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Article

Effects of *Porphyra tenera* Supplementation on the Immune System: A Randomized, Double-blind and Placebo-controlled Clinical Trial

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Abstract: Objective: The purpose of this study was to determine if *Porphyra tenera* extract (PTE) has immune-enhancing effects and is safe in healthy adults. **Methods:** Subjects ($3x10^3 \leq$ peripheral blood leukocyte levels < $8x10^3$ cells/μl) who met the inclusion criteria were recruited for this study. Enrolled subjects (n=120) were randomly assigned to either the PTE group (n=60) who were given 2.5 g/day of PTE (as *Porphyra tenera* extract) in capsule form or the placebo group (n=60) who were given crystal cellulose capsules with the identical appearance, weight, and flavor as the PTE capsules for 8 weeks. Outcomes were assessed by measuring natural killer cell (NK-cell) activity, cytokines, and upper respiratory infection (URI), and safety parameters were assessed at baseline and 8 weeks. **Results:** Compared to baseline, NK cell activity (%) increased for all effector cell to target cell ratios in the PTE group after 8 weeks, but there were no changes in the placebo group ($p<0.1$). Subgroup analysis of 101 subjects without an URI revealed that NK-cell activity in the PTE group tended to be increased for all E:T ratios (E:T=12.5:1 p=0.068; E:T=25:1 p=0.036; E:T=50:1 p=0.081) compared to the placebo group. There was a significant difference between these two groups for the E:T=25:1 ratio, which increased from 20.3±12.0% at baseline to 23.2±12.4% after 8 weeks in the PTE group (p=0.036). There was no significant difference in levels of cytokines between these two groups. **Conclusions:** PTE supplementation appears to enhance immune function by improving NK-cell activity without adverse effects in healthy adults.

Keywords: *Porphyra tenera*; immune; clinical trial; natural killer cells; cytokines

1. Introduction

Immune system imbalances can be caused by various factors, including aging, viral disease, increased stress, and environmental pollution. There is great interest in functional foods and alternative treatments to improve immunity as pharmacologically active substances in foods may boost the immune system. Mass production of sea algae using aquaculture technology has been used to produce value-added foods[1,2]. Laver (*Porphyra tenera*), a type of red algae, has long been a staple food eaten in Western Pacific Ocean regions, including South Korea, China, and Japan, and its consumption is steadily increasing not only in Southeast Asia but also around the world. Laver is low in calories but rich in carbohydrates, protein, vitamins, and minerals. Unlike other sea algae substances, laver has a lot of free sugars, such as isofloridoside and floridoside, which are major carbohydrates. In addition, it has abundant dietary fiber in the form of sea algae polysaccharides including hemicellulose, which is an insoluble polysaccharide and cell wall component, and

porphyrans, which are water-soluble acidic polysaccharides and intercellular rechargeable substances[3,4] The porphyran present in laver comprises 3,6-anhydro-L-galactose, D-galactose, ester sulfate, and 6-O-methyl-D-galactose components. This porphyran is not digestible by humans and is highly viscous, thus it can be used as a diluent agent[5,6]. Fucoidan is another component of edible sea algae (green or brown algae) and is considered a functional food, food additive, and physiologically active substance[7,8] There is increased interest in laver as a functional food; in particular, the antioxidant activities of porphyrans and polyphenols in laver[9,10]. Laver has been reported to degrade cholesterol[11], show antitumor and immune enhancement activities[12], function as an antioxidant[9,13], improve lipid metabolism[14], have anti-inflammatory activity[15], and exert anti-mutagenic effects[16]. *In vitro* and *in vivo* studies found that *Porphyra tenera* extract (PTE) administration increased survival rates of splenocytes and macrophages, increased stimulation of NF-kB, increased NO production, increased levels of the Th1 cytokines IL-2, IL-12, IFN-γ, and TNF-α, and increased immune function by promoting the secretion of i-NOS[17,18]. However, no clinical trials have verified the immune enhancement effects of PTE. In this study, we examined the effect of *Porphyra tenera* extract (PTE) supplementation on immunological indicators in healthy adults and investigated its safety.

2. Materials and methods

2.1. Test supplements

Ethanol extraction of 100 kg dried laver (*Porphyra tenera*) (Wando, Jeollanamdo Province, Korea) was performed at temperature (80±2℃) for 3 hours using 10% ethanol, and PTE was obtained using a lyophilization method after filtration using a 1 μm housing filter and concentration of the extract to 10～20 Brix at 65 – 70℃. The yield rate of PTE was 13% and the concentration of porphyra334 in this PTE was 68.45±20% mg/g. An HPLC chromatogram of the PTE is presented in **<Fig. 1>**. According to our pre-clinical studies[17,18], administering oral doses of PTE 500 and 1,000 mg/kg to 6-week-old ICR mice improved immunity-related indicators significantly by promoting the secretion of cytokines and i-NOS. This is equivalent to a human daily intake of 2.5 g per day using a dose of 500 mg/kg. All test products used in this study were provided by the Marine Biotechnology Research Center (Wando, Jeollanamdo Province, Korea) in the form of yellow-brown capsules (powder). Placebo products were made of crystal cellulose and had the same appearance, flavor, and weight as the test products **(Table 1).**

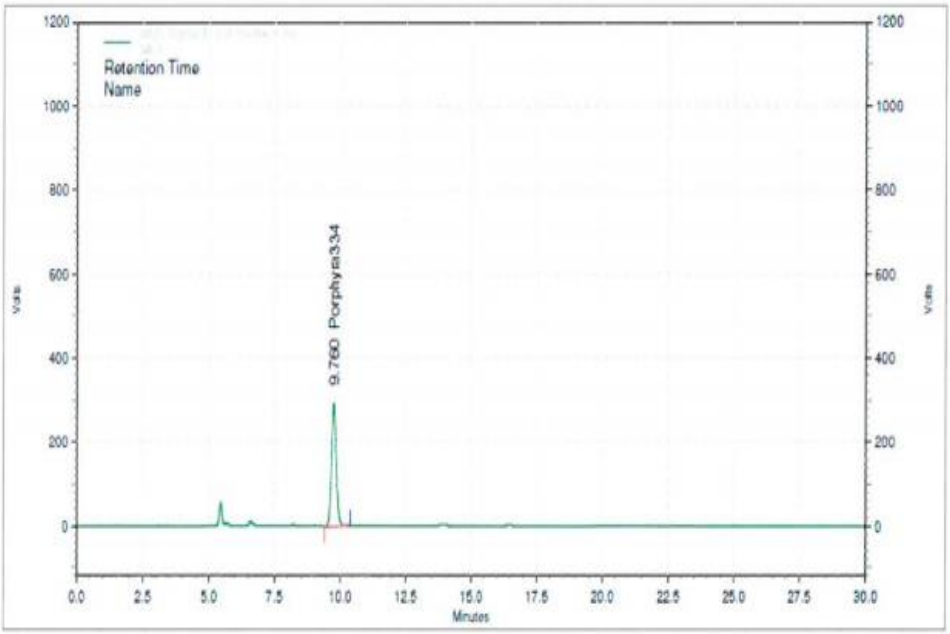

Figure 1. Representative chromatograms of PTE (HPLC analysis of porphyra34 in PTE).

Table 1. Composition of the test product as determined by high-performance liquid chromatography (HPLC).

Component	**Test capsule** ***Porphyra tenera* extract supplement (%)**	Placebo supplement (%)
Porphyra tenera extract	99.52	-
Silicon dioxide	0.48	0.2
Microcrystalline cellulose	-	99.6
Caramel coloring	-	0.2
Total	100	100

2.2. Subjects

Subjects were recruited from May 22 to August 22, 2019 through internal advertising (Internet postings on departmental home pages, brochures, and posters) at the Clinical Trial Center for Functional Foods (CTCF2) at Chonbuk National University Hospital. This study was conducted after receiving approval from the Institutional Review Board (IRB) (IRB No. CUH 2019-04-050) of Chonbuk National University Hospital, and the protocol was registered at www.clinicaltrials.gov (NCT04017988). The entire study was carried out in accordance with the Helsinki Declaration and the provisions of the Korean Good Clinical Practice (KGCP). A total of 120 participants judged to be eligible after screening tests such as questionnaires, physical examinations, and laboratory examinations were enrolled within 3 weeks before being provided with supplement after providing informed consent. Selection criteria were as follows:

1) Men and women older than 50 years at the time of the screening test;

2) Written consent to participate received prior to the start of the study;

3) A peripheral white blood cell count greater than $3x10^3$ and less than $8x10^3$ cells/μl as measured in the screening test.

Exclusion criteria were as follows:

1) Vaccination against influenza within 3 months prior to the screening test;

2) BMI less than 18.5 kg/m^2 or greater than 35 kg/m^2 at the time of the screening test;

3) Presence of a clinically acute disease or chronic cardiovascular, endocrine, immune, respiratory, hepatobiliary, kidney, urinary, neuropsychiatric, musculoskeletal, inflammatory, hematological, or gastrointestinal disease;

4) Supplementation with medicines or health functional foods related to the enhancement of immunity within 1 month prior to the screening test;

5) Administration of antipsychotics within 3 months prior to the screening test;

6) Suspected alcoholism or drug abuse;

7) Participation in other human tests within 3 months prior to the screening test;

8) The following results in diagnostic and medical tests:

- ☞ AST, ALT > 3x the normal upper limit
- ☞ Serum creatinine > 2.0 mg/dl

9) Pregnant or nursing;

10) No contraceptive use in fertile women;

11) Deemed inappropriate to participate in the research for other reasons, including the results of diagnostic and medical examinations.

2.3. Study design

The study was a random, double-blind, placebo-controlled study to assess the effectiveness and safety of PTE at enhancing immune function when taken for 8 weeks. A total of 120 subjects participated in the study, with 60 individuals each assigned to the test and placebo groups. Subjects who met the selection criteria were assigned to the test group or placebo group based on an allocation code generated using a block-based random assignment method. Subjects took the test capsule or placebo capsule twice a day (two capsules at a time) for eight weeks after breakfast and dinner.

Screening was conducted once for all subjects and they received a basic evaluation (baseline) on the day of the first visit (Week 0). After the first visit, subjects visited CTCF2 every 4 weeks (2nd visit: Week 4, 3rd visit: Week 8) to examine vital signs, drug dosage, medical condition changes, and adverse reactions. Intake of the test capsules was examined and monitored by the test manager by directly counting the number of remaining capsules to monitor compliance (intake rate, %) at the second visit (Week 4) and third visit (Week 8).

2.3. Randomization

In this study, the same block size was applied for balanced random assignment in the intake groups, and the ratio of the assigned subjects in each group was determined using a 1:1 ratio. A random assignment table was generated using the randomization program of SAS® from subject number 1 using random number permutations (random numbers of A and B). When packing the test capsules, the test capsule labels were attached according to the random assignment table and supplied to CTCF2 before starting the study. Allocation codes were managed by the study investigators and were monitored by clinical research associates until the end of the study. During the research period, all research investigators and participants were blinded to identification codes.

2.4. Outcome measurements

All subjects who visited CTCF2 underwent a safety assessment at the first visit (baseline Week 0) and third visit (Week 8).

2.4.1. Clinical and biochemical analyses

Hematology examination, blood biochemical tests, and urine tests were performed after collecting blood and urine after a 12-hour fast the day before blood collection.

2.4.2. Primary outcomes

NK cell activity was assessed using the CytoTox 96W Non-Radioactive Cytotoxicity Assay kit (Promega Co., WI, USA). After separating peripheral blood mononuclear cells (PBMCs) from heparin-treated venous blood using a density gradient centrifugation method, the cell-mediated cytotoxicity of NK-cells in peripheral blood monocytes was measured[19]. The lactate dehydrogenase (LDH) assay method[20] was used and the target cells were K562 cells (human leukemia cell line, Korean human leukemia cell line bank, Seoul, Korea). Effector cell : target cell (E:T) ratios were 50:1, 25:1, and 12.5:1. Cytotoxicity (%) was calculated as described below by measuring both natural release and maximum release at the first visit (baseline Week 0) and third visit (Week 8):

Cytotoxicity (%) = (experimental release – effector spontaneous release – target spontaneous release)/(target maximum release – target spontaneous release) * 100.

2.4.3. Secondary outcomes

Cytokines (IL-2, IL-6, IL-12, interferon-γ, and TNF-α) were measured at the first visit (baseline Week 0) and third visit (Week 8). The incidence of upper respiratory infection (URI) was assessed on the day of the visit for the screening test (1st visit, Week 0), the second visit (Week 4), and the third visit (Week 8). To analyze blood cytokines, 3 mL of blood was collected in a 5-mL SST tube (Hanil Science Industrial Co. Ltd, Seoul, Korea) and centrifuged for 10 minutes at 3,000 rpm (or 2,000*g) after leaving the tube at room temperature for 30 minutes to let the blood clot. Approximately 1 mL of supernatant was transferred to a microtube and kept frozen at –70°C until analysis. IFN-γ, IL-2,

IL-6, IL-12, and TNF-α levels in serum were assessed using the ELISA kit (Enzyme linked immuno sorbent assay, Microplate Reader, VERSA Max, Molecylar device, USA). The incidence and frequency of upper respiratory infections (URIs) was evaluated at the first visit (Week 0), the second visit (Week 4), and the third visit (Week 8) using the URI symptom assessment tool (Wisconsin Upper Respiratory Symptom Survey (WURSS)[21]). Symptom scores and duration (days) were also recorded. Examination items were signs of URI, presence or absence of URI symptoms, and types of symptoms (throat pain, sniffing, nasal congestion, sneezing, hoarseness, myalgia, earache, pyrexia, headache, coughing, phlegm, labored breathing, diarrhea, nausea, and vomiting). The severity of symptoms was assessed as follows: "0" for no sign of symptoms, "1" for insignificant symptoms, "2" for average symptoms, and "3" for serious symptoms.

2.5. Safety outcome measurements

Clinical conditions of the subjects, including adverse reactions, were evaluated and recorded in the case report list. Safety assessments comprised electrocardiograms, vital signs (blood pressure and pulse rate), and laboratory tests. In the hematological examination, white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin level, hematocrit, platelet count, and neutrophil, lymphocyte, and basophil counts were examined. In the blood biochemical examination, liver and kidney functions were investigated through total bilirubin, total protein, alkaline phosphatase (ALP), gamma-glutamyl transferase (gamma-GT), alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), glucose, and creatinine assessment. Lipid metabolic indicators were investigated by measuring levels of total cholesterol, triglycerides, high density lipoprotein-cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C).

2.6. Evaluation of diet and physical activity

To investigate changes in dietary habits, a nutritionist trained in the dietary records method explained to subjects how to prepare a dietary diary and collected data after confirming the data through face-to-face interviews when retrieving the dietary diary. During the first visit (baseline; Week 0) and the third visit (Week 8), dietary diary from the previous day were collected and analyzed using CAN-Pro 4.0®, which is a computer-aided nutritional analysis program of the Korean Nutrition Society Forum, Seoul, Korea, and average values were calculated. To investigate changes in physical activity, the global physical activity questionnaire (GPAQ:[22]) was administered on the first visit (baseline; Week 0) and the third visit (Week 8), and the metabolic equivalent task (MET) value was calculated using these data. Subjects were instructed to maintain their regular daily lifestyle, diet, and physical activity during their participation in this study.

2.7. Statistical analysis

All statistical analyses were performed using SAS® version 9.4 (SAS Institute, Cary, North Carolina, USA). All data are presented as means ± standard deviations (SD) for continuous variables, and as n (%) for categorical variables. Final results were evaluated by full analysis set (FAS) and per protocol (PP) analysis. PTE and placebo groups were compared using independent t-tests for continuous variables collected at baseline, while categorical variables and URI incidence rates were compared using the Chi-square test (Fisher's exact test). Changes in NK-cell activity and cytokine levels as continuous variables before and after intake were analyzed by paired t-test, while differences between intakes groups were analyzed by a linear mixed model. Several previous studies [23-25]have reported that URI infections change the activity of cytokines and NK cells. Therefore, we conducted subgroup analysis based on the incidence of URI. Baseline and demographic variables for heterogeneous evaluation items were calibrated with covariates for analysis of covariance (ANCOVA) testing. The significance was statistically significant at the level of $p < 0.05$.

2.8. Sample size

The primary outcome of this study was a change in NK-cell activity between the PTE and placebo groups after 8 weeks of supplement intake. A power calculation was applied based on the results and design of a previous study[26]. The change in NK-cell activity in the PTE group was assumed to be 7.4%, while the changes in the placebo group and SD were assumed to be –0.28 and 12.5%, respectively. The sample size required to maintain 80% static power at a significance level (two-tailed test) of 5% was calculated to be 42 persons per group. Therefore, a total of 120 persons, 60 persons per group, were calculated to be required assuming a dropout ratio of 30%.

3. Results

3.1. Participant demographic characteristics

After screening a total of 137 volunteers, 120 subjects were selected (20 males, 100 females) as eligible candidates, and 60 subjects were randomly assigned to the PTE and placebo groups, respectively. A total of 111 subjects completed the study per protocol. Nine subjects were excluded due to drop-out or violation of the study plan (two violators of the exclusion criteria in the PTE group, two violators of the study plan in the placebo group, and five withdrawals of consent after the first visit in the placebo group). Data from 111 subjects was used for analyses **<Fig. 2>**. There were no significant differences in compliance with regard to taking the supplements between the PTE group and placebo group (95.2±5.8% of the PTE group vs 96.2±4.1% of the placebo group, p=0.265). There were also no significant differences in age, sex, BMI, vital signs, smoking rate, smoking quantity, drinking rate, or drinking amounts between the two groups (p>0.05) **<Table 2>**. Although there were no significant differences in NK-cell activity and IL-2, IL-6, IL-12, and IFN-γ between the two groups, TNF-α was significantly higher at baseline in the placebo group than in the PTE group (p=0.017) **<Table 2>.** Statistical results of this study were therefore calibrated for this difference in baseline TNF-α.

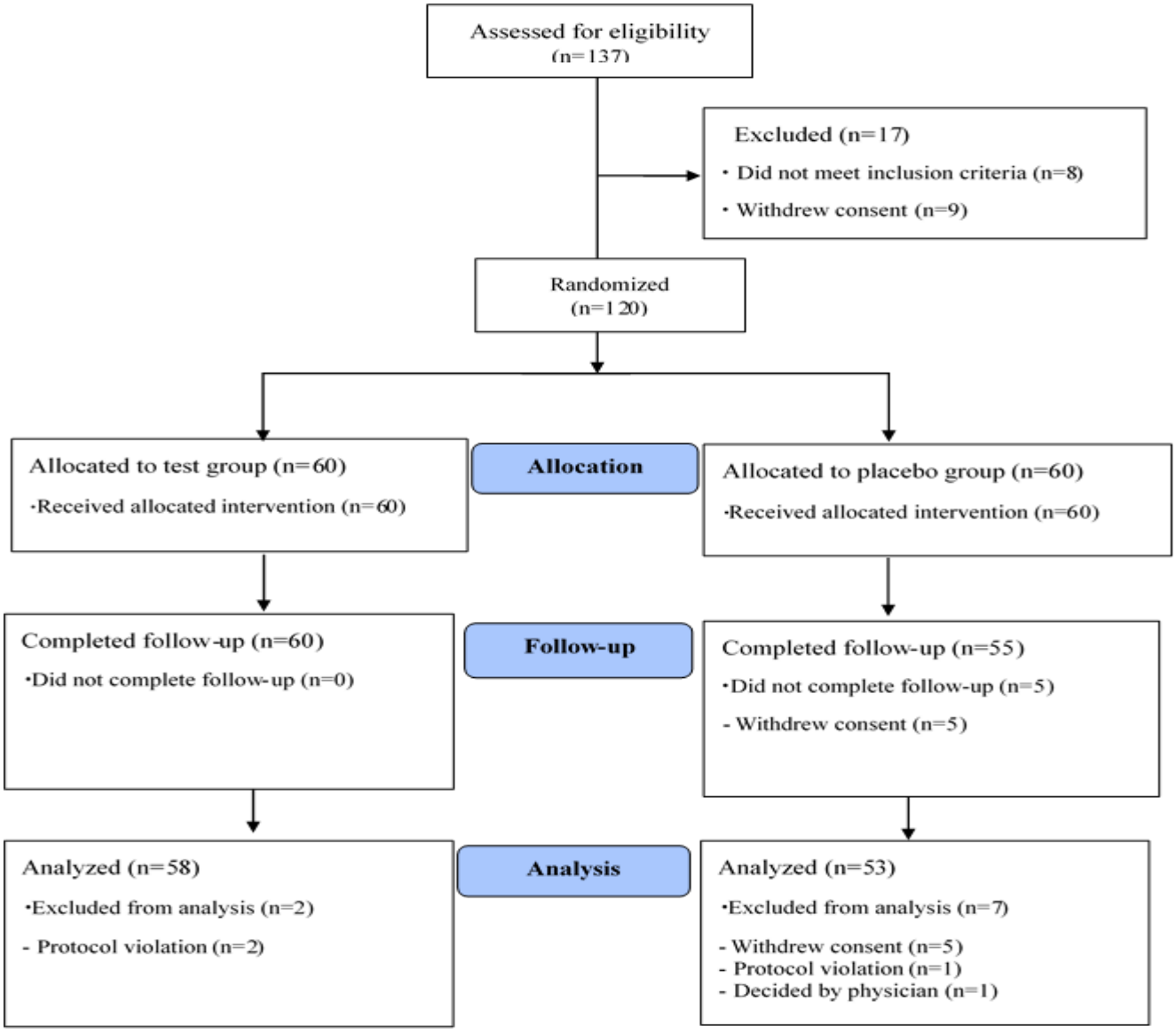

Figure 2. Flow chart of participants in this study.

Table 2. Baseline general characteristics of the subjects

	PTE group (n=60)	Placebo group (n=60)	Total (n=120)	*P*-value[1)]
Sex (M/F)(n.%)	10(16.7)/50(83.3)	10(16.7)/50(83.3)34	20(16.7)/100(83.3)	0.999[2)]
Age (years)	59.7±4.8	59.5±6.1	59.6±5.4	0.854
Height (cm)	158.6±6.6	158.17.1	158.3±6.8	0.701
Weight (kg)	60.3±8.1	59.1±9.7	59.7±8.9	0.466
BMI (kg/m^2)	23.9±2.6	23.6±2.9	23.8±2.8	0.466
SBP (mmHg)	120.9±15.4	119.1±18.2	120.0±16.8	0.560
DBP (mmHg)	70.3±10.6	68.9±12.6	69.6±11.6	0.492
Pulse (bpm)	69.0±9.7	69.3±8.8	69.2±9.2	0.852
NK-cell activity	11.4±8.4	11.8±9.1	11.6±8.7	0.828

12.5:1 (%)				
25:1 (%)	20.6±12.2	20.7±13.6	20.6±12.8	0.992
50:1 (%)	28.0±14.3	27.4±14.3	27.7±14.3	0.837
IL-2 (pg/mL)	1.27±0.85	1.51±1.51	1.39±1.23	0.281
IL-6 (pg/mL)	0.6420.68	0.64±0.83	0.63±0.75	0.982
IL-12 (pg/mL)	2.29±1.83	2.16±1.45	2.22±1.65	0.656
INF-γ (pg/mL)	8.30±8.54	7.02±6.70	7.66±7.67	0.363
TNF-α (pg/mL)	6.04±2.43	7.21±2.82	6.63±2.68	0.017
Alcohol (n, %)	25(41.7)/35(58.3)	30(50.0)/30(50.0)	55(45.8)/65(54.2)	0.360
Alcohol (unit[3])/week)	1.5±2.2	3.0±4.4	2.3±3.6	0.103
Smoking (Y/N)	0(0.0)/60(100.0)	2(3.3)/58(96.7)	2(1.7)/118(98.3)	0.496 [4]
Smoking (pieces/day)	0.00±0.00	12.5±3.5	12.5±3.5	-

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Values are presented as mean ± SD or number

[1] Analyzed by independent t test

[2] Analyzed by chi-square test

[3] Alcohol 1 unit = Alcohol 10 g = Alcohol 12.5 mL

[4] Analyzed by Fisher's exact test

Abbreviations: BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure, Natural killer cell, NK-cell; Interleukin-2, 6, 12 (IL-2, IL-6,IL-12); INF-γ, interferon gamma; TNF-α, Tumor necrosis factor alpha

3.2. Diet intake and physical activity

Dietary intake results are presented in **<Table 3>**. Carbohydrate (CHO) intake in the PTE group increased significantly after 8 weeks (p=0.002) compared to baseline, leading to a significant difference in CHO intake between the two groups at 8 weeks (p=0.0198). There were no statistically significant differences in calorie, protein, fat, or fiber intake between the two groups (p>0.05). Results are presented after calibrating for CHO intake. There were no significant changes in MET within or between groups (p>0.05).

3.3. Efficacy evaluation

3.3.1. Primary outcome

Changes in NK-cell activity level of the PTE and placebo groups are presented in <**Table 4**>. NK-cell activity (%) increased in the PTE group for all E:T ratios (E:T=12.5:1 p=0.0004, E:T=25:1 p=0.0034, and E:T=50:1 p=0.0055) compared to baseline, but not in the placebo group. Comparison of changes in NK-cell activity between the placebo and PTE groups after adjusting for baseline CHO and MET revealed an increasing tendency in the PTE group compared to the placebo group for all E:T ratios (E:T=12.5:1 p=0.0829, E:T=25:1 p=0.0587, and E:T=50:1 p=0.0832) (p<0.1). NK-cell activity level in individuals who did not have a URI (n=101) are presented in **<Table 5>**. NK-cell activity in the PTE group after 8 weeks was increased relative to baseline for all E:T ratios (E:T=12.5:1 p=0.0019, E:T=25:1 p=0.0106, and E:T=50:1 p=0.0134), but not in the placebo group. NK-cell activity of the PTE group showed a greater tendency to increase than in the placebo group for all E:T ratios (E:T=12.5:1 p=0.0683, E:T=25:1 p=0.0357, and E:T=50:1 p=0.0810) (p<0.1). NK-cell activity of the PTE group at the ratio of

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E:T=25:1 increased significantly to 23.2±12.4% after 8 weeks compared to 20.3±12.0% at baseline (p=0.0357).

3.3.2. Secondary outcomes

Cytokine concentrations in the PTE and placebo groups are presented in <**Table 4**>. There were no significant differences in serum levels of IL-2, IL-6, IL-12, or IFN-γ between baseline and after 8 weeks of supplementation in either the PTE or placebo groups for the full analysis set (n=111), and there were also no significant differences in cytokine levels between these two groups (p>0.05). Although the TNF-α concentration in the PTE group appeared to increase after eight weeks compared to the baseline, this increase was not statistically significant within the PTE group or significantly different between the two groups (p>0.05). Other cytokines showed no significant differences between the two groups (p>0.05). Sub-analysis of cytokine concentrations in subjects without a URI (n=101) are presented in **<Table 5>.** Comparison of IL-2, IL-6, IL-12, and IFN-γ levels at baseline and after 8 weeks of supplementation revealed no significant differences within or between groups (p>0.05). TNF-α level in the PTE group increased significantly (p=0.0368) after 8 weeks compared to baseline. There were no significant differences in cytokine levels between the placebo group and the PTE group (p>0.05). Among all subjects (n=111), URI infection in the PTE group decreased by 6.9% and 3.5% from baseline after 4 and 8 weeks of supplementation, respectively, but these differences were not statistically significant (p>0.05). Among subjects with a URI, URI symptom total score in the PTE group (n=8) decreased from 3.00±2.16 at baseline to 1.50±0.71 after 8 weeks of intake, but the score in the placebo group (n=2) increased from 3.00±0.0 at baseline to 4.00±0.0 after 8 weeks of intake. Thus, there was significant difference between these two groups (p=0.030) at 8 weeks **(Supplementary Table 1)**. Although the rhinorrhea symptom score among those with a URI was not significantly different between the PTE and placebo groups, the duration of rhinorrhea symptoms in the PTE group decreased significantly from baseline after 4 and 8 weeks of supplementation (p=0.0459). Thus, there was a significant difference between these two groups in duration of rhinorrhea symptoms at 8 weeks (p=0.0366). Sneeze symptom score of the PTE group decreased over time (p=0.0546), while the score in the placebo group increased over time. Thus, there was significant difference in this score between these two groups at 8 weeks (p=0.0217). The duration of sneeze symptoms in the PTE group decreased over time (p=0.0170), but was not significantly different between the two groups at 8 weeks (p=0.0562). Other symptom items such as sore throat, nasal congestion, hoarseness, myalgia, fever, headache, cough, and sputum showed no statistically significant differences between the two groups.

Table 3. Intake of major nutrients and metabolic equivalent values in the PTE and placebo groups measured at baseline and at 8 weeks.

	PTE group (n=58)				Placebo group (n=53)				
	Baseline	**Week 8**	**Change**	***P*-value[1)]**	**Baseline**	**Week 8**	**Change**	***P*-value[1)]**	***P*-value[2)]**
Energy (Kcal)	1,538.2±537.3	1,552.5±394.5	14.4±501.1	0.828	1,682.4±568.5	1,580.9±520.4	-101.6±525.5	0.165	0.237
Carbohydrates (g)	236.9±67.4	264.0±66.2	29.1±69.0	0.002	251.9±85.1	247.6±81.3	-4.33±79.6	0.694	0.020
Lipids (g)	40.3±26.9	34.6±18.9	-5.7±28.8	0.137	46.4±17.1	40.9±23.9	-5.4±31.9	0.219	0.965
Protein (g)	62.1±27.1	52.9±17.5	-9.1±26.4	0.011	64.9±23.2	61.7±29.4	-3.2±26.9	0.386	0.250
Fiber (g)	19.3±7.9	21.5±8.1	2.2±9.0	0.067	19.8±7.7	21.4±9.7	1.6±8.2	0.169	0.697
MET (min/week)	1886.9± 2055.2	3016.6± 5722.3	1149.7± 5732.8.3	0.132	2891.7± 4699.8	2307.9± 3518.7	-583.8± 4403.9	0.339	0.079

Values are presented as means ± SDs

[1)] Analyzed by paired *t*-test

[2)] Analyzed by Linear Mixed Model between groups

Abbreviation: MET, metabolic equivalent

Table 4. NK-cell activity and cytokine cluster analysis obtained prior to and after treatment in the two groups.

	PTE group (n=58)				Placebo group (n=53)					***Adj.***
	Baseline	**Week 8**	**Change**	***P*-value[1)]**	**Baseline**	**Week 8**	**Change**	***P*-value[1)]**	***P*-value[2)]**	***P*-value[4)]**
NK-cell activity (%)										

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12.5:1	11.4±8.4	14.8±8.2	3.4±6.9	**0.0004**	11.8±9.1	13.3±9.6	1.6±6.3	0.079	0.1390	**0.0829**
25:1	20.6±12.2	23.6±12.8	3.0±7.4	**0.0034**	20.7±13.6	21.1±13.1	0.5±7.0	0.638	**0.0692**	**0.0587**
50:1	28.0±14.3	30.8±14.6	2.8±7.3	**0.0055**	27.4±14.3	28.1±14.6	0.6±8.1	0.585	0.1401	**0.0832**
IL-2 (pg/mL)	1.28±0.85	1.28±0.92	0.00±0.45	0.9909	1.44±1.51	1.37±1.06	-0.08±1.02	0.5792	0.6054	0.7319
IL-6 (pg/mL)	0.64±0.69	0.69±0.89	0.06±0.79	0.5922	0.63±0.85	0.53±0.51	-0.10±0.50	0.1384	0.2022	0.2582
IL-12 (pg/mL)	2.31±1.86	2.18±1.32	-0.13±0.78	0.2007	2.08±1.49	1.89±1.05	-0.19±0.87	0.1245	0.7270	0.8367
INF-γ (pg/mL)	8.35±8.68	8.00±6.75	-0.35±2.59	0.3135	7.04±6.97	6.41±4.49	-0.63±3.53	0.2029	0.6378	0.6044
TNF-α (pg/mL)	6.05±2.47	7.01±3.53	0.96±3.76	**0.0577**	7.36±2.82	7.94±4.78	0.58±5.48	0.4420	0.6798	0.3650 0.4097 [3)]

Values are presented as means ± SDs

[1)] Analyzed by paired *t*-test

[2)] Analyzed by Linear Mixed Model between groups

[3)] Analyzed by ANCOVA after adjusting for baseline values.

[4)] Analyzed by ANCOVA after adjusting for carbohydrate and T-MET change values

Abbreviations: MET, metabolic equivalents value; natural killer cells, NK-cells; interleukin-2, 6, 12 (IL-2, IL-6, IL-12); IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha

Table 5. NK-cell activity and cytokine cluster analysis obtained prior to and after treatment in the two subgroups.

	PTE group (n=50)	**Placebo group (n=51)**

	Baseline	Week 8	Change	*P*-value[1)]	Baseline	Week 8	Change	*P*-value[1)]	*P*-value[2)]
NK-cell activity (%)									
12.5:1	11.2±8.4	14.6±8.0	3.4±7.3	**0.0019**	11.8±9.2	12.8±9.5	1.0±5.7	0.2238	**0.0683**
25:1	20.3±12.0	23.2±12.4	2.8±7.5	**0.0106**	20.6±13.8	20.5±13.0	-0.1±6.4	0.8907	**0.0357**
50:1	27.8±14.2	30.5±14.4	2.7±7.4	**0.0134**	27.3±14.4	27.3±14.5	0.1±7.5	0.9457	**0.0810**
IL-2 (pg/mL)	1.33±0.875	1.36±0.94	0.03±0.46	0.6064	1.44±1.51	1.36±1.07	-0.08±1.04	0.5885	0.4811
IL-6 (pg/mL)	0.70±0.72	0.65±0.58	-0.05±0.34	0.3395	0.64±0.87	0.53±0.52	-0.11±0.51	0.1427	0.4824
IL-12 (pg/mL)	2.51±1.92	2.34±1.34	-0.17±0.83	0.1451	2.10±1.51	1.91±1.06	-0.19±0.89	0.1263	0.9097
INF-γ (pg/mL)	8.95±9.18	8.56±7.06	-0.39±2.74	0.3175	7.13±7.08	6.50±4.55	-0.63±3.60	0.2171	0.7084
TNF-α (pg/mL)	6.00±2.56	7.09±3.60	1.13±3.72	**0.0368**	7.31±2.86	7.59±3.83	0.29±4.65	0.6606	0.3180 0.7997[3)]

Values are presented as means ± SDs

[1)] Analyzed by paired *t*-test

[2)] Analyzed by Linear Mixed Model between groups

[3)] Analyzed by ANCOVA after adjustment for baseline values

Abbreviations: MET, metabolic equivalents value; natural killer cells, NK-cells; interleukin-2, 6, 12 (IL-2, IL-6, IL-12); IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha

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3.4. Safety and adverse events

Subjects in this study showed no significant changes or differences in safety indicators such as laboratory tests, electrocardiograms, or vital signs (BP and pulse) during their participation ($p>0.05$). All laboratory test items were within the normal range and no side-effects were observed. With regard to adverse reactions, 11 cases of slight or severe abnormalities occurred in 11 subjects who ingested PTE among the total of 120 subjects. Eight of these abnormalities were in the PTE group and three in the placebo group, but this difference was not statistically significant ($p>0.05$) **(Supplementary Table 2)**. Adverse reactions included one case of abdominal discomfort, four cases of heartburns, one case of contact dermatitis (left leg), one case of left knee pain, one case of chronic dermatitis (leg) deterioration, one case of left trigger fingers, one case of an increase in liver enzyme ratio (AST/ALT), and one case of a burn (back of the left hand). Five of these cases did not have a clear causal relationship between the adverse reaction and the intake of the test product and were therefore not considered relevant, while six cases could have been caused by intake of the supplement **(data not shown)**.

4. Discussion

The purpose of this study was to evaluate the effectiveness and safety of PTE supplementation in enhancing immune function in healthy adults. To the best of our knowledge, this is the first randomized, double-blind, and placebo-controlled study to determine the immune-boosting effects of laver. We found that supplementation with 2.5 g of PTE per day for 8 weeks had beneficial immune regulation effects without adverse effects. According to several previous studies [7,24,25,27], influenza vaccination and influenza virus infection increase NK cell activity and antibody titers, which significantly affects immune parameters. Thus, we performed subgroup analysis of subjects without a URI to exclude the effects of viral infection on NK-cell activity. In both subgroup analysis of subjects without a URI (n=101) and all subjects (n=111), there was a tendency for NK-cell activity to increase in the PTE group for all E:T ratios (E:T=12.5:1, E:T=25:1, and E:T=50:1) ($p<0.1$) compared to the placebo group, but there were no significant differences between the two groups. Subjects without a URI in the PTE group showed an increase in NK-cell activity at an E:T ratio of 25.1:1 compared to the placebo group. Several previous studies [19,26,28-33] reported enhanced NK-cell activity in healthy adults only at certain E:T ratios; in this study, enhanced NK-cell activity was consistently identified in the PTE group at all E:T ratios (E:T=12.5:1, E:T=25:1, and E:T=50:1).

In general, active Th cells differentiate into two functional subclasses, Th1 and Th2, which are categorized according to the types of cytokines they secrete. Th1 cells produce Th1 cytokines (pro-inflammatory cytokines) such as IFN-γ, TNF-α, IL-2, and IL-12 that improve cellular immunity against intracellular pathogens such as bacteria. Th2 cells produce Th2 cytokines (anti-inflammatory cytokines) such as IL-4, IL-5, IL-6, and IL-10 and are involved in combating extracellular parasite infections and contributing to humoral immunity [34]. Th1 cytokines are known to affect immune responses and control NK-cell activity[35]. When NK-cells are activated, they secrete various cytokines such as IFN-γ and TNF-α and effectively exterminate target cells[36]. In preclinical studies[17,18], oral intake of PTE for 4 weeks increased the secretion of cytokines (IL-1β, IL-2, IL-4, and IFN-γ) by spleen cells and promoted iNOS expression in ICR mice. In addition, PTE stimulated T-helper cell type immune reactions and promoted the secretion of cytokines (IL-10, IL-6, TNF-α, and IFN-γ) in RAW264.7 cells and mouse splenocyte and macrophages cells, indicating that PTE has immune regulatory effects. Consistent with a previous animal study that reported increased TNF-α generation by macrophages in response to PTE[18], we observed an increase in TNF-α level after 8 weeks of PTE supplementation compared to baseline. These results are partially consistent with the study of Jiang et al. [37] who showed that the porphyran fraction of PTE inhibited NO generation in macrophages by blocking NF-kB activation [17] in macrophages. Taken together, these previous findings suggest that in our study, the porphyran component of PTE likely stimulated Th1 cells and improved cell-mediated immunity, as reflected by an increase in NK-cell activity along with the

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generation of the Th1 cytokine, TNF-α. However, we did not measure Th-2 induced cytokines (IL-4, IL-6, and IL-10) in this study. Kwak et al. **[28]** reported that changes in NK-cell activity were related to the level of serum IFN-γ. The changes in NK-cell activity and cytokines identified in this study are consistent with the results reported in several earlier studies [26,31,32] and cases [19,28,30,38,39]. Healthy adults who received *Cordyceps militaris* [31] supplements for 8 weeks or a mycelium extract of *Cordyceps* [32] for 8 weeks showed an increase in NK-cell activity without a change in cytokine levels, consistent with our findings in the current study. *Cordyceps militaris* increased IL-2 and IFN-γ levels in addition to NK-cell activity in healthy males (average age, 36.5±11.2 years) after 4 weeks of supplementation[38], but after 12 weeks of intake, only NK-cell activity was increased in healthy males and females (average age 48.9±6.7 years [31]), similar to our findings. In other studies, intake of *Chlorella [28]* by healthy adults (WBC counts 4,000-8,000 cells/μL) for eight weeks increased cytokine levels (IFN-γ, interleukin-1β) in addition to increasing NK-cell activity. Furthermore, supplementation with silk peptide [30] in non-seasonal influenza vaccine subjects for 8 weeks increased levels of cytokines (IL-2, IL-12, and IFN-γ) and NK-cell activity. Intake of Tulsi (*Ocimum sanctum* Linn.) leaf extract [39] for 4 weeks increased cytokine levels (IFN-γ, IL-4) only, while intake of β-1,3-glucan [19] by adults with severe stress levels increased the level of IL-10 level in addition to NK-cell activity. The results of these studies differ from our current findings. In the studies mentioned above, both NK cell activity and cytokine levels changed upon supplementation in healthy adults under 40 years of age [19,28,29,33,38,39]; in our study, only NK-cell activity increased [26,31,32], although our subjects were generally older than 40 years. This suggests that age moderates the effects of PTE and related supplements on cytokine levels. Factors that stimulated the increase in NK-cell activity may also have stimulated an increase in IFN-γ expression through different mechanisms. As mentioned above, the differences in cytokine activity among studies may be due to various factors such as age [19,26,28,29,31-33,38-40], inherent immunity, WBC count [28-32], availability of the influenza vaccination [24,27], seasonal factors (URI preventive inoculation season[30]), intake period of test supplements [29-33,38], and stress [19]. In the current study, the URI incidence rate (%) among all 111 participants was 10 (n=8 in the PTE group and n=2 in the placebo group); this difference was not statistically significant. When only subjects with a URI were analyzed (n=10), we found that the total URI symptom score in the PTE group improved significantly compared to that in the placebo group over time. In particular, the duration of three URI symptoms (sniveling, nasal congestion, and sneezing) decreased. In general, weakening or loss of NK-cell activity may be a sign of health issues. Given that it is the strongest predictor of immune function [25,41], supplementation with PTE may decrease the incidence of URI in subjects and shorten the duration of symptoms. No clinically meaningful adverse reactions or body changes were observed upon PTE supplementation in this study, indicating that PTE is safe to use in humans. Strengths of this study are as follows. We minimized the effect of URIs and exposure to influenza infection as much as possible to minimize the impact of URI and influenza on immune parameters (NK-cell activity, cytokines, and antibody titers [7,24,25,27]) during the research period. Specifically, after recruiting all subjects within a short period of time (one month), seasonal factors and individual variations were minimized by starting and completing the research in 3 months (May 22 - August 22, 2019). This allowed us to assess if PTE can enhance immune function in subjects without a URI by improving NK-cell activity. Limitations of this study are as follows. PTE did not change the expression of immune cytokines, suggesting that PTE may have increased NK-cell activity by facilitating the generation of the Th1 cytokine, TNF-α. Second, the improvement in URI symptoms was based on analysis of only 10 subjects with a URI (n=8 in the PTE group, n=2 in the placebo group), thus the number of subjects to be studied in the future should be expanded to confirm this finding. Third, we evaluated immune cytokine levels in the absence of trigger factors (stress, fatigue, exercise, etc.) that stimulate immune responses in healthy middle-aged people with no specific diseases. The exact effects of PTE on immune function should be investigated in future studies by considering the age at which immunity decreases, selecting subjects with high stress levels, and conducting the study outside of the influenza vaccination season.

5. Conclusions

PTE supplementation for 8 weeks increased NK-cell activity (%) compared to placebo, and there were significant increases in NK-cell activity among subjects without an upper respiratory tract infection (URI) compared to the placebo group. Total score variation of URI symptoms was significantly lower in the PTE group than the placebo group. This confirms that PTE supplementation can aid in rapid recovery from URI symptoms. During the study period, there were no clinically significant changes or adverse effects as assessed by laboratory tests, ECG, and vital signs. Together, these results suggest that 8 weeks of PTE supplementation can improve immune function through improving NK-cell activity in healthy people.

Additional file

Additional file 1: CONSORT 2010 checklist *.

Author contributions

Conceptualization and study design – SWC, SJJ, ESJ, HIB, and KCH; investigation - ESJ, SON, SWC, and HYJ; statistical analysis - HYJ and ESJ; data interpretation - SWC and SJJ; manuscript preparation - SJJ, and SWC; methodology - SWC, SJJ, SWS, THO, BJA, HIB, KCH, and SON. All authors participated in analyses of the biochemical data, interpretation of the data, and review of the paper. All authors read and approved the final manuscript.

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Conflict of interest

The authors have no competing interests to declare.

Consent for publication:

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available to protect patient confidentiality but are available from the corresponding author on reasonable request.

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