Official Title of the study:
Effect of Sea grapes-antioxidants Extract in Obese Men : 4 Weeks Randomized-Double Blind Controlled Trial

Document Type: Study Protocol with SAP and/or ICF

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INTRODUCTION

Peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1 alpha (PGC-1α) plays a role in many substantial metabolic processes, energy homeostasis and promotes muscle tissue remodeling, which is oxidative and less glycolytic (Liang & Bangsal, 2006). In conjunction with PGC-1α on mitochondrial respiration (regulating respiration) in muscle cells, this co-activator also induces gene expression for the insulin sensitive glucose transporter (Glut-4) and increases glucose uptake (Puigserver & Spiegelman, 2003; S. Yang et al., 2020). PGC-1α also induces brown adipose tissue (BAT) which usually declines in aging (Puigserver & Spiegelman, 2003). BAT which is a brown adipocyte contains many small droplets and a much higher number of mitochondria (containing iron), which gives the tissue its color (Cedikova et al., 2016). Brown fat also contains more capillaries than white fat and this supplies tissues with oxygen and nutrients and distributes the heat generated throughout the body (Cedikova et al., 2016). Noticing the function of PGC-1α in the thermogenic brown adipose tissue (BAT) program, the regulation of Glut-4 and mitochondrial oxidation in the muscle, and the dominant role of PGC-1α in hepatic gluconeogenesis along promoting muscle tissue remodeling activity, all of which suggest that this

co-activator can be a target for antiobesity, antidiabetic and antiaging drugs (Chronic Diseases and Aging) (Liang & Ward, 2006; Puigserver & Spiegelman, 2003; S. Yang et al., 2020).

Sea grape (Caulerpa racemosa) or lawi-lawi (local Indonesian term) is a species of green algae classified as *Caulerpaceae* family that had be found in the waters around Sulawesi (Pakki et al., 2020). Sea grapes are harvested intensively because they are an important source of macronutrients and micronutrients, especially in East and Southeast Asia (commercially grown in ponds and consumed in parts of the Philippines, Indonesia and Vietnam) as the main source of traditional diets (Chen et al., 2019), although it is still used for health products. Several studies have shown that sea grapes contain several bioactive components, such as protein, polysaccharides, polyphenols, flavonoids, and antioxidants (P. Yang et al., 2015; Yep et al., 2019; Taslim & Fahrul, 2021). In addition, sea grapes contain high antioxidant levels, and may be potential as functional foods or nutraceuticals (Tanna et al., 2020; Yep et al., 2019; Nurkolis et al., 2021). Doses of 150 mg/kg BW (30 mg/200g BW) sea grape extract in a previous pre-clinical trial, showed that it could improve blood glucose, total cholesterol and serum PGC-1 levels in rats fed a diet high in fat and cholesterol (Kuswari et al., 2021). In addition, you also have hepatoprotective activity (aka non-toxic) in diabetic mice (Qudus B Aroyehun et al., 2020). Previous research (Kuswari et al., 2021), is an in vivo (Pre-clinical) study that has not represented the benefits or efficacy of sea grape extract on variables tested in humans. Therefore, this clinical trial was conducted to support the effect of sea grape extract-antioxidant on blood glucose, total cholesterol, and PGC-1 levels in obese men for 4 weeks using a Randomized-Double Blind Controlled Trial.

Methods

This clinical trial study was conducted at Manado City, Indonesia.

Extract Creation

Fresh sea grape (*Caulerpa racemosa*) is collected from the shallows (5-10 meters above sea level) of Mantehage seawater, North Sulawesi, Indonesia. Botanical identification and authentication are confirmed in the department of pharmacology, faculty of mathematics and natural sciences, Sam Ratulangi University, Indonesia. Specimens are collected for future reference. Sea grapes (Whole body) is thoroughly rinsed with water, dried with air at room temperature, and in a 40^{o} C oven, then smoothed with an electric grinding. Furthermore, the extract preparation, coarse powder (1 kg) is macerated in ethanol 96% for 72 hours with each extraction carried out in triple, which results in a yield of 34%. The extract is roughly filtered with Whatman 41 filter paper. The total filtrate is glued and evaporated at 40^{o} C with the RV 8 IKA rotary evaporator under reduced pressure (100 millibars) for 90 minutes and evaporated in a 40^{o}C oven to produce the viscous extract. The extract is stored in the refrigerator at a temperature of 10°C until used in research. The extract powder is encapsulated in capsules no. 000.

Dose Conversion

The best and significant dose in a preliminary study (Pre-clinical) in Ratus novergicus rats is 150 mg/kg BW (Kuswari et al., 2021). Afterward, the doses in this clinical trial study applied the table (Table 1) "Evaluation of drug activities: pharmacometrics, ed. by D.R. Laurence and A.L. Bacharach" (Laurence & Bacharach, 2013). The conversion factor from mouse to human was 56.0. However, the dose is still within (mg/kg BW) while the conversion factor for rats must be per 200gram BW, so 150 divided (1000:200=5) then you get 30 mg/200gram BW. Therefore, it means: Human Dose = Rat Dose x Conversion Factor = 30 x 56,0 = 1680 mg/70kg BW

= **1,68 g/70kg BW**

Antioxidant Test on Sea Grape Extract

Antioxidant activity is determined by using DPPH (Batubara et al., 2015). Each sample consisting of 100 L was placed in microplate 96 and added 100 L DPPH 0.3 mM, then incubated for 30 minutes in a dark room. Sample absorption was measured using an ELISA reader at wavelength 517 nm. Antioxidant activity is calculated by equation 1 as follows:

$$Inhibition\ (\%) = \frac{(A0-A1)}{A0}\ x\ 100\%$$

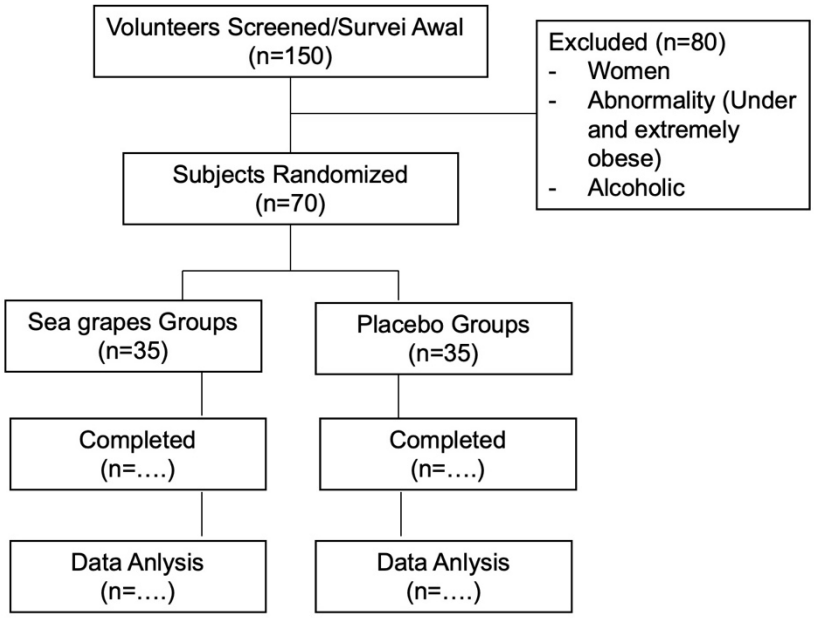
Explanation:

A0 = Blank Absorption, A1 = Standard or Sample Absorption

The results of the antioxidant activity test against DPPH resulted in a value of 45.66 ± 0.55% (Triplicates/Triplo).

Caulerpin (PubChem ID: 5326018) with molecular formula: C24H18N2O4 is a typical secondary metabolite compound belonging to the algae genus Caulerpa Sp and is a bioactive compound from a group of alkaloid compounds that also functions as antioxidants (de Souza et al., 2009; Aguilar-Santos., 1970; Gorbi et al., 2014). Therefore, a further test was carried out, namely the antioxidant-caulerpin levels in the extract (sample) using Liquid Chromatography - Mass Spectrometry (LC-MS) and obtained 398.13278 Mass (397.12257 m/z). This shows that it is true that there is an antioxidant compound-caulerpin in the sample extract.

Study Protocol with SAP and/or ICF



The study was a 4-week, randomized, double-blind, placebo-controlled clinical trial followed by a 1-week screening period. Participants who respond to the invitation and meet the entry criteria during the telephone screening interview are scheduled for the initial visit. Evaluation during the initial visit includes physical examination in the form of BMI (Body Mass Index) based on Asia-Pacific guidelines and blood parameter screening tests in the form of blood glucose, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol, and PGC-1α were performed on all participants within 1 week of the initial screening. A random number between 1 and 70 was generated for each subject and registered participants were scheduled for their first visit and randomly assigned to the sea grape extract group (n=35) or placebo (n=35). Sea grape extract/placebo tablets/capsules were given to participants every 1 week

(1 day of consumption per oral) 15 Minutes before eating (According to the diabetes drug guidline consumtion).

During the 4-week intervention period, participants were asked to continue their usual diet and not to consume functional foods or other dietary supplements. Anthropometry (BMI), Waist Hip Ratio (WHR) , blood parameters, urine profile (Marker of toxicity), and nutrient intake of both groups were measured before and after the intervention period. During the trial phase, all participants were instructed to maintain their normal diet and physical activity. Each week participants were asked to report assessments of side effects or changes in training, lifestyle, or diet; and to evaluate tablet compliance.

Subjects/Participants

The study participants were recruited during 2021 at RSUP Prof. Dr. RD. Kandou (Manado, Republic of Indonesia). A total of 150 participants agreed to participate in the study. Only individuals men who are obese (BMI $\geq$ 25 kg m^{-2} and Waist Hip Ratio (WHR) $\geq$ 0.90) according to Asia-Pacific guidelines and have not been diagnosed with other diseases were included in the study. To meet guidelines for evaluating the efficacy of functional food referring to the Korea Food and Drug Administration (because Indonesia does not yet exist and Koreas belong to one Asian region so used) (KFDA Guidline, 2021), very obese participants (BMI $\geq$ 30 kg m−2) were not included in the study.

In total, 70 participants met the research criteria (age 29.98 ± 3.26 year; weight, ... ± .... kg; BMI, ... ±.... kg m^{-2}) and were randomly divided into two groups (n = 35 each) given sea grape extract 1.68 g/70kg BB day^{-1} or placebo (1.68 g/70kg BB day^{-1}). The exclusion criteria for this study are as follows: (a) significant weight variation (over 10%) in the last 3 months; (b) a history of cardiovascular disease including arrhythmia, heart failure, or myocardial infarction, diabetes

mellitus (DM) and the use of pacemakers; (c) a history of conditions that may interfere with test products or inhibit their absorption such as gastrointestinal diseases (Crohn's disease) or surgeries that have been experienced (caesarean section or enterocele); (d) participation in other clinical trials in the last 2 months; (e) abnormal liver function; (f) a history of kidney disease (eg, acute or chronic renal failure and nephrtic syndrome); (g) undergo antipsychotic drug therapy within the last 2 months; (h) laboratory test results as well as medical or psychological conditions that may interfere with successful participation in research assessed by researchers; (i) a history of alcohol or substance abuse; and (j) allergy or hypersensitivity to any of the ingredients in the test product; (k) is neither a passive nor an active smoker. All participants give written consent before the investigation begins. The research protocol was submitted to the Health Research Ethics Commission (KEPK) of The General Hospital of Education Prof. Dr. RD. Kandou (Manado, Republic of Indonesia) online at http://sim-epk.keppkn.kemkes.go.id. All protocols referred to The Declaration of Helsinki and The Council for International Organizations of Medical Sciences (CIOMS).

Measurement of Efficacy Results

A total of 70 participants who met the research criteria were asked to visit the clinic once every 1 week (0th, 1st, 2nd, 3rd, and 4th/last week of the study period) with a total of 5 clinical visits including initial examination. During each visit, the use of the supplement is currently reviewed and symptoms or side effects are noted. During screening visits, demographic and lifestyle information is collected (age, alcohol consumption, and smoking habits). Medical history is taken and urine turbidity tests are carried out.

The following parameters are assessed; Weight, Height, WHR and BMI during each visit. Blood samples were collected after a minimum of 12 hours of fasting during initial screening as

well as in the 0, 2nd and 4th weeks of the intervention period to obtain blood glucose profiles, total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and PGC-1α. Blood samples were taken from the arm vein. The arm vein to be punctured is cleaned with 70% alcohol and allowed to dry. A tourniquet is placed on the upper arm to expose and slightly accentuate the veins. The skin is stretched over the vein with the fingers of the left hand so that the vein cannot move. The skin is pierced with a needle and syringe with the right hand until the tip of the needle enters the lumen of the vein. The tourniquet is removed and slowly withdraw the syringe suction until the desired amount of blood is obtained. A 70% alcohol swab is placed over the needle and the syringe and needle are removed. The needle is removed from the syringe and the blood sample is transferred into a vacutainer tube. The sample is then centrifuged for 20 minutes at a speed of 3000 rpm. Finally, the serum is collected for analysis of blood glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and PGC-1α. Blood glucose, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and cholesterol levels were tested using COBAS Integra® 400 plus analyzer (Roche). The sample was washed with Phosphate Buffered Saline (PBS, pH 7.4) 1% until the liquid was clear. The sample was centrifuged at 3000 rpm for 20 minutes to obtain pellets and supernatants. The supernatant is taken for PGC-1α examination. The concentration of PGC-1α is measured using the Human PGC-1α (PGC1 alpha (PPARGC1A) (NM_013261) Human Untagged Clone kit.

Evaluation of Safety and Diet

The safety of the extract is assessed by the following procedure. Urine Test Strips 10 Verify Parameters, Parameters examined are:

- Glucose (50 -100 mg / dl)
- Protein (7.5 - 15 mg / dl)

- pH (5-9)
- Leukocytes (9-15 leu / ul)
- Nitrite (0.05-0.1 mg/ dl)
- Urobilinogen (0.2-1.0 mg / dl)
- Blood (5-10 Ery/ul)
- Ketones (2.5-5 mg/dl)
- Bilirubin (0.4-1.0 mg/ dl)
- Specific Gravity (SG) (1,000-1,030)

Test the urine parameters above using *Urinalysis 10U Reagent Strips Verify* (REF U031-102 exp. 2022-03-05). Pulse rate and blood pressure are measured on each visit after a 5-minute break using intelliVue MP70 (Philips, Netherlands). Personal reports are also recorded at these times. We kept the subjects maintaining their usual diet and activity, and all participants completed diet records on each visit to the clinic during the intervention period to evaluate their energy intake and diet quality. Food intake data were analyzed by a nutritionist (Melvin Junior Tanner, S.Gz).

Statistical Analysis

Statistical analysis is performed using SPSS software, version 26 (IBM Corporation). Fixed effects include treatment groups, treatment visits, and interactions between treatment and visit groups. The value $p<0.05$ is considered statistically significant.

Table 1. Dose Conversion of Experimental Animals with Humans

	Mice 20 g	Rat 200 g	Guinea Pig 400 g	Rabbit 1,5 kg	Cat 2 kg	Ape 4 kg	Dog 12 kg	Human 70 kg
Mice 20 g	1,0	7,0	12,25	27,8	29,7	64,1	124,2	387,9
Rat 200 g	0,14	1,0	1,74	3,9	4,2	9,2	17,8	56,0
Guinea Pig 400 g	0,08	0,57	1,0	2,25	2,4	5,2	10,2	31,5
Rabbit 1,5 kg	0,04	0,25	0,44	1,0	1,08	2,4	4,5	14,2
Cat 2 kg	0,03	0,23	0,41	0,92	1,0	2,2	4,1	13,0
Ape 4 kg	0,016	0,11	0,19	0,42	0,45	1,0	1.9	6,1
Dog 12 kg	0,008	0,06	0,10	0,22	0,24	0,52	1,0	3,1
Human 70 kg	0,0026	0,018	0,031	0,07	0,076	0,16	0,32	1,0

SPECIAL MEDICAL APPROVAL LETTER (INFORM CONSENT)

I Signed Below:
Name:
Gender (M/F):
Age/Date of Birth:
Address:
Phone:
Stating in fact from *Myself/*As a Parent/*Husband/*Wife/*Child/*Guardian of:
Name:
Gender (M/F):
Age/Date of Birth:
Address:
Phone:

Hereby agree/refuse to be involved as a volunteer or participant in the study "Supplementation of sea grape extract on blood glucose levels, total cholesterol, and PGC-1".
From the explanation given, I have understood everything related to the disease, as well as the medical measures that will be taken and the possibility of post-action that can occur according to the explanation given.

Manado, ……..…2021

Doctor,	Subjects,
Sign	Sign
(……)	(……..)

* Strikethrough The Unnecessary

KOMITE ETIK PENELITIAN KESEHATAN
HEALTH RESEARCH ETHICS COMMITTEE
RSUP PROF. DR. R. D. KANDOU MANADO
RSUP PROF. DR. R. D. KANDOU MANADO HOSPITAL

KETERANGAN LAYAK ETIK
DESCRIPTION OF ETHICAL APPROVAL
"ETHICAL APPROVAL"

No.142/EC/KEPK-KANDOU/VIII/2021

Protokol penelitian yang diusulkan oleh :
The research protocol proposed by

Peneliti utama *Principal In Investigator*	:	Fahrul Nurkolis
Nama Institusi *Name of the Institution*	:	Universitas Islam Negeri Sunan Kalijaga Yogyakarta

Dengan judul:
Title

"Pengaruh Ekstrak Antioksidan Anggur Laut pada Pria Obesitas : Randomized-Double Blind Controlled Trial 4 Minggu"

"Effect of Sea grapes-antioxidants Extract in Obese Men : 4 Weeks Randomized-Double Blind Controlled Trial"

Dinyatakan layak etik sesuai 7 (tujuh) Standar WHO 2011, yaitu 1) Nilai Sosial, 2) Nilai Ilmiah, 3) Pemerataan Beban dan Manfaat, 4) Risiko, 5) Bujukan/Eksploitasi, 6) Kerahasiaan dan Privacy, dan 7) Persetujuan Setelah Penjelasan, yang merujuk pada Pedoman CIOMS 2016. Hal ini seperti yang ditunjukkan oleh terpenuhinya indikator setiap standar.

Declared to be ethically appropriate in accordance to 7 (seven) WHO 2011 Standards, 1) Social Values, 2) Scientific Values, 3) Equitable Assessment and Benefits, 4) Risks, 5) Persuasion/Exploitation, 6) Confidentiality and Privacy, and 7) Informed Concent, referring to the 2016 CIOMS Guidelines. This is as indicated by the fulfillment of the indicators of each standard.

Pernyataan Laik Etik ini berlaku selama kurun waktu tanggal 23 Agustus 2021 sampai dengan tanggal 23 Agustus 2022.

This declaration of ethics applies during the period August 23, 2021 until August 23, 2022.

August 23, 2021
Professor and Chairperson,

Prof. Dr. dr. Max F. J. Mantik, Sp.A(K)

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