Supplementation with extract of *Gynostemma pentaphyllum* leaves reduces anxiety in healthy subjects with chronic psychological stress: A randomized, double-blind, placebo-controlled clinical trial

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ABSTRACT

Background: The ethanol extract of *Gynostemma pentaphyllum* Makino leaves (EGP) has been reported recently to have anxiolytic effects on chronically stressed mice models.

Purpose: We aimed to investigate the efficacy and safety of EGP on anxiety level in healthy Korean subjects under chronic stressful conditions.

Study design: Double-blind, placebo-controlled trial.

Methods: This study was conducted with 72 healthy adults who had perceived chronic stress and anxiety with a score on the State-Trait Anxiety Inventory (STAI) from 40 to 60. Participants were randomly assigned to receive either EGP (200 mg, twice a day, $N = 36$) or placebo ($N = 36$). All participants were exposed to repetitive loads of stress by performing the serial subtraction task for 5 min every second day during the 8-week intervention. Primary outcome of Trait-STAI and secondary outcomes of State-STAI, total score of STAI, Hamilton Anxiety Inventory (HAM-A), Beck Anxiety Inventory (BAI), blood norepinephrine and adrenocorticotropic hormone (ACTH), salivary cortisol and alpha-amylase, cardiovascular autonomic nervous system (ANS) functional test, and heart rate variability (HRV) test were measured before and after intervention.

Results: After the 8-week intervention, the EGP significantly lowered the score of the Trait Anxiety Scale of the STAI (T-STAI) by 16.8% compared to the placebo ($p = 0.041$). The total score on the STAI decreased by 17.8% in the EGP group and tended to improve compared with that of the placebo group ($p = 0.067$). There were no significant differences in the changes in score of S-STAI, HAM-A, BAI, and other parameters from baseline between the two groups. There was no causal relationship between the ingestion of EGP and adverse drug reactions.

Conclusion: We found that supplementation with EGP reduced "anxiety proneness" in subjects under chronic psychological stress, as shown by a decrease in the score of T-STAI and the tendency for decrease in the total

score of STAI. This result suggests that EGP supplementation can be used as a regimen to safely reduce stress and anxiety; however, more studies are needed to establish the long-term safety and effectiveness.

Introduction

Psychological stress has been demonstrated to be correlated with risk of several diseases. It predisposes people to a wide range of diseases including anxiety and depression (Monroe and Simons, 1991), cardiovascular disease (Rozanski et al., 1999), and cancer (Antoni et al., 2006) and is deleterious to the immune system (Segerstrom and Miller, 2004). Stressful events are thought to influence the pathogenesis of physical disease by causing negative affective states (e.g., feelings of anxiety and depression) that, in turn, exert direct effects on biological processes or behavioral patterns that influence risk of physical disease (Cohen et al., 2007). Another pathway through which stressors influence disease risk is the stressor-elicited endocrine response (Flier et al., 1998). It is widely known that organisms including humans have a physiological adaptive response called the "fight-or-flight" response. Both the hypothalamus-pituitary-adrenocortical axis (HPA) and the sympathoadrenomedullary (SAM) system are activated in response to perceived stressful stimuli and mediate early stages of the adaptive response (Goldstein, 1987; Herman et al., 2003). Psychological stressors cause the HPA and the SAM system to release cortisol and catecholamines for an extended period, which leads to deleterious consequences for an organism, resulting in increased risk for physical and psychiatric disorders (Chrousos and Gold, 1992; Flier et al., 1998). Thus, it is necessary to find ways to reduce the behavioral and biological sequelae of psychological stress by conducting randomized clinical trials.

Gynostemma pentaphyllum (GP) Makino, a climbing vine of the Cucurbitaceae family, which is indigenous to South Korea, Japan, and southern regions of China, has been used as an herbal tea or medicine. It contains various gypenosides including dammarane-type saponins, phyllodulcin, flavonoids, and carotene as main components (Xie et al., 2011). Recently, an extract of GP Makino leaf (EGP) was reported to have anti-stress and anxiolytic effects on chronically stressed mice (Choi et al., 2013; Zhao et al., 2015).

In this regard, the purpose of this study was to conduct a randomized, placebo-controlled clinical trial to determine whether EGP has positive effects on reducing anxiety levels measured by psychometric questionnaires, blood and salivary stress markers, and the ANS functional test in healthy Korean subjects under chronic stressful conditions.

Materials and methods

Ethics approval

The study was conducted according to the Helsinki Declaration and the Guideline for Good Clinical Practice by the International Conference on Harmonization (ICH GCP). The study protocol and informed consent form were reviewed and approved by the Functional Foods Institutional Review Board (IRB) of our hospital (FFIRB number 2013-02-001). Written informed consent was provided by all volunteers before the study began. An independent contract research organization (Healthcare Claims and Management Co., Ltd., South Korea) was responsible for monitoring the study according to the ICH GCP. The protocol was registered in www.clinicaltrials.gov (NCT03277833).

Subjects

The eligible participants were males and females aged 20 to 64 years who were mentally and physically healthy and who reported perceived chronic stress and anxiety. To show positive effects of EGP, participants with high anxiety level showing STAI over the cutoff value of 40 were included. Thus, participants who showed an anxiety STAI score more than 40 and less than 60 at the screening visit were defined as having elevated anxiety and were enrolled.

Subjects were excluded from the study if they had any of the following conditions: (a) depression or other mental disorder diagnosed by DSM-IV Axis 1, (b) chronic physical fatigue, e.g., a night-shift worker or a person with two jobs, (c) any ongoing medical illness or history of chronic disease in the previous 3 years, (d) participation in any other clinical trial with consumption of investigative medicinal products within the past 2 months, (e) allergy or hypersensitivity to any of the ingredients in the test products, (f) conditions that could interfere with successful participation in the study or that could risk subject safety in the opinion of the investigators: women pregnant or breastfeeding; history of alcohol or drug abuse; abnormal laboratory tests; medical or psychological conditions.

Study design

This study was an 8-week, randomized, double-blind, placebo-controlled trial conducted at Clinical Trial Center for Functional Foods (CTCF2) in a tertiary hospital. Treatment arms were comprised of a test group and a placebo group, and 1/1 randomization was performed according to a computer-generated randomization list. The test group ($n = 36$) consumed 400 mg of EGP per day (200 mg, twice a day) for 8 weeks, while the control group ($n = 36$) consumed a placebo in the same way.

The subjects visited the CTCF2 four times during the 8-week intervention period: at the screening visit, visit 1 (randomization), visit 2 (after 4 weeks), and visit 3 (after 8 weeks, end of the study). To verify eligibility of participants at the screening visit, medical history taking; comprehensive physical examination; and questionnaire assessments of STAI, electrocardiography (ECG), laboratory blood tests, and anthropometric measurements were performed. The primary outcome measure was the Trait Anxiety Scale of State-Trait Anxiety Inventory (T-STAI) score, and secondary outcome measures were the scores on the State Anxiety Scale of State-Trait Anxiety Inventory (S-STAI), total score of STAI, Hamilton Anxiety Inventory (HAM-A), Beck Anxiety Inventory (BAI), blood norepinephrine and adrenocorticotropic hormone (ACTH), salivary cortisol and alpha-amylase, and standardized classical Ewing's test battery and heart rate variability (HRV) test, which were performed at the screening visit or visit 1 (baseline). All participants were exposed to repetitive loads of stress by arithmetic tasks during the 8-week intervention. After the 8-week supplementation of test products with the repetitive loads of stress caused by arithmetic tasks, all outcomes were measured again. Repetitive loads of stress were carried out every other day from the day following the study visit to the day before the next visit. An average of 13 repetitive stress load cycles were carried out between visits 1 and 2 and visits 2 and 3 using telephone. The outcome measurements were performed on the visit day, which was one day or two days after the day of stress load. Administration of EGP and control was done two times a day every day during the 8-week intervention period. Safety parameters including adverse events, vital signs and physical examination results (at visits 1, 2, and 3), and ECG and laboratory tests (at the screening visit and visit 3) were assessed. Adverse events are defined as any unwanted medical event that had been tested in participants who ingest a test product at least once. Adverse events include the date and time of onset, the extent and consequences of adverse events, the measures taken in relation to the test product / placebo product, the causal relationship between the

test product and the placebo product, name of any suspected drug used other than the test product, and whether and how adverse reactions were tested. Information on adverse events was voluntarily reported by the subjects and was confirmed through examiner interviews at regular visits.

All participants were asked to maintain their usual lifestyle patterns (e.g., diets, daily physical activities, and sleeping habits) during the 8-week intervention period. Before and after the intervention, dietary intakes and physical activities were investigated in detail by the 3-day record method and WHO international physical activity questionnaire (IPAQ), respectively. All of the above were reviewed at visits 2 and 3. Test products were provided to participants at visits 1 and 2, and the remnants were returned at the subsequent visit and counted for assessment of pill compliance.

Test supplement

The test product, *Gynostemma pentaphyllum*, was obtained from Wonkwang Food Manufacturing Co. (Geochang, Korea), and a voucher specimen of the herbal leaves of GP was deposited at the herbarium of the College of Pharmacy, Chungbuk National University (Cheongju, Korea). The dried leaves of GP were extracted twice with 80% ethanol at 75–85 °C for 3 h, and then the extract was evaporated to dryness (9.6%,w/w) (Novarex, Ochang, Cheongju). The marker compound, ombuoside, was isolated from 80% ethanol extract of *G. pentaphyllum* and identified by comparison with the authentic compound (greater than 98% pure ombuoside). For standardization, the marker compound was ombuoside, and the extract of dried leaves of GP was standardized to a concentration of 1.4 to 2.1 mg/g of ombuoside. The HPLC chromatograms of the 80% ethanol extract of *G. pentaphyllum* and ombuoside are provided in Figs. S1 and S2. The main compounds gynosaponin TN-2 and ombuoside are also indicated in Fig. S1. The compositional profile is presented in Table 1.

Repetitive loading of stress and psychiatric questionnaire survey

Repetitive loads of stress – During the 8-week intervention, every participant was exposed to repetitive loads of stress by performance of arithmetic tasks (Vuksanović and Gal, 2007). Investigators phoned the participants and asked them to perform serial subtraction tasks for 5 min every second day. The questions were different each time, e.g., subtract 7 from 1000 consecutively in the first week and subtract 9 from 1000 in the next week.

The State - Trait Anxiety Inventory (STAI) – This tool consists of separate self-report scales for measuring two distinct anxiety concepts of state anxiety and trait anxiety (Knight et al., 1983). The State Anxiety Scale of STAI (S-STAI) evaluates the current state of anxiety, and the Trait Anxiety Scale of STAI (T-STAI) evaluates relatively stable aspects of individual differences in "anxiety proneness." The STAI has 40 items, 20 for each of the two subscales, and the range of scores for each

subscale is 20–80. Scores on the STAI have a direct interpretation: high scores on both subscales indicate higher anxiety level, while low scores represent lower anxiety. The cutoff value of STAI to detect clinically significant symptoms for the state anxiety scale has been suggested as 39–40 (Knight et al., 1983).

The Hamilton Anxiety Inventory (HAM-A) - The HAM-A is a 14-item clinician-rated instrument designed to assess and quantify severity of anxiety during the previous week. Each item is rated on a five-point Likert-type scale ranging from 0 to 4, with higher scores indicating more severe anxiety. A total score between zero and five represents no anxiety, six to 14 suggests minor anxiety, and scores of 15 or higher indicate major anxiety (Shear et al., 2001).

The Beck Anxiety Inventory (BAI) – This instrument is a 21-item self-report questionnaire designed to briefly measure the frequency of anxiety symptoms such as nervousness, dizziness, and inability to relax, with a focus on somatic symptoms of anxiety over the past weeks (Beck et al., 1988). The total score ranges from 0–63, with 0–9 indicating normal or no anxiety; 10–18, mild to moderate anxiety; 19–29, moderate to severe anxiety; and 30–63, severe anxiety (Julian, 2011).

Biochemical and functional analyses

Venous blood samples were collected after a 12 h overnight fasting and were centrifuged at 3000 rpm for 10 min. Parameters were composed of hematology and other laboratory tests (WBC, RBC, hemoglobin, hematocrit, platelet count, total protein, albumin, ALP, γ-GTP, AST, ALT, BUN, creatinine, glucose, lactate dehydrogenase, creatine kinase, Na^+, K^+, Cl^-, calcium, phosphorus, total cholesterol, triglycerides, and urinalysis). Plasma samples for measuring norepinephrine level were stored at -70 °C until further analysis, and their levels were measured using High Performance Liquid Chromatography (Bio-Rad, USA). Plasma ACTH level was measured the day of sampling using radioimmunoassay by Gamma Counter (PerkinElmer, USA). Saliva samples for measuring salivary cortisol and alpha-amylase levels were collected using a cotton ball between the upper and lower molars. Salivary cortisol level was measured using enzyme-linked immunosorbent assay (ELISA) by ER HS SALIVARY CORTISOL kit (Salimetrics, USA), and salivary alpha-amylase level was measured ELISA by Salivary Alpha-Amylase Assay kit (Salimetrics, USA). The standardized classical Ewing's test battery (Ewing and Clarke, 1986) and HRV (1996) were measured using an automatized machine (DiCAN, South Korea) for the assessment of cardiovascular autonomic changes.

Statistical analyses

Statistical analyses were performed using SAS version 9.2 (SAS Institute, USA). Data are presented as mean ± SD to detect a difference in score of 6.3 (SD = 7.6) in T-STAI between groups with 80% power and a 2-tailed α of 0.05. The sample size of each group was determined to be 36 participants, allowing for a 15% dropout rate. Efficacy parameters were analyzed in the per-protocol (PP) group, and safety parameters were analyzed in the safety group. A Chi-square test was performed to determine differences at baseline in frequencies of categorized variables between the groups. Student's paired *t*-test was used for continuous measures to assess differences between before and after the 8-week intervention period. A linear mixed-effects model was applied to repeated measures data for each continuous outcome variable. Fixed effects were treatment group, treatment visit, and interaction between treatment group and visit. A difference was considered statistically significant when the probability value (*p*-value) was less than 0.05.

Table 1
Composition of test products provided per tablet.

	EGP	Placebo
Component	Content (%)	Content (%)
Extract of GP	50.00	0.00
Crystallized cellulose	49.00	94.49
Gardenia green color	0.00	2.28
Cacao color	0.00	1.82
Turmeric Oleoresin	0.00	0.41
Magnesium Stearate	1.00	1.00
Total	100.00	100.00

EGP: extract of *Gynostemma pentaphyllum*; GP: *Gynostemma pentaphyllum*.

Results

Study population

Among the 75 volunteers screened, 72 who fulfilled the inclusion criteria were enrolled and randomized into two treatment arms. Participants were randomly assigned to either a test group (200 mg of EGP, twice a day, $N = 36$) or a control (200 mg of placebo, twice a day, $N = 36$). The participants were characterized as mentally and physically healthy and having been in chronic stressful conditions with an S-STAI score of 50.50 $\pm$ 6.21 and T-STAI score of 50.18 $\pm$ 7.29 at the commencement of this study. There were no statistical differences between groups at baseline for demographic, anthropometric, or psychometric parameters (Table 2).

During the study, three participants of the EGP group were excluded for consent withdrawal. Among a total of 69 participants who completed the study, two in the EGP group and one in the placebo group were excluded from the data analyses because of lack of pill-compliance and outlier data, respectively. Pill-compliance was 92.4 $\pm$ 10.1% in the EGP group and 91.2 $\pm$ 8.3% in the placebo group, with no statistical difference between groups ($p = 0.452$). Patient flow through the study is depicted in Fig. 1.

There were no significant differences in any of the baseline parameters of dietary intake and physical activity between groups. No values of dietary intake changed within groups, and there were no significant differences between groups in any changes of the above values throughout the 8-week intervention period.

Efficacy

After the 8-week intervention, the average decrease in T-STAI score in the EGP group was almost twice that in the placebo group (-8.74 ± 9.80 and -4.14 ± 8.08, respectively). There was a statistical difference in changes from the baseline to the end of the study between groups ($p = 0.041$) (Table 3). The average decrease in total score of STAI in the EGP group was also almost twice that in the placebo group (-18.55 and -10.63, respectively). However, there was only a tendency for difference between groups in change in total score of STAI from baseline to the end of the study ($p = 0.067$) (Table 3). The scores of S-STAI, HAM-A, and BAI decreased in both EGP and placebo groups and did not show statistical differences between groups ($p = 0.155$, 0.583, 0.307) (Table 3).

The levels of plasma norepinephrine, plasma ACTH, and salivary cortisol did not change in the EGP group after the 8-week intervention

and did not show statistical differences between groups in changes from baseline to the end of the study ($p = 0.694$, 0.628, 0.532) (Table 4). The level of salivary alpha-amylase significantly decreased in both EGP and placebo groups from baseline to the end of the study, without a significant difference between groups ($p = 0.486$) (Table 4).

The scores of the ANS functional test battery and the results of the HRV test did not change in the two groups and did not show statistical differences between groups from baseline to the end of the study.

Safety

No serious adverse events were noted in the safety population. There were 22 mild adverse events in 13 subjects (EGP group: 14 events of 7 subjects; placebo group: 8 events of 6 subjects). None of the adverse events were related to consumption of the supplements or caused study drop-out. All values of vital signs, ECG, hematology and other laboratory tests, and anthropometric data were within the normal ranges throughout the 8-week intervention period. Also, no changes in the above values from baseline to the end of the study were statistically different between groups ($p = 0.219$).

Discussion

In this study, EGP supplementation at a dosage of 400 mg/day for 8 weeks significantly lowered the score of the T-STAI and tended to decrease the total score of STAI compared to the placebo group. No corresponding changes were seen for the S-STAI, HAM-A, BAI, blood and salivary stress markers, cardiovascular ANS functional, and HRV tests between the EGP and placebo groups.

The Spielberger STAI was based upon the theoretical conception of anxiety as having two facets. The T-STAI questionnaire was designed to measure general, consistent, and long-standing feelings of anxiety, whereas the S-STAI measures the current state of anxiety, how the respondents feel "right now, at this moment" (Julian, 2011; Vuksanović and Gal, 2007). According to the results of this study, EGP supplementation significantly reduced the generalized propensity to be anxious or "anxiety prone" including general states of calmness, confidence, and security, without influence on the current and transitory state of anxiety including subjective feelings of apprehension, tension, nervousness, worry, and activation/arousal of the autonomic nervous system in subjects with chronic stress. In this regard, the results of this study suggest that administration of EGP can have a positive effect on reducing an individual's personal anxiety level not influencing the anxiety under stressful condition.

Table 2
Baseline demographic, anthropometric and psychometric characteristics.

	EGP group (n = 36)	Placebo group (n = 36)	Total (n = 72)	p-value[a]
Age (years)	35.28 $\pm$ 10.38	35.33 $\pm$ 10.28	35.31 $\pm$ 10.26	0.982
Sex (M/F)	12/24	14/22	26/46	0.624[b]
Height (cm)	164.22 $\pm$ 8.25	164.28 $\pm$ 9.77	164.25 $\pm$ 8.98	0.979
Weight (kg)	61.35 $\pm$ 12.56	61.91 $\pm$ 13.26	61.63 $\pm$ 12.83	0.855
BMI (kg/m^2)	22.63 $\pm$ 3.67	22.78 $\pm$ 3.29	22.70 $\pm$ 3.46	0.856
T-STAI score	50.08 $\pm$ 7.66	50.28 $\pm$ 7.00	50.18 $\pm$ 7.29	0.911
S-STAI score	51.19 $\pm$ 6.71	49.81 $\pm$ 5.69	50.50 $\pm$ 6.21	0.347
total score of STAI	101.28 $\pm$ 13.25	100.08 $\pm$ 11.54	100.68 $\pm$ 12.35	0.685
BAI score	14.67 $\pm$ 8.34	12.47 $\pm$ 8.17	13.57 $\pm$ 8.27	0.263
HAM-A score	14.72 $\pm$ 8.73	13.33 $\pm$ 7.42	14.03 $\pm$ 8.08	0.470

M: male; F: female; BMI: body mass index; T-STAI: Trait version of State-Trait Anxiety Inventory; S-STAI: State version of State-Trait Anxiety Inventory; BAI: Beck Anxiety Inventory; HAM-A: Hamilton Anxiety Inventory.
Data are presented as mean $\pm$ SD.
[a] p-value was calculated with independent t-test.
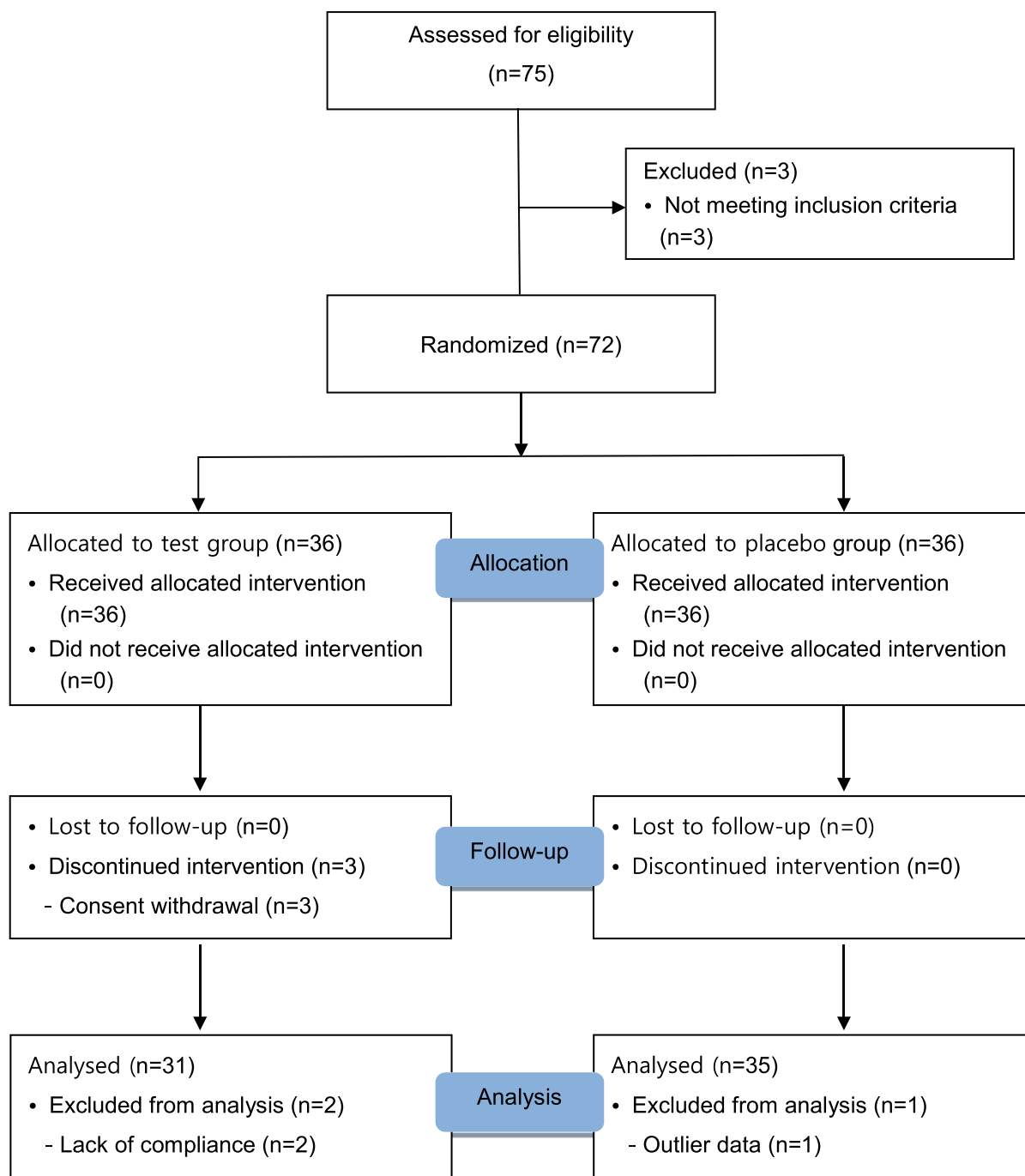[b] p-value was calculated with Chi-square test.

Fig. 1. CONSORT flow diagram.

There are several reasons why the S-STAI, unlike the T-STAI, did not change after the 8-week intervention. First, chronic stress loading during 8-weeks might have not been sufficient to elevate the subjects' stress responses in this study. Previous studies have reported that trait scores remain relatively stable under stressful and non-stressful testing conditions, while state scores show a significant change in the expected direction (Kendall et al., 1976). If there was a sufficient load of chronic stress, at least the S-STAI score would have been increased in the control group; however, in the present study, it decreased even in the control group.

Second, there was no stress load shortly before measuring the S-STAI on the day of visiting the hospital, although every participant was exposed to repetitive loads of stress caused by arithmetic tasks every other day through the 8-week intervention. For example, a previous study on EGP performed the elevated plus-maze and marble burying test after 30 min, followed by exposure to EF stress (Zhao et al., 2015). Another study performed the STAI test immediately after the attentional tasks (Higashiyama et al., 2011). In this study, not measuring the STAI immediately on the last day of the 8-week arithmetic task might have influenced the S-STAI result according to the subjects' individual stress levels on that day.

Recently, ethanol extract of *Gynostemma pentaphyllum* Makino leaf was reported to have anti-stress and anxiolytic effects on mice chronically stressed by electric footshock, elevated plus-maze, and marble burying test (Choi et al., 2013). Mechanisms explaining the anti-stress or anxiolytic effects of the EGP are not clear yet. EGP was reported to modulate the dopamine and serotonin neuron activity, as well as the expression of c-Fos in the brain and the serum level of corticosterone,

Table 3
Effects of EGP supplementation on psychiatric measures.

	EGP group (n = 31)				Placebo group (n = 35)				P-value[b]
	Week 0	Week 8	Difference	P-value[1]	Week 0	Week 8	Difference	P-value[a]	
T-STAI	52.06 ± 8.25	43.32 ± 6.49	−8.74 ± 9.80	<0.0001***	48.46 ± 6.34	44.31 ± 6.82	−4.14 ± 8.08	0.005**	0.041*
S-STAI	52.10 ± 6.53	42.29 ± 8.19	−9.81 ± 9.27	<0.0001***	49.17 ± 5.80	42.69 ± 7.28	−6.49 ± 9.44	0.0001***	0.155
Total score-STAI	104.16 ± 13.46	85.61 ± 14.09	−18.55 ± 17.97	<0.0001***	97.63 ± 11.24	87.00 ± 13.12	−10.63 ± 16.50	0.0006***	0.067
HAM-A	14.74 ± 8.57	11.03 ± 8.54	−3.71 ± 9.23	0.033*	13.71 ± 7.74	11.06 ± 8.46	−2.66 ± 6.10	0.015*	0.583
BAI	15.10 ± 8.21	8.94 ± 8.77	−6.16 ± 10.06	0.002**	12.23 ± 8.32	8.14 ± 7.55	−4.09 ± 6.02	0.0003***	0.307

Data are presented as mean ± S.D.
T-STAI: Trait version of State-Trait Anxiety Inventory; S-STAI: State version of State-Trait Anxiety Inventory; HAM-A: Hamilton Anxiety Inventory; BAI: Beck Anxiety Inventory
[a] p-value was calculated with Paired t-test for within-treatment differences
[b] p-value was calculated with linear mixed effect model for between-treatment differences
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

which has been suggested to be associated with the paraventricular nuclei (PVN) regions and HPA axis (Choi et al., 2013). The levels of dopamine and serotonin in the whole brain, which were significantly decreased by electric footshock stress, were recovered by treatment with EGP for 14 days. In addition, the serum levels of corticosterone and c-Fos expression in the PVN regions, which were markedly increased by electric footshock stress, were reduced by treatment with EGP for 14 days. Our study was conducted to investigate the anxiolytic effect of EGP in humans and to support the anxiolytic effect of previous animal studies (Choi et al., 2013; Zhao et al., 2015).

In this study, stress markers including the levels of plasma norepinephrine, plasma ACTH, salivary cortisol, and salivary alpha-amylase, the scores of Ewing's test battery, and the results of the HRV test did not show any significant differences between groups after an 8-week intervention. The potential explanations for these results are as follows. First, there were no stress loads shortly before sample collection, as well as no serial time-course assessment of plasma norepinephrine or salivary alpha-amylase in this study. Catecholamines including norepinephrine are very quickly released from neuronal endings in the sympathetic nervous system, and the alpha-amylase level is increased just after the beginning of a stressful event (Takai et al., 2004). Second, catecholamines are released by a wide range of stimuli including systemic and local factors (e.g., body temperature, pH), and significant psychosocial studies on α-amylase responsiveness are difficult due to the human body's complexities. Thus, environmental conditions for assessment of plasma norepinephrine and salivary alpha-amylase have to be controlled strictly. However, all participants were treated as outpatients on the day of visiting the hospital, and the environmental conditions could not be controlled strictly as in animal experiments, although the collection of

blood and salivary specimens was performed under standard conditions including overnight fasting, refraining from smoking and caffeine-containing beverages, and collection in a quiet room at a temperature of 19–22 °C in the morning.

Salivary cortisol level was closely correlated with serum cortisol level and thus reliably reflects HPA activity (Kirschbaum and Hellhammer, 1994). Many studies have shown that psychological stress such as public speaking, academic examinations, and suspense film viewing activates the HPA axis to release cortisol and consequently increases the level of salivary cortisol above the resting level (Bassett et al., 1987; Hubert and de Jong-Meyer, 1989; Nejtek, 2002). However, contrary to our predictions, there were no significant decreases in the levels of plasma ACTH and salivary cortisol in the EGP group compared to the placebo group in this study (Table 4). The potential explanations for these results are as follows. First, collection of the specimens for assessment of plasma ACTH and salivary cortisol was performed in an outpatient setting, and only basal levels were assessed. Second, the effect of anti-stress on the level of salivary cortisol remains obscure (Takai et al., 2004) in that only a few results are available on the possible effects of anti-stress behaviors such as relaxation or meditation (Jin, 1992), although several researchers have clearly suggested that various stressful stimuli increased salivary cortisol level (Bassett et al., 1987; Hubert and de Jong-Meyer, 1989; Maes et al., 1998; Nejtek, 2002).

In the revised version of the STAI in 1983 (Vuksanović and Gal, 2007), state scores were 35.7 (SD 10.4) for working men and 35.2 (SD 10.6) for women, and mean trait scores were 34.9 (SD 9.2) for men and 34.8 (SD 9.2) for women. Typical scores for people with diagnosed anxiety was reported in the range 47 to 61 (Antony et al., 2001). Considering that the mean state anxiety score in those with generalized

Table 4
Effects of EGP supplementation on blood and salivary stress markers.

	EGP group (n = 31)				Placebo group(n = 35)				P-value[b]
	Week 0	Week 8	Difference	P-value[a]	Week 0	Week 8	Difference	P-value[a]	
Norepinephrine (pg/ml)	222.58 ± 125.92	187.57 ± 68.60	−35.02 ± 132.02	0.150	222.63 ± 91.21	176.23 ± 58.76	−46.39 ± 101.60	0.011*	0.694
ACTH (pg/ml)	27.91 ± 8.85	27.00 ± 8.64	−0.97 ± 9.27	0.582	30.18 ± 13.78	30.94 ± 11.98	0.76 ± 16.98	0.794	0.628
Cortisol (μg/dl)	0.22 ± 0.09	0.19 ± 0.16	−0.03 ± 0.16	0.331	0.20 ± 0.09	0.20 ± 0.13	−0.01 ± 0.11	0.709	0.532
α-Amylase (U/ml)	64.47 ± 49.19	28.98 ± 18.04	−35.49 ± 43.04	<0.0001***	58.19 ± 42.60	29.41 ± 30.82	−28.79 ± 34.71	<0.0001***	0.486

Data are presented as mean ± S.D.
ACTH: adrenocorticotropic hormone.
[a] p-value was calculated with Paired t-test for within-treatment differences.
[b] p-value was calculated with linear mixed effect model for between-treatment differences.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

anxiety disorder was 52 ± 13, while the trait anxiety score was 55 ± 10, and the mean state anxiety score was 48 ± 12 in those with panic disorder, while the trait anxiety score was 50 ± 10 (Kennedy et al., 2001), subjects in this study showed elevated levels of state and trait anxiety even though they were normal healthy subjects without a diagnosis of anxiety or depression disorder. The state score and trait score of the subjects in this study were quite high compared to the values from a previous study, similar to those with generalized anxiety disorder and panic disorder and lower than those with major depression disorder (Kennedy et al., 2001). This study aimed to determine whether the personal characteristics and high level of state anxiety of a subject with high anxiety proneness could be decreased under chronic stressful conditions after administration of EGP. Therefore, these healthy subjects with elevated anxiety level were included.

In this study, the changes in HAM-A and BAI, which are also psychometric questionnaires for measuring the level of anxiety (Beck et al., 1988; Shear et al., 2001), were not significantly different in the EGP group compared to the placebo group after an 8-week intervention. The scores of HAM-A and BAI decreased in the placebo group and EGP group without statistical difference. The subjects showed relatively high levels of state and trait anxiety, while the scores of BAI and HAM-A showed mild anxiety at baseline evaluation. This discrepancy might be because the STAI and BAI load onto separate factors in factor analysis, suggesting that they represent separate concepts (Higashiyama et al., 2011). Convergent validity between STAI and BAI ranged from 0.47 to 0.64 (Higashiyama et al., 2011), and Pearson's correlation coefficient between STAI and HAM-A was 0.536 for state anxiety and 0.434 for trait anxiety in patients with generalized anxiety disorder (Kennedy et al., 2001).

This study has some limitations. First, the subjects were followed for physical activity, body mass index, and laboratory study before and after the 8-week intervention, but there was no objective measurement of an individual's stress level other than the arithmetic test exposure. Objective measurement of individual stress level such as perceived stress scale (Cohen et al., 1994) would help to show to how much stress the subjects were exposed during the intervention period. Unlike animal studies, stress load in a human study can be highly influenced by individual's daily life situations; therefore, environmental control cannot be as complete as in an animal study, resulting in inter-subject variation regarding stress load.

Second, there were no stress loads shortly before measuring the S-STAI on the day of visiting the hospital. State anxiety scores reflect a significant change according to stressful condition and can detect longitudinal changes, evaluating how respondents feel "right now, at this moment." However the S-STAI was performed at the follow up visit day after the 8-week intervention. Subjects were exposed to chronic stress for 8 weeks, but measurement of state anxiety failed to show the predicted elevation of state anxiety according to exposed stress at the follow up evaluation.

Conclusion

Supplementation with EGP has an effect of decreasing anxiety proneness in subjects prone to high anxiety due to chronic psychological stresses.

Conflict of interest

The authors declare they have no competing financial interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.05.002.

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