NK cells in the TA group and the reverse in the P group between week 0 and week 4. TA water extract(LJ100/Physta®) contains >30% polysaccharide and >40% glycosaponins. Polysaccharides from medicinal plants have been reported to improve NK cell levels (Nair *et al.,* 2004).

There were no significant differences between the both groups for naïve/memory ratio and T cell proliferative index, even though naïve and total T cells significantly increased in the TA group. In fact the T cell proliferative activity was strongly significantly higher in the TA group ($p < 0.001$) compared to that of the P group ($p < 0.05$). This could be because of the homeostasis balance in the body and short time of supplementation (1 month) in a small sample size which did not allow for drastic in between group differences in these areas. With the activation of adaptive immunity, the immunological age got younger by 4 years in the TA group and the overall significant improvement in SIV and immunological grade which were derived from these subscores.

Possible mechanism of immune improvement via ingestion of TA

The antioxidative effects of eurycomanone, a compound in the extracted TA, (Varghese *et al.,* 2013) might have also caused improvements in immunity (Hirokawa *et al.,* 2009). In addition to aging, oxidative stress plays an important role in the progression of immunological decline, and antioxidant compounds can enhance antioxidant-producing enzymes (Hirokawa *et al.,* 2009) to slow the decline. In almost all living systems, cells require adequate levels of antioxidant defense to avoid the harmful effects of excessive reactive oxygen species productions and to prevent immune cell damages.

Safety

There were no severe AEs reported by the subjects or observed through clinical parameters (Table 3 and Supplementary Tables 1–3). The AEs were mild in the both groups and not related to the products. Based on the biochemical results which had some statistical differences between both the groups, these were not clinically significant as the changes were all within normal levels. Serum lipids such as HDL cholesterol and triglycerides were improved in the TA group although it was not significant in triglycerides. The mechanism is not well understood though this phenomenon have been reported in testosterone treated hypogonadic subjects (Tambi *et al*., 2011; Yassin *et al.,* 2014). A 200 mg/day oral dose of a water extract of TA is considered as safe under the conditions of this study.

Limitations and foresight

The current study was conducted, performed, and measured with a primary focus on acquired immunity, as determined by total T cells and T cell subsets. Further studies should be performed to investigate the effects of the TA water extract on other components of immunity, such as innate immunity, intestinal immunity, and cytokine and/or chemokine production. This study recruited only middle-aged subjects, and it would be useful to evaluate the effects of TA in younger subjects in order to investigate the effect of TA on other age groups. Furthermore, another inclusion criterion such as allergic subjects could also be included to investigate the immunomodulatory effect of TA. This study was conducted for 4 weeks as previous studies on immunity using other food ingredient showed immune improvements after 4-week ingestion (Suzuki *et al*., 2012, unpublished data). A longer intake should help in the understanding of TA's immunomodulating activity in the long term.

CONCLUSION

This clinical study was aimed to evaluate whether the comprehensive immune functions of middle-aged men and women with comparatively lower levels of immunity would be improved by the ingestion of a 200 mg/day dose of the water extract of TA for 4 weeks. In conclusion, comprehensive immune measurements (SIV and Immunological grade) revealed significant improvement following TA ingestion. Immunological grade in the TA group was improved from the warning zone (Grade II), which is required to improve the immunity, to the observation zone (Grade III). According to these findings, intake of the TA might contribute immunological improvement, which results in maintenance and improvement of health. Our findings indicate for the first time the immune-related improvements following the ingestion of the propriety water extract of TA (LJ100/Physta®).

Acknowledgements

The authors would like to thank all subjects participating in this study. We would like to thank A. Kazama and Ask Intercity Co., Ltd. for facilitating communications and local arrangement of TA and P encapsulation under GMP in Japan. We are also grateful to S. Ichikawa (Mie University) for his helpful advice with regard to statistical analyses.

This study was funded by the NKEA Research Grant Scheme (NRGS) EPP#1 under Ministry of Agriculture and Agro-Based Industry, Malaysia.

AUTHORS' CONTRIBUTION

AG, NS, ABA, KM, and TT designed the study. NS, MU, KH, and TT performed the clinical measurements and analyzed samples. NS statistically analyzed the data. All authors discussed and wrote the manuscript.

Conflicts of Interest

AG and ABA are employed by Biotropics Malaysia Bhd, sponsor of the study. NS is an employee of ORTHOMEDICO Inc. KM (Ph.D.) is an Emeritus Professor of Meiji Pharmaceutical University. MU (Ph.D.) and KH (M.D., Ph.D.) are staff in Tokyo Medical and Dental University and also belong to a contract research organization for immune measurements. TT (M.D.) is the Principal Investigator.

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