University of Guadalajara

Health Sciences University Center

High Resolution Microscopy Laboratory

Olive leaf extract effects on meta-inflammation and anxiety symptoms in women with excess weight

Document date: March 20th, 2024.

Approval date: April 17th, 2024.

Abstract

Background: During anxiety, inflammation and oxidative stress (OS) occur in the brain. OS and inflammation create a positive feedback loop that prolongs anxious symptomatology. A low, sustained and similar inflammatory process occurs in overweight individuals, called meta-inflammation. The excessive presence of weight and the associated meta-inflammation leads to changes in brain architecture at the organic and psychological level. Being a woman increases the probability of suffering from anxiety and overweight women have double chances of suffering from anxiety disorders due to psychological and physiological characteristics. In Guadalajara's metropolitan area (GMA), 75.9% of women suffer from excessive weight (BMI ≥25 kg/m^2). The overweight prevalence (≥25-<30 kg/m^2) represents 36.7% and obesity's (≥30 kg/m^2) prevalence 39.2% from that 75.9%; hence, they maintain a higher prevalence of anxiety disorders compared to men.

Due that excessive weight and anxiety disorders are modifiable risk factors on the rise in our population, different innovative ways have emerged to seek to treat them, such as supplementation with natural products and functional foods with antioxidant and anti-inflammatory effects, among them, olive leaf extract (OLE); Studies report good results of its anxiolytic effect in animal models; in addition, antioxidant and anti-inflammatory effects are attributed to it in both animals and humans.

Objective: Based on this information, the present study aims to evaluate the effects from the supplementation of OLE on meta-inflammation and anxiety symptoms in GMA women living with excessive weight, in their comorbidities and associated components.

Methodology: A randomized double-blind placebo clinical trial is proposed. The study groups will be conformed by women living with excessive weight from the GMA who meet the following criteria: age ≥18- ≤40 years, body mass index (BMI) ≥25- ≤40 kg/m2, blood pressure ≤ 129 mmHg systolic and ≤80 diastolic; anxiety symptomatology score 6 to 14 (mild) for the Hamilton Anxiety Scale (HAS).

70 women at the University Center for Health Sciences (CUCS) who meet the inclusion criteria will be invited to participate. Two groups will be randomly formed consisting of 35 women with excessive weight and anxious symptoms each (in accordance with the BMI and HAS score, respectively); one will receive cellulose as a placebo and the other 750 mg/day of OLE orally. The Beck Anxiety Inventory (BAI) and the HAS will be administered to all participants every 30 days for 3 months; On the other hand, adherence to treatment will be reviewed on a biweekly basis. To evaluate meta-inflammation, the levels of leptin, IL-6, TNF-α and cortisol in serum will be quantified using the ELISA technique; weight, height, age and components associated with excessive weight such as percentage of fat and muscle, lipid profile (LDL cholesterol; LDL-c, total cholesterol; TC, triglycerides; TG and HDL-cholesterol; HDL-c) and glycosylated hemoglobin will be also evaluated; these evaluations will be taken at the beginning and at the end of the study, in order to determine if OLE has effects on them.

Based on the reported literature, in this trial, it's expected to observe a reduction in levels of proinflammatory cytokines, cortisol and leptins; consequently, this decrease would also reflect lower anxiety symptomatology scores. Regarding the components associated with excessive weight, a reduction in LDL-c, TC and TG levels is likely to be observed.

Background

Anxiety and stress

The term anxiety can be defined as a set of diffuse emotions caused by a situation that is potentially harmful with the probability of harm occurring being uncertain. When anxiety is sustained for a long time or is produced by non-threatening stimuli, is considered pathological (Daviu, 2019); It is accompanied by nervousness, worry, uneasiness, anticipation and mental tension (Bystritsky, 2014).

Stress is one of the main causes of anxiety and can produce inflammation. It is characterized by a poor relationship between the individual and the environment, in which the former is perceived to be surpassed by

the latter and its well-being or balance is at risk. Stress can lead to anxiety and anxiety can also be considered a type of stressor (Saccaro, 2021).

Anxiety, inflammation and oxidative stress; molecular mechanisms.

Anxiety disorder is closely associated with inflammatory processes. The onset of central inflammation in anxiety occurs due to disruption of the blood-brain barrier (BBB). Stress decreases tight and adherent junctions; increases the production of pro-inflammatory components (TNF-α, IL-6, IL-1β, IL-17A and IL-23) that activate microglia, astrocytes and affect endothelial permeability (Welcome, 2020).

The increase in endothelial permeability of the BBB, the production of pro-inflammatory cytokines and stress damage favors the production of lipid peroxides and reactive oxygen species (ROS), generating oxidative stress (Welcome, 2020; Salim, 2012). Oxidative stress further promotes the production of proinflammatory cytokines by astrocytes and microglia through the activation of the transcription factor NF-kβ (its activation in the hippocampus, amygdala and locus coeruleus (LC) is related to anxious behavior) (Salim, 2012; Berríos-Cárcamo, 2020); cytokines perpetuate the activation of these glial cells and promote the production of oxidative stress. Thus, oxidative stress and inflammation generate a positive feedback loop that perpetuates anxious behavior (Berríos-Cárcamo, 2020).

Excessive weight and meta-inflammation

With excessive weight, a chronic, low-intensity, inflammatory process occurs, defined as meta-inflammation (Blancas-Flores, 2010). It happens with the increase in adipose tissue and two processes are identified: increase in its size (hypertrophy) and quantity (hyperplasia). The inflammatory response begins in adipocytes and subsequently they recruit macrophages, mainly of the M1 type, among other immune cells, which favor the production of pro-inflammatory molecules and increases the recruitment of more macrophages, producing a positive-feedback loop (Blancas-Flores, 2010; Xu, 2013). Among the substances that adipose tissue can release are proinflammatory molecules, hormones, acute phase proteins and leptins (Manzur, 2010; Calder, 2011); it is capable of producing IL-1, an antagonist of the IL-1 receptor, IL-6, IL-7, IL-18, IL-10 and TNF-α. (Calder, 2011).

In turn, the increased number of adipocytes favors the deoxygenation of cells furthest from the vascular zone. This promotes stress in the endoplasmic reticulum and produces oxidative stress (Blancas-Flores, 2010). Thus, similar mechanisms of oxidative stress and inflammation can be known between the external stressors that produce anxiety and those present in excessive weight.

Anxious symptoms and excessive weight in women

Women are twice as likely to suffer from anxiety (Catuzzi, 2014) and the lifetime prevalence of an anxiety disorder is 60% higher in them than in men (Donner, 2013).

They may present greater hormonal fluctuations that spread anxiety disorders (Hantsoo, 2017); They have greater activation of the central amygdala in relation to negative emotions, more sensitivity to threatening stimuli, and tend to overestimate threat more than men. This, combined with inflexibility to explore a diversity of possible responses to potentially dangerous situations, lays the foundation for suffering from anxiety disorders (Catuzzi, 2014; Donner, 2013).

Regarding obesity, according to ENSANUT 2018, the prevalence of excessive weight in the state of Jalisco, in women, is 75.9%, where 36.7% correspond to overweight and 39.2% to obesity, respectively. The frequency of anxiety diagnosis in obese women is twice that of obese men; Likewise in women, anxiety and depression are more likely to reflect increased appetite and weight gain. This is associated with being the result of stress to favor the consumption of foods with high palatability (Fulton, 2022).

Olive leaves and their polyphenolic compounds.

Leaves from olive tree, *Olea europaea*, are rich in phenolic compounds in even greater quantities than the fruit, or olive oil (Sabry, 2014). The main phenolic compounds from olive leaves are: oleuropein, tyrosol, hydroxytyrosol, ligstroside and caffeic acid (Acar-Tek, 2020); being oleuropein the most important. It can inhibit the formation of free radicals and exert antioxidant activity by giving up hydrogen ions (Acar-Tek, 2020; Khalatbary, 2013; Sabry, 2014); Also, it is mainly responsible for the anti-inflammatory effect (Qabaha, 2018)

Among the main effects attributed to olive leaf extract, in studies with animal models, are improvement of glucose metabolism, increase in antioxidant function and reduction of oxidative stress and inflammation (Table 1), as well as reduction in anxious-type behaviors (Table 2).

Likewise, studies with humans report lower expression of genes associated with inflammation and a decrease in the expression of proinflammatory cytokines, as well as improvement in glucose metabolism. It also has hypotensive effects and can lower fat mass as well as plasma levels of TC, LDL-C and TG (Table 3).

Subjects	Methodology	Results	References
Korean regional mice. OLE at 3g, 6g and 30g/kg.	Groupss: -Control. -Diabetes (D). -D+ low dose OLE. -D+ mid dose OLE. -D+ high dose OLE.	Decrease in glucose and insulin plasma levels; increase in antioxidant enzymes and decrease in proinflammatory cytokines.	Park, J. H. *et al.* (2013).
Wistar rats of 3 months and 20 months of age. OLE 500 and 1000 mg/kg. 2 months	Groups: -Young rats. -Old rats. -Old rats + OLE (500mg/kg). -Old rats + OLE (1000mg/kg)	Reduction of malonyl aldehyde (MDA; oxidative stress) in all aged rats with 1000 mg.	Çoban, J. *et al.* (2014).
Dark Agouti male rats (2-3 months). OLE 1024mg/kg (45.96mg/kg oleuropein). 20 and 30 days. Experimental autoimmune encephalomyelitis (EAE).	Groups: -EAE ctrl 20d. -EAE+ OLE 20d. -EAE ctrl 30d. -EAE+OLE 30d.	Decreased TBARS (oxidative stress) and increased GPx expression; attenuated myelin destruction and increased microglia differentiation to M2 phenotype.	Giacometti, J., & Grubić-Kezele, T. (2020).
Male Wistar rats. 6 weeks of treatment. STZ: streptozotocin. MT: metformin (600mg/kg). OLE: 200 and 400mg/kg. DM: Diabetes Mellitus	Groups: -DM -DM+MT -DM+OLE 200 -DM+OLE 400 -Control	OLE decreased glucose, MDA and AGEs levels and increased SOD and nitric oxide (NO) levels.	Khattab, H. A. *et al.* (2020).
6-week-old Sprague Dawley rats. OLE 20, 50 and 100mg/kg. Ibuprofen (IBU) 40mg/kg. 21 days of treatment. PTSD: post-traumatic stress.	Grupos: -Ctrl. -PTSD. -PTSD+OLE 20. -PTSD+OLE 50. -PTSD+OLE 100. -PTSD+IBU.	OLE inhibited the increases in CORT and reduced the levels of IL-1β and TNF-α; increased CREB and BDNF levels in the hippocampus; attenuated the memory deficit.	Lee, B. *et al.* (2018).

Table 1. Olive leaf extract (OLE) effects in studies with animal models. Among the main effects attributed to it are improvement of glucose metabolism, improvement of antioxidant function and reduction of oxidative stress and inflammation.

Subjects	Methodology	Results	References
Wistar rats. All groups except Ctrl and sham had Parkinson's induced through 6-hydroxydopamine. -SS: Saline solution.	Groups: -Ctrl. -Sham (SS). -Parkinson (SS). -OLE 50mg/kg. -OLE 100mg/kg. -OLE 150mg/kg.	OLE reduces anxiety-like behavior in rats administered 6-OHDA.	Hosseini, S., & Hajizadeh, A. (2015).
Male Sprague Dawley rats. OLE: 20, 50 and 70mg/kg. Fluoxetine (FLX): 10mg/kg. PTSD: post-traumatic stress. SS: saline solution.	Groups: -SS. -PTSD. -PTSD+OLE20. -PTSD+OLE50. -PTSD+OLE70. -PTSD+FLX.	OLE reduced anxiety-like behavior and CORT levels. The expression of 5-HT (serotonin) and NP-Y were increased by it.	Lee, B. *et al.* (2018).
Male Wistar rats. Lead (Pb): 250mg/l of water. EHO: 0.1%(w/v).	Groups: -Ctrl. -Pb. -Pb+OLE.	OLE reduced blood and hippocampal Pb and caspase 3 levels, also, DNA fragmentation. It inhibited TNFα, IL-1β and reversed anxiety-like behaviour.	Seddik, L., *et al.* (2011).
Male mice. Cortisol (Cort): 40mg/kg. FLX: 20mg/kg. Oleuropein (OE): 8, 16, 32 mg/kg. FLX or OE was injected 30min prior Cort. SS: saline solution	Groups: -SS -Cort+SS. -Cort+FLX. -Cort+OE 8. -Cort+OE 16. -Cort+OE 32.	OE reduced anxious-like behavior; increased GSH (antioxidant function) and reduced oxidative stress; attenuated the Cort-induced decrease in 5-HT and dopamine.	Badr, A. M., *et al.* (2020).

Table 2. Olive Leaf Extract (OLE) effects, in animal models, on anxiety-like behavioral disorders.

Regarding OLE toxicity, in vitro studies with doses of 320, 800, 2000, 5000 ng/mL do not reveal promutagenic effects on *salmonella typhimurium* and *E.Coli* in the bacterial reverse mutation test. Similarly, concentrations of 250, 500, 750, 1000 and 1250 µg/mL do not affect the number of aberrant cells and polyploid range in guinea pig lung cells in the chromosome aberration test (Romero-Márquez, 2023).

There was also no genotoxicity in male Wistar rats due to the consumption of olive leaf extract at doses of 100, 200, 400 and 2000 mg/kg supplemented for 28 days; they did not show signs of toxicity, mortality, behavioral or physical alterations, or abnormalities in the liver or kidney. In another study in which 300, 600 and up to 1,000 mg/kg were given for three months, similar results were observed. Daily consumption of 250 mg by elderly women for 1 year also did not reveal adverse effects (Romero-Márquez, 2023; Acar-Tek, 2020; Dekanski, 2014; Filip, 2015).

Subjects	Methodology	Results	References
N=29 men. OLE: 20ml/day (121mg oleuropein/day and 6.4mg hydroxytyrosol/day).	Double-blind, placebo-randomized control trial (RCT). Groups: -Placebo. -EHO. 8 weeks.	Less expression from essential genes in inflammation (OSM, Cox-2, IL-8), IKKB, Myd88 and the phospholipase A pathway.	Boss, *et al* (2016).
N=32 (22 women and 10 men). OLE: 20g of leaves (100m oleuropein) per day. Placebo: gluten-free starch. URI: upper respiratory infection.	RCT Groups: -Placebo. -OLE. 2 months.	Shorter duration of URIs 28%, mainly in women. It had no effect on the incidence.	Somerville, V., *et al*. (2019).
N=46 overweight men. Pol: 51mg oleuropein and 9.7 hydroxytyrosol.	RCT. Groups: -Placebo. -Pol. 12 weeks of tx, 6 weeks off and 12 weeks of crossover tx.	Insulin sensitivity improved by 15%, with no changes in TNF-α and C-reactive protein (pCr).	De Bock, M. *et al.* (2013).
61 subjects 24-72 years old. OLE: 20ml/day (136mg oleuropein and 6.4mg hydroxytyrosol). TC: total cholesterol. TAG: triglycerides. LDL-c: LDL cholesterol.	RCT. Groups: -Placebo. -OLE. 6 weeks tx, 4 weeks off and 6 weeks of crossover tx.	OLE decreased systolic and diastolic pressure during the day; as well as plasma levels of TC, LDL-c, TG and IL-8.	Lockyer, S., *et al.* (2017).
N=18 (9 women and 9 men). OLE: 400mg (51.12mg oleuropein and 9.67 hydroxytyrosol) in a single dose. Placebo: matched control.	RCT. -Placebo. -OLE. Two evaluation visits 4 weeks apart (crossover tx).	The hydroxytyrosol derivatives reached their peak between 4-8 hrs and those of oleuropein at 24 hrs. Ex vivo, IL-8 production was reduced.	Lockyer, S *et al.* (2015).
N=60 (26 men and 34 women). 55±8.8 years of age. OLE: 500mg/day (.62mg luteolin and 16% oleuropein). Placebo.	RCT. -Placebo. -OLE. 12 weeks.	OLE reduced levels of IL-6, TNF-α and IL-8.	Javadi, H., *et al.* (2019).
N=70 women. Age ≥18 years. BMI ≥30-≤40 kg/m2 OLE: 500mg/day (50mg of oleuropein). Placebo: 125mg starch. WRD: weight reduction diet.	RCT. Groups: -Placebo+WRD -WRD+OLE. 8 weeks.	OLE significantly reduced weight, BMI, fat mass, fasting glucose, LDL, leptin, adiponectin and free fatty acids compared to Placebo+WRD.	Haidari, F., *et al.* (2021).

N=64 women. Age 49-69 years. OLE: 250mg/day with 40% polyphenols. Ca: 1g/day of Calcium.	RCT Grupos: -Ca. -Ca+OLE. 1 year.	No adverse effects associated with tx were reported; OLE increased osteocalcin levels by 32% and reduced TC, LDL and TG.	Filip, R., *et al.* (2015).
N=148 (126 women and 22 men) with stage 1 hypertension. Age 25-60 years without medication for hypertension (HTN). OLE: 500mg/day. Captopril: 12.5mg/day.	RCT Groups: -Captopril. -OLE. 8 weeks.	OLE lowered blood pressure levels more than captopril; also improved the TG, TC and LDL-c profile.	Susalit, E., *et al.* (2011).
N=79 (51 men and 28 women) with diabetes mellitus (DM). 18-79 years old. BMI <40kg/m2, HbA1c <10%. OLE: 500mg/day.	RCT Groups: -Placebo. -OLE. 12 weeks.	Lower levels of HbA1c and fasting insulin were observed in the group supplemented with OLE.	Wainstein, J., *et al.* (2012).

Table 3. Effects of olive leaf extract (OLE) supplementation in clinical trials in humans.

Problem statement

According to the National Self-Reported Wellbeing Survey, in Mexico women showed a lower mood balance in all age groups compared to men (mainly between 33-40 years old). Regarding anxiety, 19.3% of the population revealed severe symptoms; 31.3% manifested symptoms of minimal or some degree of anxiety. For the female population the percentages were 23.2% and 32.8%, respectively. In general, this disorder affects the selective transport function at the blood-brain barrier (BBB), which favors the translocation of peripheral proinflammatory cytokines and the production of reactive oxygen species, which increases oxidative stress (OS).

OS and inflammation generate a positive feedback loop in which the preservation of one another is favored, and thus, perpetuate anxiety-like behavioral alterations and sometimes aggravate them. This increases in OS and inflammation are also associated with excessive weight due to the meta-inflammation produced by it. The intake of olive leaf extract (OLE) in different subjects or animal models has been shown to reduce the damage generated by oxidative stress and the inflammatory state. Since these harmful processes are present in anxiety disorder, OLE emerges as a possible modulator of anxious symptomatology.

Rationale

The prevalence of an anxiety disorder is 60% higher in women than in men. In Jalisco, 5 out of every 20 individuals have a mental disorder, the most frequent being anxiety, with 16%; In the Guadalajara Metropolitan Area (GMA) these alterations have a prevalence of 15%, where the majority of those who suffer from it are adult women between ages from 25-50 years. Furthermore, obesity and overweight increases the probability of suffering from an anxiety disorder by 30-40%; and the frequency of this disorder is double in obese women. According to ENSANUT 2018, the prevalence of excessive weight in the state of Jalisco, in women, is 75.9%, of which 36.7% corresponds to overweight and 39.2% to obesity.

Based on the above, we consider that OLE represents a good option to help with symptoms from anxiety disorder by using it as a dietary supplement, due to its known beneficial effects, mainly on oxidative stress and inflammation, processes that converge in both anxiety and excessive weight.

Research question

What is the effect from supplementation with olive leaf extract (OLE) on the concentration of mediators of meta-inflammation (TNF-α, IL-6, leptin, cortisol) and anxiety symptomatology in adult women with excessive weight?

Hipothesis

Dietary supplementation with olive leaf extract (OLE) reduces meta-inflammation and anxiety symptoms in adult women with excessive weight.

General and specific objectives

General:

To evaluate the effects from the consumption of olive leaf extract as a dietary supplement on anxiety symptoms, meta-inflammation and metabolic comorbidities in women with excessive weight from the Guadalajara Metropolitan Area (GMA) in Jalisco, Mexico.

Specific:

1. To evaluate the presence/absence of changes at the 3rd month, with respect to the beginning of the study prior to supplementation, in the expression patterns of the lipid profile, glycated hemoglobin (HbA1c) and inflammatory mediators (TNF-α, IL- 6, Cortisol and leptins) as indicators of meta-inflammation in both study groups (supplemented with olive leaf extract or placebo).
2. Evaluate changes in weight and percentage of fat and muscle monthly.
3. Analyze the dietary-nutritional component of the participants during the study period to know the possible influence it may have on the indicators to be evaluated.
4. Analyze the effect of supplementation with the extract or placebo in levels of anxiety symptomatology, at the end of each of the three months of the study.
5. Identify if there are significant differences, in the evaluated parameters, between the two study groups (supplemented with OLE or placebo).

Tipe of study

- Longitudinal clinical trial, double-blind randomized placebo.

Research location

- University Center for Health Sciences (CUCS) of the University of Guadalajara (UdeG) (recruitment of participants).
- Resolution Microscopy Laboratory, CUCS, UdeG and Guadalajara Metropolitan Area (GMA) (laboratory analysis and subjects' recruitment, respectively).
- Neuroscience Care Unit, Department of Neuroscience, building L, Door 18, CUCS, UdeG (evaluation office).

Study period

The scientific literatura review pase began in February 2024 and the study is expected to conclude in January 2026.

Study subjects

Adult women with excessive weight from the GMA, Jalisco, Mexico.

Inclusion criteria:

- Females.
- Residing in GMA.
- Initial age ≥18-≤ 40 años
- Initial Body Mass Index (BMI) ≥25-≤40 kg/m^2
- Blood pressure: ≤129mmHg systolic and ≤80 diastolic.
- Anxious symptomatology score 6 to 14 (mild) through the Hamilton anxiety scale (HAS).

Non-inclusion criteria:

- Use of psychoactive drugs, suffering from major psychiatric disorders (depression, attention deficit hyperactivity disorder, bipolar disorder, eating disorders), pregnancy, breastfeeding, hypotension, alcohol use disorder (AUD).
- Suffer from liver, kidney or thyroid disease or cancer.
- Olive allergy.
- Consume any medication that influences weight
- Consume some other type of food supplement from organic origin.

Exclusion criteria:

- Less than 70% adherence frequency to the consumption of the extract/placebo.
- Getting pregnant during the study.

Sample size.
Sample size was chosen based on similar studies in the literature that identified statistically significant differences. Studies that evaluated inflammatory markers and the effect of olive leaf polyphenol supplementation on proinflammatory cytokines generally used samples between 20-35 subjects per group (intervention group and control group) (Javadi, 2019; Lockyer, 2017). The calculation of the comparison of means taking as reference the study by Javadi et. to the. 2019 (Control 78.4±13.2ng/L vs Experimental 69.7±9.8 ng/L IL-6; post-intervention with polyphenols) through the OpenEpi statistical calculator suggests recruiting 29 participants in total to demonstrate a moderate effect (>0.60) for the groups comparison to intervene with statistical significance and power of 80% (α= 0.05). We decided to use 35 participants (n=70) per group due to possible eliminations.

Sample aleatorization
Block randomization was performed using *Random Allocation Software*. Subsequently, participants will be stratified according to their Body Mass Index (BMI). From this stratification, 7 blocks of 10 individuals will be produced. According to their correspondence, the codes in Figure 1 will be distributed into two equal containers separated by denomination (case or placebo) that the participants in each block will take. The correspondence of an individual to a certain group will be blinded for the researcher and the participants.

0001: **Case** 0002: **Placebo**	0003: **Case** 0004: **Placebo**	0005: **Placebo** 0006: **Case**	0007: **Placebo** 0008: **Case**	0009: **Placebo** 0010: **Case**
0011: **Placebo** 0012: **Placebo**	0013: **Placebo** 0014: **Case**	0015: **Placebo** 0016: **Placebo**	0017: **Case** 0018: **Case**	0019: **Case** 0020: **Case**
0021: **Case** 0022: **Placebo**	0023: **Case** 0024: **Case**	0025: **Case** 0026: **Placebo**	0027: **Placebo** 0028: **Placebo**	0029: **Case** 0030: **Placebo**
0031: **Case** 0032: **Placebo**	0033: **Case** 0034: **Case**	0035: **Placebo** 0036: **Placebo**	0037: **Placebo** 0038: **Placebo**	0039: **Case** 0040: **Case**
0041: **Case** 0042: **Placebo**	0043: **Case** 0044: **Placebo**	0045: **Placebo** 0046: **Case**	0047: **Case** 0048: **Placebo**	0049: **Placebo** 0050: **Case**
0051: **Case** 0052: **Placebo**	0053: **Case** 0054: **Placebo**	0055: **Placebo** 0056: **Placebo**	0057: **Case** 0058: **Placebo**	0059: **Case** 0060: **Case**
0061: **Case** 0062: **Case**	0063: **Placebo** 0064: **Case**	0065: **Placebo** 0066: **Case**	0067: **Placebo** 0068: **Placebo**	0069: **Placebo** 0070: **Case**

Figure 1. Block randomization, of 10, delivered by the *Random Allocation Software*.

Group Division

Participation in the study will be promoted inside CUCS from UdeG through information flyers and official digital media, with prior authorization. 70 women from the GMA who meet the inclusion criteria will be recruited. They will be given an informed consent document and, if they accept, they will be included in the study. Each of the participants will be assigned in a double-blind and randomized manner to one of two groups: supplemented with olive leaf extract (EHO) (n=35) or placebo group (cellulose; n=35). They will be treated as indicated in Figure 2. Most measurements will be carried out at the beginning of the study and three months after it, when the clinical trial concludes. Anxious symptomatology will be evaluated using alternately the Hamilton Anxiety Scale (HAS) and the Beck Anxiety Inventory (BAI) (at the beginning and subsequently every 30 days, for 3 months); Patients who present scores that refer to moderate/severe anxiety will be referred to the relevant medical care service free of charge by direct contact (Department of Neurology of the Fray Antonio Alcalde Civil Hospital and Neuroscience Care Unit, Dept. of Neurosciences building L, CUCS , UdeG). Adherence to the protocol will be verified every 15 days and a 24-hour dietary recall and food frequency will be applied to keep a record of eating patterns throughout the clinical trial. Weight and fat and muscle percentages will be recorded monthly using an Omron® HBF-514C scale. ELISA assays will be used to measure levels of TNF-α, IL-6, Cortisol and leptins; height will be measured through a stadiometer and, finally, lipid profile and glycosylated hemoglobin will be measured through blood chemistry; All these parameters will be taken at the beginning and at the 3rd month of the trial (height will only be taken at the beginning).

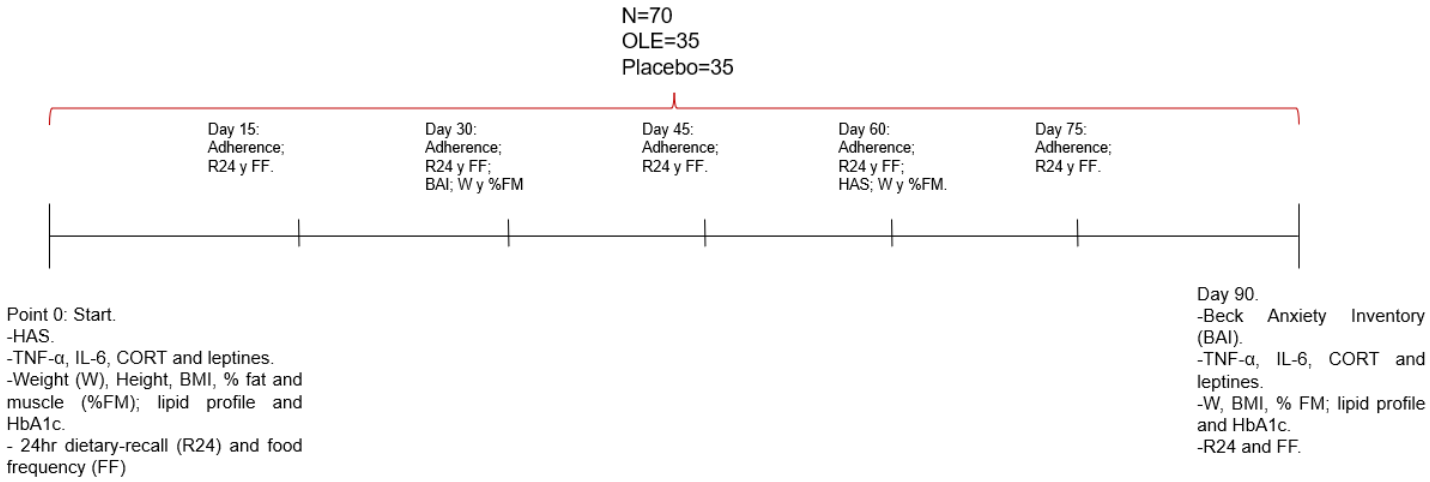

Figure 2. Division, treatment and evaluation for each participant group.

Dependent and independt variables

Independent variable:

olive leaf extract (OLE), placebo and anxiogenic state.

Intervener variable:

Age and dietary-nutritional component.

Dependent variables:

TNF-α, IL-6, cortisol, leptines, weight, BMI, muscle and body fat percentage, lipid profile, glycosylated hemoglobin and anxious symptomatology.

Biosafety considerations

The procedures to be followed are specified in accordance with NOM-087-ECOL-SSA1-2002, NOM-054-SEMARNAT-1993 and NOM-052-SEMARNAT-2005; the competence in biosafety of the researchers responsible for the High-Resolution Microscopy Laboratory, University Center for Health Sciences (CUCS), UdeG, and the equipment available to carry out the experiments, in ANNEX 4.

For the management of chemical waste, NOM-118-STPS-2000 and the manual for the management of hazardous chemical waste (CRETI) will be considered, both attached in the following link: https://drive.google.com/drive/folders/16mo95bffasZs7ug0Gweyanl8I8x3F_xg

Ethical considerations

This project adheres to the requirements by the mexican Regulation of the General Health Law on Health Research (mainly articles 13, 14, 15, 16, 17, 20 to 27) and the CIOMS guidelines (1st to 6th, 8th to 15th and 18th). To safeguard the rights and autonomy of the participants, they will be made aware of the objectives, risks and benefits expected in this study through an informed consent (ANNEX 1); right there they will be informed of the right to withdraw without any opposition. The purpose of this research is supported by previous scientific studies. Regarding its social value, if successful, it could improve the quality of life of women who suffer from meta-inflammation and anxiety; Its cost is not much higher than the drug buspirone, one of the most used in the treatment of anxious behavior disorders, however, the field of its beneficial effects is broader. Likewise, olive leaves, which can represent up to 5% of the mass of olives in olive mills, could be revalued as raw materials (Garro, 2023). Also, participants will be given personalized nutritional advice for 3 months after their participation in the study, in the form of compensation and gratitude for their participation. On the other hand, if at any time during the study they present moderate/severe levels of anxiety and/or physical damage resulting from this research, they will be referred free of charge to the corresponding medical service by direct telephone contact (Dr. José Luis Ruíz Sandoval, head of the service of neurology, Hospital Civil Fray Antonio Alcalde; with free access to the clinical care unit of the Dept. of Neurosciences, CUCS).

The study presents equity in the distribution of research burdens, the risks reported by the consumption of olive leaf extract are not significant and are outweighed by the known benefits. The use of cellulose as a placebo is because it will allow us to discern whether the extract has a significant therapeutic effect when comparing both groups. Likewise, current treatments for anxiety disorders are mainly through neuromodulators derived from

benzodiazepines that have a delayed action and may have unwanted side effects, plus, their effect on meta-inflammation is unknown.

For its part, although some studies in animal models report a neuromodulatory effect of olive leaf extract, its main action is through an antioxidant and anti-inflammatory effect; It is through these mechanisms that an anxiolytic action is attributed to it in these same studies; Through its anti-inflammatory action, we will evaluate its possible anxiolytic effect, as well as its effect on meta-inflammation caused by excessive weight (processes associated with each other).

This research also adheres to the WMA Declaration of Helsinki-Ethical Principles for Medical Research on Human Beings 64th Assembly, Fortaleza, Brazil, October 2013. In accordance with article 17 of the Regulations of the General Health Law on Matters of Health Research, the risk of the research is minimal since it contemplates blood extraction, application of psychological tests and administration of a nutritional supplement (olive leaf extract) whose dose to be used does not exceed the manufacturer's recommendation and does not report adverse effects in the literature. The informed consent, the data collection format (contained in the nutritional clinical history) and the conflict-of-interest report are attached at the end of this document (ANNEX 1, 2 and 3, respectively). The confidentiality of the data will be given by identifying each participant by means of a code (randomization number) that only the main researcher responsible for the study will have access to decipher (name-code agreement).

Methodology

Cytokine quantification to asses meta-inflammation

Enzyme-linked immunosorbent assay (ELISA) kits will be used according to the manufacturer's instructions (Table 4) and will be interpreted based on reference values for each molecule. The procedure for blood collection is mentioned in ANNEX 4.

Table 4. ELISA assay specifications

Kit	Detection range	Sensitivity
LEGEND MAX™ Human IL-6 ELISA kit	7.8 pg/mL – 500 pg/mL	1.6 pg/mL
LEGEND MAX™ Human TNF-α ELISA kit	15.6 pg/mL – 1000 pg/mL	3.5 pg/mL
Invitrogen™ Kit de ELISA human cortisol	100-3,200 pg/mL	17.3 pg/mL
Invitrogen™ Leptin Human ELISA Kit	15.6-1000pg/mL	<3.5 pg/mL

Asses of nutritional parameters

A nutritional clinical history will be prepared that contains information on eating habits (24-h recall and food frequency), clinical history and family history. Anthropometric parameters such as weight, height, age, BMI will be recorded; percentage of fat and muscle through bioimpedance (ANNEX 2). The lipid profile and glycated hemoglobin will be evaluated by biochemical tests. The procedure for blood collection is mentioned in ANNEX 4.

Olive leaf and placebo treatment

Nutricost ® 20% oleuropein olive leaf extract will be administered for 3 months, 1 capsule per day, at a dose of 750mg daily. The placebo group will receive 100 mg of microcrystallized cellulose during the same period.

Anxious symptomatology

It will be measured through the Hamilton Anxiety Scale (HAS) and the Beck Anxiety Inventory (BAI) adapted to the Mexican population (ANNEX 6; “Clinical Practice Guide GPC, IMSS 392-10”. Complete at: https://drive.google.com/drive/folders/16mo95bffasZs7ug0Gweyanl8I8x3F_xg). HAS will be applied at the beginning and individuals with anxiety scores between 6-14 will be included; BAI will be applied to support the results obtained through the EAH, every 30 days alternately, for three months. This evaluation period is based

on the cumulative effect of the consumption of the extract to produce a possible metabolic readjustment that improves the state of meta-inflammation and consequently the anxiety symptomatology. The alternation of the tests (HAS and BAI) is done to avoid bias in patients that allows them to recognize answers to obtain better scores.

If any participant presents moderate/severe anxiety scores, they will be referred to relevant medical care (Dr. José Luis Ruíz Sandoval, head of the neurology service, Hospital Civil Fray Antonio Alcalde; free access to the clinical care unit of the Department of Neurosciences, CUCS).

Adherence
It will be carried out biweekly from the return of the container where the respective treatment was delivered; The adverse effects questionnaire, the 24-hour food recall and food frequency will also be applied here (ANNEX 5).

Statistical analysis
It will be carried out through a descriptive and inferential analysis, taking $p<0.05$ as the significance level. The Shapiro-Wilk test will be used to observe the distribution of the data.

- Descriptive analysis: measures of central tendency (arithmetic mean or median) and measures of dispersion (deviation or standard error of the mean).

- Inferential analysis: Parametric tests (“Student's t”, ANOVA). If the data do not follow a normal distribution, non-parametric tests will be reported (X”, Kruskal-Wallis, Mann-Whitney U).

- Post hoc: If parametric tests are used, the Sidak analysis will be performed. If non-parametric tests are used, the Tukey test will be carried out after the ANOVA test.

Project viability

Funding
The responsible researchers have the adequate budget to start part of the proposed techniques and supplementation (OLE/placebo); likewise, the project will be submitted to the PIN 2023-IV financing call.

Infrastructure
The project will be carried out in the High-Resolution Microscopy Laboratory, the Dept. of Neurosciences, CUCS, UdeG and GMA. The equipment the laboratory has is specified in ANNEX 4.

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ANNEX 1 **Informed consent**

"Olive leaf extract effects on meta-inflammation and anxiety symptoms in women with excess weight"

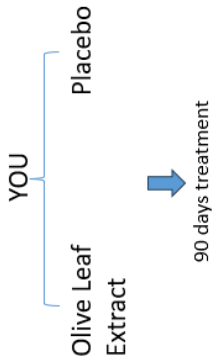
Research objective

This study seeks to see if olive leaf extract (OLE) has any effect that could help lowering the number of molecules in your blood that increases due to excessive weight and promotes the symptoms of mild anxiety.

Why were you included?

Because you're a woman who lives in the metropolitan area of Guadalajara, between 18 and 40 years old, with excessive weight and normal or slightly elevated blood pressure.

Study diagram



You will be chosen at random, by drawing, to take for 90 days (3 months) one of two supplements: olive leaf extract or cellulose (placebo), a fiber very different from olive leaf extract.

Neither you nor the other researchers, except Dr. Ma. Sonia Luquín, will know which supplement you received. The extract is what will help you, in theory, reduce the effects of the molecules that increases with excessive weight and provokes anxiety symptoms.

Cellulose can't help you with that kind of thing, but it can improve your digestion because it helps your intestine movility.

Neither of these two compounds have reported serious ill effects.

How can you be excluded from the study?

1. If you add 13 days of not taking the supplement during the 3 months.

2. If you get pregnant.

3. If you need to take some type of medication for depression, other behavioral disorders or a more serious chronic illness (diabetes, cancer, liver and kidney diseases, etc.).

4. If you present any negative effect from the supplement or if you start taking another type of organic herbal supplement in conjunction with participating in the study.

Research justification

Olive leaves are used in many food preparations. They have compounds that reduces the increased negative molecules in your blood that happens when you have excessive weight and anxiety symptoms. Anxiety is closely related to stress. Cortisol levels (a molecule in your blood) are used to evaluate the presence of stress; Cytokines (other molecules in your blood) are used as an object to understand inflammation. Excessive inflammation is bad; It happens when you have excessive weight or anxiety. More cytokines equals more inflammation. Finally, leptin levels (another molecule in your blood) tell us about how fat reserve in your body behaves. From these measurements plus the profile of other fats (LDL, HDL cholesterol levels, triglycerides and total cholesterol), glycated hemoglobin (to know if you have diabetes), body mass index (to know if you have excessive weight), weight, height and the percentages of fat and muscle in your body, a complete evaluation will be made on the possible benefits that the extract may generate in you, on excessive weight and anxiety symptoms.

Researcher objectives

1. Know the anxious symptoms by asking questionnaires called: Hamilton Anxiety Scale (HAS) and Beck Anxiety Inventory (BAI)

2. Evaluate inflammation, fat reserve and stress due to excessive weight and anxiety symptoms by measuring proinflammatory cytokines (TNF-α and IL-6), leptin and cortisol in your blood.

3. Know the profile of other fats and glycated hemoglobin (blood sugar levels during the last 3 months).

4. Measure Weight, Height, BMI, body muscle and fat percentage.

5. Supplement for 3 months with olive leaf extract (OLE) or cellulose according to the corresponding selection.

6. Know every 15 days your adherence to the treatment, the presence of bad effects from taking it and if you wish to continue in the study; apply the 24-hour food reminder and food frequency.

Procedures

When participating in the trial, you may be given to take every day, for 3 months, a capsule with olive leaf extract (750 mg/day) or a capsule with cellulose (100 mg; a substance without pharmacological effects but that helps improve bowel movement). This will depend on a random selection, that is, by a draw without any preference per person.

1.- Objective 1: To evaluate anxious symptoms, two questionnaires will be applied at different times of the study. The first, the Hamilton Anxiety Scale, consists of 14 questions that address your current mood and which you will answer with “absent, mild, moderate, severe and very severe”; The second questionnaire, the Beck Anxiety Inventory, consists of 21 questions that refer to situations from the last two weeks of your life to which you will answer with “no; mild; moderate; severe” according to how you identify yourself with respect to the question. Applying the two questionnaires lasts approximately 25 minutes in total. They will be applied every 30 days alternately for 3 months.

Risks: When answering these questionnaires the risks are very low. It may present stress, confusion, sadness, among other negative emotions.

2.-Objective 2 and 3: To evaluate the levels of cytokines, cortisol, leptins, profile of other fats and glycated hemoglobin, a small puncture will be made in the middle of the arm and approximately 20 ml of blood will be extracted in total. 20 ml will be taken before starting the supplementation and after 3 months, when you finish supplementing, another 20 ml will be taken. This means that you will receive two injections (with 20ml of blood each) throughout the study.

The person drawing your blood should use a new syringe or needle, gloves, a gown, and a face mask; he will need to clean the punction site with an alcohol swab before and after drawing blood. If the person does not has and do all of these, you can refuse to have blood drawn.

Risks: There are few associated risks, but there may be excessive bleeding, fainting or a feeling of dizziness, accumulation of blood under the skin (bruises), and occasionally more than one sting if the vein is not found; In very unlikely cases the punction can become infected.

3.-**Objective 4**: Some body parameters will be evaluated. You will be required to provide us with your age. Your weight, height, percentage of fat and muscle will be recorded with a special scale in which you only need to stand on and hold a bar integrated into the scale with both hands. To know your body mass index, you only need to know your weight and height. Measurement of weight and percentage of fat and muscle will be carried out monthly.

This processes will be carried out by nutricionist Mario Hernández Garibay.

Risks: There are no significant risks associated with obtaining this information.

4.- **Objective 5**: By participating in this research you agree to ingest daily (every day) for 3 months a capsule of olive leaf extract or cellulose, a non-digestible fiber, according to the selection made at random, by lottery. The capsule is taken with a little water; This may be a little uncomfortable, until you get used to it.

Risks: The associated risks are few, if you do not tolerate the ingestion of the olive leaf extract or cellulose you could experience headache, muscle pain, low fever (low-grade fever), sweating, nausea, sore throat, runny nose and dizziness, in which case the ingestion of the capsule is suspended and if it is considered necessary, you will be referred to the corresponding medical attention (Dr. José Luis Ruíz Sandoval, 3331973293).

5.-**Objective 6:** To find out your adherence to the treatment, you will be asked to return the container where the corresponding compound was last given and we will see how many capsules you took; at that time you will be given more capsules. You will be given a questionnaire to find out if you have had adverse (bad) effects that you attribute to the treatment and, as mentioned, if you need medical attention, you will be referred to it (Dr. José Luis Ruíz Sandoval, 3331973293). You will be given a 24-h food reminder, and you will be given the option to continue or abandon the study. This will be done in the Department of Neurosciences at the University Center for Health Sciences (CUCS) of the University of Guadalajara, biweekly, for 3 months. In the event that it is difficult or uncomfortable for you to travel to CUCS, it can be done at home if you do not have any inconvenience. If you have any inconvenience, the evaluation can be rescheduled in nearby days.

This processes will be carried out by nutricionist Mario Hernández Garibay (3316052068).

Risks: There are no significant risks associated with obtaining this information.

Expected benefits

Apart from the aforementioned benefits on inflammation and anxiety symptoms, olive leaf extract reduces blood sugar levels and levels of total cholesterol, LDL and triglycerides (this is associated with a lower risk of suffering from diabetes and chronic diseases); It also reduces blood pressure and is capable of improving the function of the immune system, which is the system responsible for defending the human body against invading microorganisms.

Finally, the main objective of consuming this compound is to help reduce the anxiety symptoms that often motivate the greater consumption of foods with a lot of fat or sugar.

As a thank you for your participation, at the end of the trial you will be given personalized nutritional assistance for three months free of charge (Nutritionist Mario Hernández Garibay 3316052068).

Once the months of participation in the study have ended, you will NOT be given more olive leaf extract.

Information privacy

Your name will only be used to sign this consent. For privacy purposes, a numerical identification method will be used according to the draw for your group assignment (extract or placebo) in the study and not your name; Your signature (without your name) will also be used when answering the treatment adherence questionnaire. Regarding personal information, you will be asked to provide your age, marital status, your cooperation in recording the parameters mentioned above (BMI, height, weight, % fat and muscle; as well as the aforementioned analyzes for which we will be taking your blood) and information to know nutritional aspects; Next, you will be shown and explained the nutritional questionnaire that will be applied to you. The information you provide us will be used solely for the purposes of this research; You will be informed of all your results once obtained.

If we would like to use your information for another research, other than this one, your permission will be requested again; Just as if your data needs to be transferred to another researcher, you will be required to agree. You are free to reject if you wish.

Knowledge of your name and data will be exclusive to the main researcher, Dr. Ma. Sonia Luquín de Anda and no one else. The responsibility and privacy of the data provided will fall on her. Your data will NOT be shared with any type of company or individual unrelated to the research.

The other researchers will know the results of the evaluations carried out, but they will not be able to identify them by name, they will only identify them by means of the numerical code assigned to them.

Doubts and clarifications

You will be in direct communication with the responsible researchers at the times when the aforementioned data and blood extraction is required; as well as three to four times a week depending on availability and need, throughout the study.

Dr. Ma. Sonia Luquín de Anda 3334408913.

Dr. Joaquín García Estrada 3332016340.

Nutricionist Mario Hernández Garibay 3316052068.

You will have the possibility to clarify any type of doubt that may arise associated with this research, as well as receive the medical attention of Dr. José Luis Ruiz Sandoval (3331973293), necessary in case you present any type of negative health response associated with participation in this research, such as those discussed above. To schedule an appointment with the Doctor, you will contact him, and he will give you the date and place of the medical consultation. Free medical care will not be given for illnesses that you suffer from before starting the study.

Any new information on the extract that indicates the need to immediately stop the study or specifically exclude you will be informed to you and will be carried out promptly under the responsibility of the principal investigators.

If you have any bad results in any of the blood tests that need to be told to you soon, you will be informed as soon as possible.

You can leave the investigation at any time you wish. Participation or non-participation, as well as abandoning the research, will not represent any type of negative repercussion.

Participating in the study will not result in any additional financial expense; In the event that one day you cannot attend, or it is inconvenient for you to go to the CUCS for the respective evaluation, this can be done at home if you consider it convenient or, failing that, it can be rescheduled according to your availability, on nearby days. The negative side effects known from consuming olive leaf extract or cellulose are generally not serious. As mentioned, if you experience any type of adverse effect, you will be referred for medical attention to Dr. José Luis Ruíz Sandoval, for whom you have contact information.

Freedom of consent

By signing this document, you agree to be part of this research. It is important to remind you that you have the freedom, at any time if you wish, to stop participating.

I repeat that your participation in this research will not generate any cost to you; If adverse effects occur due to participation in this study, medical attention will be provided (Dr. José Luis Ruíz); It is reaffirmed that you are free to accept as well as free to stop participating at any time.

Finally, I remind you that, as a thank you for your participation, if you wish, you will be given individualized nutritional attention at no cost at the end of the study, biweekly, for a period of 3 months by Nutricionist Mario Hernández Garibay.

By signing this document, you are agreeing to participate in this study and be part of everything described above:

_____

Date, Name and sign from the participant

_____ _____

Witness 1. Date, name and sign. Witness 2. Date, name and sign.

_____

Responsable researcher:

Dr. Ma. Sonia Luquín de Anda

sonia.luquin@academicos.udg.mx

3334408913

_____ _____

Dr. Joaquín García Estrada

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3332016340

Nutricionist Mario Hernández Garibay

marioahg93@gmail.com

3316052068

ANNEX 2

Nutriotional History

Date: _____

Expedient: _____

Age: _____ Sex: _____

Birthdate: _____

Scolarship:_____ Occupation: _____

CLINICAL INDICATORS

Diarrhea: _____ Constipation: _____ Gastritis: ____ Nausea: _____ Pyrosis: _____ Threw up: _____ Colitis: _____

Teeth: _____ Other: _____

Observations (signs and symptoms):

Has a diagnosed illness: _____

Has suffered a major illness: _____

Take some medication: Wich: Dose: Since:

Family background:

Obesity: DBM: AHT: Cancer: Hypercolesterolemia: Hypertrigliceridemia: Thyroid diseases:

Other:

Physical Activity

Tipe: Intensity: Frecuency: Timing:

Reasons:

Arterial tension (left arm)

Time: Score:

Waist: _____ BMI:_____ Weight: _____ Height: _____

% Body fat: _____ % Muscle: _____

Hip: _____

Waist hip index: _____

Men	Women	Health risk
Less than 0.95	Less than 0.80	Very low
0.96 to 0.99	0.81 to 0.84	Low
≥ 1	≥ 0.85	High

24 hr-food recall.

	Time	Place	Food	Portions	Preparation method
Breakfast					
Collation					
Meal					
Collation					
Dinner					

Food frequency					
	0	1	2 a 3	4 a 5	6 o más
Vegetables					
Chard, cabbage, spinach, lettuce, alfalfa germ.					
Broccoli, beet, cauliflower.					
Squash, carrot, onion, tomato, mushroom, pumpkin flower, cactus.					
Fruits					
Strawberries, pineapple, guava, watermelon, blueberries, blackberries, peaches, tangerines, lemon, lime, apple, grapefruit, mango, banana, orange.					
Fat-free cereals					
Amaranth, rice, oatmeal, bolillo, sweet potato, commercial cereal, corn, pasta, Maria cookies, granola, macaroni, midnight, popcorn, potato, corn tortilla.					
Cereals with fat					
Baked corn or nopal toast, telera, commercial cereal bars, croissants, fried foods (commercial potatoes), commercial cookies, sweet bread, tamale, cake, flour tortilla.					
Legumes					
Chickpea, bean, kidney bean, broad bean, soybean.					
Protein with very low fat content					
Clam, fresh or water-drained tuna, beef steak, shrimp, jerky, smoked chop, egg whites, beef brisket, fish fillet, machaca, chicken breast, fish, commercial low-fat cheeses.					
Protein with low fat content					
Skirt steak, tuna in oil, pork chop, pork fillet, pork loin, chicken or beef liver, ground beef, white fish, salmon, light commercial cheeses.					
Protein with moderate fat content					
Pork crackling, egg, chicken with skin, turkey sausage, turkey salami, commercial reduced-fat cheeses.					
Protein with high fat content					
Beef jerky, backbone, ham, pork or beef tongue, moronga, chicken nugget, pigeon, crispy chicken, yellow cheese, Vienna sausage and cheeses in general.					
Dairy					
Milk (specify presentation), yogurt (specify presentation) and ice cream.					
Oils and fats					
Cooking oil, olives, salad dressing, coconut, cream, shortening, margarine, mayonnaise, butter, avocado, bacon, cream cheese.					
Oils and fats with protein					
Almond, hazelnut, peanut, chilorio, chistorra, chorizo, peanut butter, serrano ham, walnut, sesame, pistachio, pepitas, pâté, sunflower seed.					

ANNEX 3

DECLARATION OF CONFLICT OF INTEREST

Through this document I state that the intervention called "**Olive leaf extract effects on meta-inflammation and anxiety symptoms in women with excessive weight**" has NOT been financed in whole or in part by any company with economic interests associated with the products, equipment or similar referred to here.

Likewise, none of the responsible researchers have relationships with any associated company that could be perceived as a potential conflict of interest.

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ANNEX 4

Waste type	Physical state	Management	Packing	Color to discard	Storage
Blood (labeled “Liquid Biological Hazardous Waste”)	Liquid	Lab coat, gloves, safety glasses, closed shoes and face mask.	Hermetic (yellow or red lid). To discard, it must be neutralized with chlorine previously.	Red	-70°C
Sharps (labeled “Biological-infectious sharps hazardous waste”)	Solid	Lab coat, gloves and face mask.	Dispose of in rigid polypropylene containers	Red	------
Globes	Solid	They should not have any holes or damage when used.	Discard in red bag.	Red	Room temperature.
ELISA plate 96 wells	Solid	Adequate ventilation, gloves, safety glasses, face mask and gown.	Rinse with plenty of water before discarding or in case of accidental spillage, collect with mechanical methods and then dispose of in a red bag.	Red	Cool, dry and well-ventilated place. Store in properly labeled containers.

In accordance with NOM-087-ECOL-SSA1-2002

- Blood sampling will be carried out in the Department of Neurosciences, of the University Center for Health Sciences (CUCS) in an area equipped for this process, where a puncture will be performed in the middle of the arm and 20 ml of blood will be extracted. A new rigid polypropylene sharps waste container with needle separator and storage opening will be brought. Subsequently, the container will be transported in a polyethylene bag, to ensure its resistance, to the High-Resolution Microscopy Laboratory, University Center for Health Sciences, University of Guadalajara. In the laboratory, the sharps container will be treated accordingly.
- The blood collected will be labeled in the manner referred to and with the participant's respective identification code. It will be transported in a disinfected hermetic container that allows it to be kept at 4°C with a splint that prevents compaction of the tubes where it was packaged.
- In the laboratory, the collected blood will be stored in an isolated area that maintains it at -70°C with its respective labeling and for no more than 30 days.
- The laboratory will be classified as Level I in the generation of RPBI since it will handle samples between 1 and 50 per day.
  Nitrile or latex gloves, a gown and wrap-around lenses will be mandatory for handling and processing the samples.

During the processing of the samples through the ELISA assay, if any contingency associated with its reagents arises, the procedure will be followed in accordance with the manufacturer's recommendations, which are mentioned in the attached safety sheets (https://drive.google.com/drive/folders/16mo95bffasZs7ug0Gweyanl8I8x3F_xg).

In accordance with NOM-052-SEMARNAT-2005 and NOM-054-SEMARNAT-1993, blood and some of the components used in the compounds of the ELISA tests refer to incompatibility with other residues, a hazard code (CPR) and specific handling. shown in the following table:

Component	CPR	Group and incompatibility	Storage by compatibility	Management	Minimization
Blood	B; infectious biological.	---	-70°C	Nitrile gloves, gown, face mask, safety glasses and closed shoes.	Inactivation with chlorine and subsequent disposal to the drain.
Hydrogen chloride.	C; corrosive.	Group 1. Incompatible with sulfuric acid and methanol.	Cool, dry, well-ventilated, corrosion-resistant area. Away from flammable or oxidizing substances, organic substances, alkalis or near metals.	Ventilated area, nitrile gloves, gown, face mask, safety glasses and closed shoes.	Neutralize with sodium hydroxide or calcium carbonate and pour into the drain.
Tritón x-100.	Tea; Environmental toxicity.	---	Cool, dry, well-ventilated area. Away from oxidizers, oxidants and strong acids.	Nitrile gloves, gown, face mask, safety glasses and closed shoes.	Disposal of the container at a hazardous waste collection point. Do not pour into drain.
3,3', 5, 5' Tetramethylbenzidine dihydrochloride.	Tt; Chronic toxicity.	---	Cool, dry and well-ventilated place. Far from oxidizers.	Nitrile gloves, gown, face mask, safety glasses and closed shoes.	Disposal of the container at a hazardous waste collection point. Do not pour into drain.
Sulfuric acid.	Tt and C; Chronic and corrosive toxicity.	Group 2. Incompatible with hydrogen chloride (group 1). Generates heat and solubilizes metals.	Keep tightly closed in a dry place. Away from metals, acids, water, hydrogen peroxide and halogenated hydrocarbons.	Nitrile gloves, gown, face mask, safety glasses and closed shoes.	Disposal of the container at a hazardous waste collection point. Do not pour into drain.
Metanol.	I and Tt; Flammable and chronic toxicity.	Group 4. Incompatible with hydrogen chloride and sulfuric acid (group 1 and 2). Generates heat, flammable, violent polymerization and toxic gases.	Well-ventilated place protected from sunlight, away from oxides, acids, halogenated hydrocarbons, flames and hot surfaces.	Ventilated area, nitrile gloves, gown, face mask, safety glasses and closed shoes.	It can be filtered and distilled. Small quantities can be allowed to evaporate or diluted with plenty of water down the drain. Disposal at hazardous waste collection point.
Dimethyl sulfoxide	Tt; chronic toxicity.	---	Inert, dry and cool place. Away from water, hot places, flames, oxidizers, acid chlorides, strong acids, phosphorus oxides and inorganic components.	Nitrile gloves, gown, face mask, safety glasses and closed shoes.	Disposal of the container at a hazardous waste collection point. Do not pour into drain.

Among the equipment available at the High Resolution Microscopy Laboratory, University Center for Health Sciences, University of Guadalajara are:

- Centrifuge for 10 ml Vacutainer tube.
- Refrigerated centrifuge for microtube.
- 2 analytical balances
- Incubator for cell culture.
- Ultramicrotome.
- Stirring and heating grill.
- Digital potentiometer.
- 2 horizontal freezers.
- Cold camera
- Motic 3-channel fluorescence microscope.
- Simple light microscope.
- 3-laser confocal microscope.
- Inverted Leica fluorescence microscope.
- Electrophoretic chambers with power source.
- Leica brand microtome and vibratome.
- Ultra freezer.

Among those responsible for the laboratory who have taken biosafety training are Dr. Rocío Elizabeth González Castañeda and Dr. Sonia Luquín de Anda. They will train the personnel who will participate in the laboratory activities in terms of risks, handling, disposal and safety codes of the different chemical substances. The laboratory has space for storage capacity for reagent compatibility.

The safety protocol to follow in case of emergency within the laboratory is in accordance with what is suggested in the “Safety protocol in the CENAPRED environmental samples laboratory”, attached in the following link: https://drive.google.com/drive/folders/16mo95bffasZs7ug0Gweyanl8I8x3F_xg.

There is access to the emergency contact number of the Toxicological Information Center, Cruz Verde de Guadalajara, (33) 36-50-30-60.

ANNEX 5

Adherence

Participant number: _____ Date: _____

Consumed capsules: _____

Non consumed capsules: _____

Reason(s):

_____
_____
_____
_____

After taking the capsule, have you experienced:

Headache: _____ Muscle pain: _____ Sore throat: _____ Low fever: _____ Sweating: _____ Nausea: _____ Runny nose: _____ Dizziness: _____ Reflux: _____ Heartburn: _____ Constipation: _____ Colitis: _____ Diarrhea: _____

Others:_____
_____
_____
_____
_____

Do you wish to continue in this research? Yes_____ No_____

Reason (s):

_____
_____
_____
_____

	Time	Place	Food	Portions	Preparation method
Breakfast					
Collation					
Meal					
Collation					
Dinner					

Food frequency					
	0	1	2 a 3	4 a 5	6 o más
Vegetables					
Chard, cabbage, spinach, lettuce, alfalfa germ.					
Broccoli, beet, cauliflower.					
Squash, carrot, onion, tomato, mushroom, pumpkin flower, cactus.					
Fruits					
Strawberries, pineapple, guava, watermelon, blueberries, blackberries, peaches, tangerines, lemon, lime, apple, grapefruit, mango, banana, orange.					
Fat-free cereals					
Amaranth, rice, oatmeal, bolillo, sweet potato, commercial cereal, corn, pasta, Maria cookies, granola, macaroni, midnight, popcorn, potato, corn tortilla.					
Cereals with fat					
Baked corn or nopal toast, telera, commercial cereal bars, croissants, fried foods (commercial potatoes), commercial cookies, sweet bread, tamale, cake, flour tortilla.					
Legumes					
Chickpea, bean, kidney bean, broad bean, soybean.					
Protein with very low fat content					
Clam, fresh or water-drained tuna, beef steak, shrimp, jerky, smoked chop, egg whites, beef brisket, fish fillet, machaca, chicken breast, fish, commercial low-fat cheeses.					
Protein with low fat content					
Skirt steak, tuna in oil, pork chop, pork fillet, pork loin, chicken or beef liver, ground beef, white fish, salmon, light commercial cheeses.					
Protein with moderate fat content					
Pork crackling, egg, chicken with skin, turkey sausage, turkey salami, commercial reduced-fat cheeses.					
Protein with high fat content					
Beef jerky, backbone, ham, pork or beef tongue, moronga, chicken nugget, pigeon, crispy chicken, yellow cheese, Vienna sausage and cheeses in general.					
Dairy					
Milk (specify presentation), yogurt (specify presentation) and ice cream.					
Oils and fats					
Cooking oil, olives, salad dressing, coconut, cream, shortening, margarine, mayonnaise, butter, avocado, bacon, cream cheese.					
Oils and fats with protein					
Almond, hazelnut, peanut, chilorio, chistorra, chorizo, peanut butter, serrano ham, walnut, sesame, pistachio, pepitas, pâté, sunflower seed.					

Participant sign: _____. Researcher name and sign: _____.

ANNEX 6

Hamilton Anxiety Scale (HAS) adapted for the Mexican population

CUADRO 1. ESCALA DE HAMILTON PARA ANSIEDAD (HARS)		
Esta escala especifica la severidad de los síntomas ansiosos en aquellos pacientes diagnosticados con alguno de los trastornos de ansiedad. En su diseño, la escala es precedida de unas breves instrucciones para el médico o el entrevistador en las que se precisa el rango de puntuación según la intensidad de los síntomas a saber: ausente = 0; leve = 1 ; moderado = 2; severo = 3; y grave o totalmente incapacitado = 4.		
1.- Humor ansioso	Inquietud, espera de lo peor, aprehensión, (anticipación temerosa), irritabilidad	0 1 2 3 4
2.- Tensión	Sensación de tensión, fatigabilidad, sobresaltos, llanto fácil, temblor, sensación de no poder quedarse en un solo lugar, incapacidad de relajarse	0 1 2 3 4
3.- Miedos	A la oscuridad, a la gente desconocida, a quedarse solo, a los animales, al tráfico, a la multitud	0 1 2 3 4
4.- Insomnio	Dificultad para conciliar el sueño. Sueño interrumpido, sueño no satisfactorio con cansancio al despertar, sueños penosos, pesadillas, terrores nocturnos.	0 1 2 3 4
5.- Funciones intelectuales	Dificultad en la concentración, mala memoria.	0 1 2 3 4
6.- Humor depresivo	Falta de interés, no disfrutar ya con los pasatiempos, tristeza, insomnio de madrugada, variaciones de humor durante el día.	0 1 2 3 4
7.- Síntomas somáticos (musculares)	Dolores y cansancio muscular, rigidez, sacudidas mioclónicas, chirrido de dientes, voz poco firme, tono muscular aumentado.	0 1 2 3 4
8.- Síntomas somáticos generales (sensoriales)	Zumbido de oídos, visión borrosa, sofocos o escalosfríos, sensación de debilidad, sensación de hormigueo.	0 1 2 3 4
9.-Síntomas cardiovasculares	Taquicardia, palpitaciones, dolores en el pecho, latidos	0 1 2 3 4

	vasculares, sensación de desmayo, extrasístoles	
10.- Síntomas respiratorios	Peso u opresión torácica, sensación de ahogo, suspiros, disnea	0 1 2 3 4
11.-Síntomas gastrointestinales	Dificultad para deglutir, meteorismo, dolor abdominal, náusea, vómitos, borborigmo, sensación de estómago vacío, pérdida de peso, estreñimiento.	0 1 2 3 4
12.-Síntomas genitourinarios	Micciones frecuentes, urgencia de micción, amenorrea, menorragia, desarrollo de frigidez, eyaculación precoz, pérdida del apetito sexual, disfunción eréctil.	0 1 2 3 4
13.- Síntomas del sistema nervioso vegetativo	Boca seca, accesos de rubor, palidez, transpiración excesiva, vértigo, cefalea por tensión, erectismo piloso.	0 1 2 3 4
14.- Comportamiento agitado durante la entrevista	Agitado, inquieto o dando vueltas, manos temblorosas, ceño fruncido, facies tensa, suspiros o respiración agitada, palidez, tragar saliva, eructos, rápidos movimientos de los tendones, midriasis, exoftalmos.	0 1 2 3 4
	TOTAL	

No existen puntos de corte para distinguir población con y sin trastornos de ansiedad, dado que su calificación es de 0 a 56 puntos, el resultado debe interpretarse cualitativamente en términos de intensidad y de ser posible diferenciar entre la ansiedad psíquica (ítems 1, 2, 3, 4, 5, 6 y 14) y la ansiedad somática (items 7, 8, 9, 10, 11, 12 y 13)

- Una puntuación mayor o igual a 15 corresponde a ansiedad moderada/grave (amerita tratamiento)
- Una puntuación de 6 a 14 corresponde a ansiedad leve
- Una puntuación de 0 a 5 corresponde a ausencia o remisión del trastorno.

Beck Anxiety Inventory (BAI) adapted for the Mexican population

Cuadro 2. Inventario de Ansiedad de Beck (BAI)				
Instrucciones : Abajo hay una lista que contiene los síntomas más comunes de la ansiedad. Lea cuidadosamente cada afirmación. Indique cuánto le ha molestado cada síntoma durante la última semana, inclusive hoy, marcando con una X según la intensidad de la molestia.				
Señale una respuesta en cada uno de los 21 rubros	Poco o nada (0)	Más o menos (1)	Moderadamente (2)	Severamente (3)
1.- Entumecimiento, hormigueo				
2.- Sentir oleadas de calor (bochorno)				
3.- Debilitamiento de las piernas				
4.- Dificultad para relajarse				
5.- Miedo a que pase lo peor				
6.- Sensación de mareo				
7.- Opresión en el pecho, o latidos acelerados				
8.- Inseguridad				
9.- Terror				
10.- Nerviosismo				
11.- Sensación de ahogo				
12.- Manos temblorosas				
13.- Cuerpo tembloroso				
14.- Miedo a perder el control				
15.- Dificultad para respirar				
16.- Miedo a morir				
17.- Asustado				
18.- Indigestión o malestar estomacal				
19.- Debilidad				
20.- Ruborizarse, sonrojamiento				
21.- Sudoración no debida al calor				
No existe punto de corte aceptado para distinguir entre población normal y ansiedad. La puntuación media en pacientes con ansiedad es de 25.7 y en sujetos normales es de 15.8.				

CUCS/CINV/0061/24

DICTAMEN DE EVALUACIÓN

Teniendo a la vista los pre-dictámenes aprobados por los Comités de Investigación, Ética en Investigación y de Bioseguridad de este Centro Universitario, respecto al protocolo que a continuación se describe:

Número de registro**: 24-11**
Título del protocolo de investigación: "**Efectos del extracto de hojas de olivo sobre la metainflamación y sintomatología ansiosa en mujeres con exceso de peso"**
Investigador responsable: **Dra. María Sonia Luquín de Anda**
Fecha de la última versión: **20 de marzo de 2024**
Institución en donde se llevará a cabo: **Centro Universitario de Ciencias de la Salud (CUCS) de la Universidad de Guadalajara (UdeG).**

Se emite el presente **DICTAMEN** con el número ***CI-02224*** en virtud de que el protocolo de investigación y el consentimiento informado presentan los elementos necesarios de contenido y calidad por lo que se **APRUEBA** para su realización.

El presente dictamen estará vigente por cuatro años a partir de la fecha, debiendo presentar avances de la investigación cuando menos una vez al año y notificar cualquier cambio o suspensión de la investigación ante esta instancia.

Este protocolo fue aprobado por los asistentes a la sesión conjunta ordinaria de los Comités de Investigación y Ética en Investigación del día 20 de marzo del 2024 y de la sesión ordinaria del Comité de Bioseguridad el 15 de febrero del 2024.

A T E N T A M E N T E
"Piensa y Trabaja"
"30 años de la Autonomia de la Universidad de Guadalajara y su organización den red
Guadalajara, Jalisco; 17 de abril de 2024

Dra. en C. Edith Oregon Romero
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