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Supplementation of Natto-Pressed Candy (CHOLESWISE Pressed Candy) Improved Blood Pressure: A Randomized, Controlled Trial

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ABSTRACT

The application of plant-based health supplements for reducing blood pressure represents a natural and comprehensive approach involving various botanical extracts aimed at maintaining cardiovascular health. Commonly found in these supplements, such as natto, Haematococcus pluvialis, garlic, and linseed oil, are believed to provide certain benefits in lowering blood pressure. However, comprehensive clinical research on their combined usage must still be available. This trial aimed to explore whether Natto-pressed candy (product name: CHOLESWISE Pressed Candy) can regulate blood pressure. First, using Natto-pressed candy (0.0625%, 0.125%) treated smooth muscle cells and vascular endothelial cells, and then analyzed vascular health genes. Second, the trial recruited 60 subjects with blood pressure (SBP >120 mmHg / DBP >80 mmHg). Subjects took 2 tablets of pressed candy daily for 8 weeks, and blood pressure and blood samples were collected at weeks 0, 4, and 8. The results showed that Natto-pressed candy can increase beneficial vascular health genes, and decrease harmful vascular health genes compared to mock group in vitro. In clinical trial, the average systolic blood pressure was significantly decreased by 9 mmHg compared to baseline (week 0), and significantly decreased by 6.4 mmHg compared to the placebo group. The fibrinogen concentration in the blood was significantly decreased by 13.4% compared to baseline (week 0). Natto-pressed candy, which included natto, *Haematococcus pluvialis*, garlic, and linseed oil, reduced blood pressure, fibrinogen and regulated vascular health genes.

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Introduction

Hypertension is a significant contributor to cardiovascular diseases and can lead to various complications. The probability of developing complications increases with higher blood pressure levels across all age groups, with an average blood pressure exceeding 130/80 mmHg being indicative of potential hypertensive complications [1]. Proper pharmacological management of hypertension in patients can effectively reduce the incidence of ischemic heart disease [2]. Elevated blood pressure levels can lead to endothelial damage, which, in combination with cholesterol deposition or smoking habits, accelerates arterial sclerosis, resulting in less elastic and more prone-to-rupture blood vessels [3]. Simultaneously, the current societal trend involves the development of natural plants as health or nutritional supplements to ameliorate the adverse effects of synthetic pharmaceuticals [4].

Natto is a traditional Japanese fermented food that ferments boiled soybeans with Bacillus natto. It is sticky and has a strong smell and taste. Once fermented, natto produces plenty of active components, including protein, linolenic acid, isoflavones, folic acid, lecithin, vitamin B, B2, E, K, calcium, zinc, magnesium, potassium, and iron. Further, a fibrinolytic enzyme found in natto is called nattokinase. Nattokinase is reported to reduce and prevent blood clot. Natto is a functional food full of physiological functions, including thrombolysis, diabetic prevention, osteoporosis, anti-hypertension, physiology regulation, and gastrointestinal protection. Clinical studies indicated that taking nattokinase for 8 weeks can reduce systolic blood pressure (SBP) and diastolic blood pressure (DBP). *Haematococcus pluvialis* is a unicellular green alga inhabiting freshwater environments, with the capability to accumulate a substantial amount of astaxanthin, resulting in its distinctive red coloration. It is also commonly referred to as "red snow algae." Astaxanthin (AST), a natural pigment in *Haematococcus pluvialis*, is a lipophilic carotenoid belonging to xanthophyll family. The study indicated that administering AST orally may lead to improvements in lipid profile, hypertension, and cognitive function symptoms among individuals with type 2 diabetes. The study indicated that *Haematococcus pluvialis* can prevent LDL from oxidizing, increase HDL, reduce the formation of plaque deposits, restore blood vessel elasticity, and improve blood flow, thereby reducing the chances of high blood pressure, stroke, and heart attack. Garlic is a natural food known for its blood pressure-lowering properties. Garlic is rich in nutritional content and, contains various bioactive compounds, and exhibits blood pressure-lowering effects in hypertensive individuals compared to those with normal blood pressure. The organic sulfur compound allicin, present in garlic, was considered a primary contributor to its blood pressure-lowering properties. Allicin can

inhibit the production of angiotensin II, a compound known to cause vasoconstriction and consequently lead to elevated blood pressure. Additionally, the anti-inflammatory and antioxidant properties of garlic may also contributed to its ability to lower and prevent increases in blood pressure. Flaxseed oil, also known as linseed oil, is a colorless to pale yellow oil obtained from the dried mature seeds of the flax plant (Linum usitatissimum). It is a desirable edible oil that can serve as a nutritional supplement and is a source of alpha-linolenic acid (ALA), an omega-3 fatty acid. ALA improved lipid pattern and insulin sensitivity, attenuate oxidative stress, and decrease prothrombotic and proinflammatory potentials [5-21].

However, there was currently limited research on the comprehensive regulation of blood pressure through natto-pressed candy, which include natto, *Haematococcus pluvialis*, garlic, and linseed oil. The study investigated the use of pressed candy (product name: CHOLESWISE Pressed Candy from Melaleuca, Inc.) to assess its potential for improving blood pressure. First, using Natto-pressed candy (0.0625%, 0.125%) treated smooth muscle cells and vascular endothelial cells, and then analyzed vascular health genes. Second, the trial recruited 60 subjects with elevated blood pressure (SBP >120 mmHg / DBP >80 mmHg). Subjects took 2 tablets of pressed candy daily for 8 weeks, and blood pressure and blood samples were collected at weeks 0, 4, and 8.

Materials and Methods

Cell Culture

Eahy926 cells were cultured in RPMI1640 media (purchased from Gibco, Langley, OK, USA) containing hypoxanthine, aminopterin and thymidin (HAT), 1% penicillin-streptomycin and 10% fetal calf serum. The HASMCs were cultured in smooth muscle cell growth medium supplied with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, USA) and antibiotics (100 U/mL penicillin/streptomycin). The Eahy926 and HASMCs were maintained at 37°C in a humidified cell incubation chamber with 5% CO2.

Quantification of Gene Expressions by Real-Time PCR

The treated Eahy926 and HASMCs were harvested, and total RNA was isolated from cells using an RNA purification kit (Geneaid, Taiwan). DNA-free total RNA was reversely transcribed to cDNA using a SuperScript™ Reverse Transcriptase kit (Invitrogen, Life Technologies Co., CA, USA). Quantitative real-time PCR was conducted using an ABI StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc., CA, USA) and the SYBR Green Master Mix (KAPA Biosystems, MA, USA) for transcript measurements. The gene-specific primers used in this study are listed in Table 4. The GAPDH gene was used as a normalization control.

Clinical Trial Design

This was a randomized, double-blind, placebo-controlled trial. The study was registered in clinicaltrials.gov (No. NCT05182788), and was performed under a protocol approved by the Ethics Committee of Shanghai Fengxian District Central Hospital (Approval Number: 2021-KY-24-01). The study recruited 60 men and women aged ≥ 30 years. The subjects had SBP >120 mmHg / DBP >80 mmHg. Screening procedures included a physical examination, and routine laboratory tests. Eligible subjects provided informed consent before entering the study. 60 subjects were recruited, excluding 9 subjects who were diagnosed with COVID-19 during the trial, and were finally randomly assigned to the placebo group (n = 27) or the pressed candy group (n = 24). All subjects were followed for 8 weeks. Blood chemistry (AST, ALT, BUN, Creatinine, Fibrinogen) were collected at week 0, week 4, and week 8 of the study. All subjects were instructed to adhere to their usual diets, exercise patterns, and lifestyles during the study. Supplements or any other foods specially designed for health purposes were not permitted. Subjects visited the clinic for examinations at week 0, 4, 8 after consuming the placebo or pressed candy. Blood pressure was determined at each visit, and blood samples were obtained for analysis at week 0, 4, 8.

Supplement Formulation

Natto-pressed candy (CHOLESWISE Pressed Candy, Melaleuca, Inc) was included natto, *Haematococcus pluvialis*, garlic, linseed oil, microcrystalline cellulose, maltodextrin, magnesium stearate, water. Placebo group was included microcrystalline cellulose, maltodextrin, magnesium stearate, water.

Blood Pressure Measurement

Blood pressure was measured using an OMRON HEM-7252G-HP automatic blood pressure monitor according to the guidelines for home blood pressure monitoring. Daytime casual blood pressure measurements were performed twice at week 0, 4, 8 after study entry. Before measurement, the subjects rested for 20 min in a sitting position in a warm and quiet room. Trained medical staff who were blinded to the allocation measured blood pressure and pulse rates more than two times at 2-min intervals. Stable and consecutive measurements were averaged.

Measurement of Serum Fibrinogen

Approximately 10 mL of peripheral venous blood was sampled and serum was collected by centrifugation for 15 min at 1000× g at room temperature to measure fibrinogen. The serum was aliquoted and stored at −80 °C until use. According to the manufacturer's instructions, the fibrinogen levels were measured using an enzyme-linked immunosorbent assay kit (Human fibrinogen SimpleStep ELISA kit, Abcam, Boston, MA, USA).

Statistical Analysis

The comparison of measurement results were analyzed by Paired t-test or one-way ANOVA followed by GraphPad Prism, as $p < 0.05$ was considered statistical significance.

Results

Natto-Pressed Candy Regulated Genes Related to Vascular Health In Vitro

Calcification of blood vessels caused the blood vessel walls to become hardened and stiff, which increased the resistance of the blood vessels and caused the heart to need more force to push blood throughout the body, thereby raising blood pressure. Calcium deposits formed a hard material in the walls of arteries, known as atherosclerosis, which further exacerbated the risk of high blood pressure. Studies had shown that the use of β-glycerophosphate (BGP) stimulation could cause calcium ion deposition in smooth muscle cells in the arterial intima and media layers. The bone morphogenetic protein 2 (BMP2) was abundantly expressed in sites of hypertension or atherosclerotic vascular calcification. In this study, BGP was used to stimulate human vascular smooth muscle cells to induce calcium ion deposition, and then treated Natto-pressed candy (0.0625%, 0.125%), and then BMP2 was used as an indicator to detect the content of calcium deposition. The results indicated that Natto-pressed candy can reduce BMP2 expression compared to the mock group (Figure 1). We also detected other vascular health genes, in beneficial vascular health genes such as prostaglandin I2 Synthase (PTGIS), nitric oxide synthase 3 (NOS3), plasminogen activator (PLAT), protein C (PROC). In harmful vascular health genes such as

endothelin 1(EDN1), serine protease inhibitor family E member 1 (SERPINE1), fibroblast growth factor 2 (FGF2), IL-8, vascular cell adhesion molecule 1 (VCAM1). The results indicated that Natto-pressed candy can increase beneficial vascular health genes expression, and decrease harmful vascular health genes expression compared to mock group (Figure 2). These results showed that Natto-pressed candy had cardiovascular protective effects.

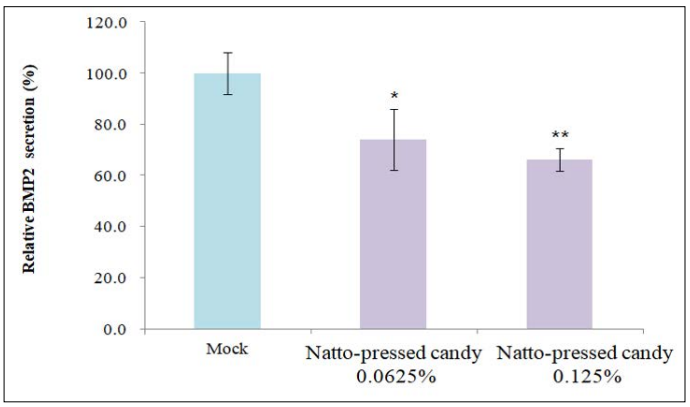

Figure 1: Natto-pressed candy reduced BMP2 expression in vitro. Using β-glycerophosphate (BGP) stimulation calcium ion deposition in smooth muscle cells (HASMC) for 24h, and then treated Natto-pressed candy (0.0625%, 0.125%) for 24h, then analyzed bone morphogenetic protein 2 (BMP2) by quantitative PCR. *, compared with mock group (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$)

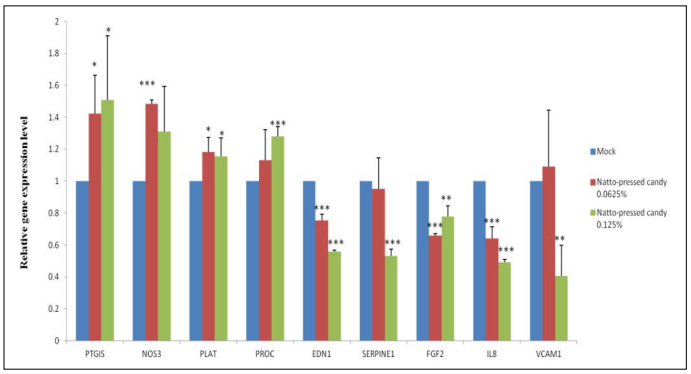

Figure 2: Natto-pressed candy can regulate vascular health genes in vitro. Using Natto-pressed candy (0.0625%, 0.125%) to treat in vascular endothelial cells (EA.hy926) for 24h, and then analyzed vascular health genes. by quantitative PCR. prostaglandin I2 Synthase (PTGIS), nitric oxide synthase 3 (NOS3), plasminogen activator (PLAT), protein C (PROC). In harmful vascular health genes such as endothelin 1(EDN1), serine protease inhibitor family E member 1 (SERPINE1), fibroblast growth factor 2 (FGF2), IL-8, vascular cell adhesion molecule 1 (VCAM1). *, compared with mock group (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$)

Safety Assessment of Natto-Pressed Candy

The subjects had no evidence of gastrointestinal discomfort or other discomforts. Table 1 shows the results of the biochemical analysis. In addition, the values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CRE) were not significantly changed. There was no significant difference between the groups.

Table 1: Subjects' Blood Pressure and Blood Biochemical Values (N=51)

Items	Group	week 0	week 4	week 8
SBP (mmHg)	Placebo Group	134.5±12.7	127.4±11.4***	127.6±3.9*
	Natto Group	130.2±12.5	121.8±12.8**	121.2±9.8**, #
DBP (mmHg)	Placebo Group	86.0±7.1	82.8±8.4**	79.6±6.7***
	Natto Group	85.4±8.4	82.0±11.9*	80.0±7.7***
FIB (g/L)	Placebo Group	2.43±0.47	3.14±3.02	2.58±0.52*
	Natto Group	2.41±0.55	2.53±0.58	2.42±0.09
AST (U/L)	Placebo Group	27.3±10.6	-	26.3±10.9
	Natto Group	23.5±9.3	-	30.9±28.9
ALT (U/L)	Placebo Group	27.0±17.3	-	29.9±23.2
	Natto Group	27.9±23.1	-	42.5±56.4
BUN (mmol/L)	Placebo Group	4.8±1.1	-	5.3±1.7
	Natto Group	5.0±1.3	-	4.9±0.9
CRE (μmol/L)	Placebo Group	64.9±15.9	-	65.9±16.4
	Natto Group	68.4±12.2	-	66.8±11.8

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FIB: Fibrinogen; AST: Aspartate aminotransferase; ALT: Alanine Transaminase; BUN: Blood urea nitrogen; CRE: Creatinine;
Significantly different from the baseline : *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
Significantly different from the placebo : #, $p < 0.05$.

Natto-Pressed Candy Improved Blood Pressure

Next, explore whether natto-pressed candy can regulate blood pressure. After the subjects took pressed candy for 8 weeks, we found that the average systolic blood pressure was significantly decreased 9 mmHg compared to baseline (week 0), and significantly decreased by 6.4 mmHg compared to the placebo group (Table 1 and Figure 3A). The results showed that taking natto-pressed candy can reduce systolic blood pressure. Interestingly, for subjects with systolic blood pressure >130 mmHg, it was found that after taking natto-pressed candy for 8 weeks, average systolic blood pressure was significantly decreased 17.1 mmHg compared to baseline (week 0), and slightly decreased 1.5 mmHg compared to placebo group (Table 2 and Figure 3B). The results indicated that natto-pressed candy can help people with hypertension regulate blood pressure.

Table 2: The Blood Pressure Values of the Subjects with Systolic Blood Pressure Above 130 Mmhg (N=24)

Items	Group	week 0	week 4	week 8
SBP (mmHg)	placebo Group	142.3±9.9	133.1±7.5**	129.5±9.8***
	Natto Group	145.1±9.3	129.4±13.8*	128.0±3.8**
DBP (mmHg)	Placebo Group	87.9±5.8	84.4±8.5**	80.6±5.4***
	Natto Group	91.5±9.6	87.8±15.0	83.1±8.4**

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Significantly different from the baseline : *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

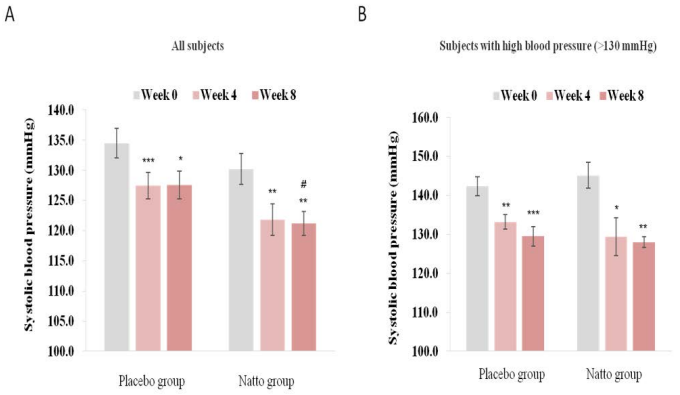

Figure 3: Natto-pressed candy decreased blood pressure. (A) All subjects took pressed candy for 8 weeks, and then analyzed average systolic blood pressure. (B) Subjects with high blood pressure (>130 mmHg) took pressed candy for 8 weeks, and then analyzed average systolic blood pressure. *, compared with baseline (week 0) (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). #, compared with placebo group (#, $p < 0.05$)

Natto-Pressed Candy Decreased Fibrinogen

Elevated plasma fibrinogen level was positively associated with prevalent hypertension both among men and women, and excessive fibrinogen concentration in the blood may cause arterial or venous embolism, increasing the risk of myocardial infarction and stroke. To explore whether natto-pressed candy can decrease fibrinogen, we collected blood and analyzed fibrinogen expression by Enzyme-linked immunosorbent assay (ELISA). Compared with the placebo group, taking natto-pressed candy for 8 weeks can help maintain the fibrinogen concentration in the blood and prevent vascular embolism (Table 1 and Figure 4A). At the same time, for subjects with high Fibrinogen (Fibrinogen > 3.5 g/L), it was found that the concentration of fibrinogen in the blood was significantly decreased by 13.4% compared to baseline (week 0) (Table 3 and Figure 4B).

Table 3: The Blood Fibrinogen Levels of the Subjects with Fibrinogen above 3.5 G/L (N=5)

Items	Group	week 0	week 4	week 8
FIB (g/L)	placebo group	3.29±0.18	3.36±0.49	3.46±0.46
	Natto group	3.82±0.57	3.28±0.31	3.31±0.05

FIB: Fibrinogen

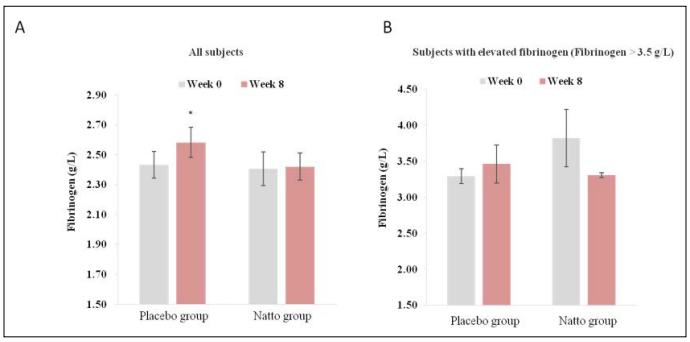

Figure 4: Natto-pressed candy decreased fibrinogen. (A) All subjects took pressed candy for 8 weeks, and then analyzed fibrinogen. (B) Subjects with elevated fibrinogen (Fibrinogen > 3.5 g/L) took pressed candy for 8 weeks, and then analyzed fibrinogen. *, compared with baseline (week 0) (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). #, compared with placebo group (#, $p < 0.05$)

Table 4: Gene, primer orientation, primer sequence (5′to 3′)

Gene	Primer	Sequence (5′to 3′)
Prostaglandin I2 Synthase (PTGIS)	Forward	GGCAGACGGGCGAGAAT
	Reverse	CCCCCCAGGGCATGTT
Nitric Oxide Synthase 3 (NOS3)	Forward	GTTCACCTACATCTGCAACCACAT
	Reverse	AATGCAGAGCTCGGTGATCTC
Plasminogen Activator (PLAT)	Forward	CAGCCTCAGTTTCGCATCAA
	Reverse	CGGTATGTTCTGCCCAAGATC
Protein C (PROC)	Forward	CAAGTAGATCCGCGGCTCAT
	Reverse	CAGCGCCGCAGGTCATAC
Endothelin 1(EDN1)	Forward	CACGTTGTTCCGTATGGACTTG
	Reverse	CCTTTCTTATGATTATTCCAGTCTTTCTC
Serine Protease Inhibitor Family E Member 1 (SERPINE1)	Forward	GTGGAGAGAGCCAGATTCATCAT
	Reverse	CTGCCGTCTGATTTGTGGAA
Fibroblast Growth Factor 2 (FGF2)	Forward	TGTGCTAACCGTTACCTGGCTAT
	Reverse	TTCTGCCCAGGTCCTGTTTT
IL-8	Forward	ACTGAGAGTGATTGAGAGTGGAC
	Reverse	AACCCTCTGCACCCAGTTTTC
Vascular Cell Adhesion Molecule 1 (VCAM1)	Forward	GTTGAAGGATGCGGGAGTATATG
	Reverse	TCATGTTGGCTTTTCTTGCAA

Discussion

In this study, we found that natto-pressed candy, which included natto, Haematococcus pluvialis, garlic, and linseed oil, reduced blood pressure and fibrinogen, and regulated vascular health genes. Furthermore, it was particularly effective in significantly lowering blood pressure in individuals with hypertension. Currently, there are numerous plant extracts believed to possess blood pressure-regulating effects. Some of these plant extracts had been extensively researched and were considered to have the potential to lower blood pressure, such as enzyme inhibitors and vasodilators. Therefore, using plant extracts to regulate blood pressure is an effective strategy.

It was demonstrated that nattokinase, derived from natto, exhibited greater fibrinolytic activity in vivo compared to plasmin, and demonstrated the ability to hydrolyze fibrin [22]. The oral administration of nattokinase was found to facilitate the release of tissue plasminogen activator from vascular endothelial cells and inhibit the levels of plasminogen activator inhibitors [23]. Furthermore, a recent study provided a novel insight, suggesting that nattokinase could prevent arteriosclerosis and thrombosis through its anti-inflammatory effects [24].

AST, a carotenoid commonly encountered in marine environments, was most abundantly present in Haematococcus pluvialis (at concentrations ranging from 10,000 to 40,000 mg/kg) and various species of salmon (at concentrations ranging from 1 to 58 mg/kg) [10]. Extensive investigations conducted through animal and cell culture studies consistently indicated that AST demonstrated superior antioxidant properties compared to other carotenoids, such as zeaxanthin, lutein, canthaxanthin, and β-carotene, exhibiting an approximately tenfold increase in potency [25]. Furthermore, its antioxidant capability exceeded vitamin E's by approximately 500 times [26]. A randomized, double-blind, placebo-controlled trial revealed that a 12-week 20 mg/d of AST supplementation regimen reduced LDL-C and ApoB levels in overweight individuals [27]. Another randomized controlled trial (RCT) focused on type 2 diabetic patients, which documented the capacity of AST to improve lipid profiles by lowering levels of TG, TC, and LDL-c. Additionally, AST exhibited an ancillary benefit by reducing levels of inflammatory cytokines, including IL-6, tumor necrosis factor-α, and von Willebrand factor [28]. Moreover, several meta-analyses were conducted to scrutinize the collective impact of AST in combination with other bioactive compounds such as folic acid, berberine, policosanol, and coenzyme Q10 [29].

The ability of garlic to reduce blood pressure was attributed to its production of hydrogen sulfide and its allicin content [17]. Allicin was reported to exhibit inhibitory effects on angiotensin II and vasodilatory properties [30]. Garlic was reported to have reduced blood pressure by influencing various mechanisms, such as the prostaglandin system, renin-angiotensin system, and renal tubular transport mechanisms [31]. Gammaglutamylcysteines, compounds found in garlic, have shown the potential to lower blood pressure by inhibiting angiotensin-converting enzyme [32].

Alpha-linolenic acid (ALA), an omega-3 fatty acid found in linseed oil, has been studied for its potential mechanisms in blood pressure regulation [33]. ALA reduces inflammation by modulating leukocyte activity and decreasing the production of inflammatory mediators, thereby alleviating the inflammatory state of blood vessel walls [34]. This is crucial in preventing atherosclerosis and vascular damage and maintaining normal blood pressure levels. ALA in linseed oil may exert its effects by promoting the generation of nitric oxide (NO) [35]. NO is a potent vasodilator, facilitating the relaxation of blood vessel walls, increasing vascular capacity, and consequently reducing blood pressure [36]. ALA may influence NO synthesis and release in endothelial cells, promoting vasodilation and regulating blood pressure [37].

Furthermore, components in linseed oil may impact kidney function, participating in plasma volume and renal sodium excretion regulation, thereby influencing blood pressure. Specifically, it may inhibit the renin-angiotensin-aldosterone system in the kidneys, affecting tubular sodium reabsorption and modulating fluid balance [38]. Consistent with our results, natto-pressed candy made with natto, Haematococcus pluvialis, garlic, and linseed oil demonstrated the ability to lower blood pressure and fibrinogen levels. Notably, its efficacy significantly lowers blood pressure in patients with hypertension.

In summary, this unique natto-pressed candy provides a delightful taste and combines a variety of natural ingredients to form a health-promoting treat. Its distinctive formula is designed to lower blood pressure without causing harm to the human body, making it an enjoyable and healthy snack option.

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