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The psychopharmacological outcomes of Citrus sinensis and Citrus aurantium essential oils after human inhalation

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ABSTRACT

Essential oils (EOs) from *citrus* peels are primarily volatile terpenoids (VTs). They are complex substances with actions on Central Nervous System (CNS), inducing sedative, anxiolytic, and other effects. The objective was to evaluate the psychopharmacological effects of inhaling mixed *citrus* EOs - rich in VTs - in humans. These EOs are traditionally used by the Brazilian population. A double-blind randomized controlled (RCT) trial with 75 volunteers divided into three groups was carried out. The experimental group inhaled a mixture of *citrus* EOs, composed of sweet orange peel *Citrus sinensis* (L.) Osbeck and bitter orange leaves *Citrus x aurantium* L. The study was conducted for six months, with healthy adults in Porto Alegre, Brazil. The volunteers were evaluated through psychometric protocols and biochemical parameters by saliva analysis. The CONSORT Flow Diagram was employed to report RCTs of aromatic interventions. Improvement in some of psycho-emotional health symptoms was demonstrated, correlated with increased salivary levels of Leptin, and decreases in IL-2, IL-6, and TNF-alpha. The results suggested the existence of an improvement in biochemical parameters and psycho-emotional symptoms after inhalation of *citrus* EOs rich in VTs. This study comes in line with traditional usage and the empirical knowledge of ancestral cultures Brazilian population. It may contribute to supporting the idea that the inhalation of natural products can improve psycho-emotional states with positive repercussions on the immune system. [Approved: network International Clinical Trials Registry Platform, ICTRP, RBR-6ktp6t9 - UTN: U1111-1332-7952, Ethical Committee Certificate of Presentation of Ethical Appreciation, number CAAE: 46098615.5.0000.5347, Federal University of Rio Grande do Sul, UFRGS, Brazil. Date August 24, 2015]

Keywords: *Citrus* essential oil, volatile terpenoids, human health, immune system, inhalation, psychopharmacological, saliva Luminex™

1. INTRODUCTION

Essential oils (EOs) are complex substances extracted from different plant species. When they are from *citrus* peels are primarily volatile terpenoids (VTs). It is known that these compounds are a large group of secondary metabolites with actions on Central Nervous System (CNS), inducing sedative, anxiolytic, antinociceptive, anticonvulsant, proconvulsant, and other effects (1). The use of VTs from EOs for therapeutic purposes is called Aromatherapy. There are several application methodologies, the most common in clinical setup being massage and inhalation (2-4).

The EO, through their terpenic components, seems to be an option or a complementary method for promotion of psychological well-being and healthy physiological effects (5, 6). The psychological effects of scent inhalation by humans, indicating that fragrances alter human behavior, were compiled in a review. The studies documented that odor influences sympathetic and parasympathetic nervous systems and neurophysiological brain activity. In addition, they can act on neuroendocrine system, neurotransmitters, and neuromodulators. This influences psychological behavior as well as biochemical parameters and their function (7).

In 2004, Nobel Prize in Physiology or Medicine was awarded jointly to Richard Axel and Linda Buck. These scientists discovered the olfactory receptors (OR), the organization of olfactory system, and their importance for the balance of whole organism (8-10). The effect of EO through inhalation route was observed even when this pathway was damaged (11). Although mapping human olfactory receptors is still under extensive research phase, researchers already know many of them. There are OR inside and outside of olfactory system, this last are

called human ectopic olfactory receptors (EOR). The therapeutics actions of VT are attributed to the OR-VT and EOR-VT bonds (12, 13).

The inhalation of odorant molecules modulates the biochemical parameters pathways then these findings suggest that olfactory system plays a role in the function of CNS in addition to that of smell. It was observed that odorant compounds influenced stress biomarkers, dehydroepiandrosterone, oxidative stress, estradiol, Dopamine, skin barrier, sebaceous secretion, and immune skin system in humans (7).

The terpenic structures present in EO affect neurotransmitter system gamma-aminobutyric acid GABAergic, glutamatergic, dopaminergic, and opioid neurotransmitter systems. Some of these receptors are dopaminergic receptors-linalool and anxiogenic receptors-limonene, present in rats, mice, and humans (1, 14). Linalool is one of components that frequently integrate EO composition. Some of biological action properties of linalool are sedative, anxiolytic, analgesic, anticonvulsant, anti-inflammatory, and local anesthetic. The chiral isomers of linalool also have biological activity, however the mechanisms of its activity are still not fully understood (15, 16).

The connection between the immune system, through interleukins, and mental health has been focusing of research where it appears that there is an interdependence, still in search of elucidation of this complex mechanism (17-21). After antidepressant treatment in human patients, pro-inflammatory interleukin IL-2 levels decreased in serum. However, anti-inflammatory interleukin IL-6 levels increased. The proposed is that antidepressant therapy has an immunomodulatory effect (22).

A review that covers studies of over 20 years found normalization of IL-6 and cytokine tumor necrosis factor alpha (TNF-alpha) levels through use of antidepressant medication, demonstrating an association between antidepressant action, and functioning of cytokines in body. The conclusion was that a better understanding of specific pharmacological and pharmacogenetic mechanisms of depression and immune system is needed (23). In addition to anti-inflammatory markers, Leptin is another biochemical parameter that is most often interrelated with psycho-emotional state of anxiety (24-26).

This hormone provides information about energy balance for the regulatory center of brain and its release is associated with promotion of satiety since states of anxiety, depression, and stress can lead to food compulsion (27, 28). The researchers observed that stress and depression scores were highly correlated, and depressive symptoms were correlated with TNF-alpha, IL-6, and anti-inflammatory interleukin IL-10.

The measurement of salivary Cortisol as a stress marker is an indicator of plasma concentration of free Cortisol (29-31). Dopamine has been shown to correlate with neurological diseases, depression, mood, well-being, and anxiety (32, 33). Some studies have quantified Dopamine in biological materials such as urine, plasma, and brain (34-36).

In clinical trials, one reason that contributes to the low number of participating volunteers is the necessity of blood collection. This invasive procedure requires qualified personnel to perform it. In this case, saliva comes as a non-invasive form of a biological sample. Saliva is representative of measuring pro and anti-inflammatory cytokines and others, which includes electrolytes, mucus, antibacterial compounds, enzymes, hormones, and growth

factors, due to their correlation with the plasmatic levels of these parameters (22, 37-41).

A single EO has many VT components. The therapeutic action of one EO considers the joint interaction of these VT components. When the effect is greater than that of its individual components, we call it a synergistic effect. When the effect is less than that of its individual components, we call it an antagonistic effect (42). A study shows a different effect on human physiological parameters and self-ratings of arousal, when inhaled sandalwood EO than its major component alpha-santalol (43-45). The synergistic interactions are important to explain the efficacy of apparently low doses of active constituents in an herbal product (46). In aromatherapy, it is common to use more than one EO together, and effect is characterized by the interactions of all components of blend EO, which act differently from when they are isolated (47, 48).

This study used blend of *Citrus sinensis* EO and *Citrus aurantium* EO because the authors have conducted in-depth research on them. The VTs composition of 14 *citrus* EO was studied by authors, and they met a complexes composition included bioactive enantiomers of limonene and linalool (49). After the authors carried out a preclinical study with 2 these EOs previously (50). The chosen EOs are traditionally used by the Brazilian population through the empirical knowledge of ancestral cultures, demonstrating that the orange EO aroma's action provides emotional well-being and prevents infections (51), and they are easily accessible and low cost in Brazil. These essential oils, EOs from sweet orange *Citrus sinensis* peel and from bitter orange *Citrus aurantium* leaves, have chemical compounds that act on the psyche and physiology (52, 53). The

authors expect that there will be a joint action of the components of the EO used, with positive effects on human health.

The anti-inflammatory action of *citrus* EOs, especially from sweet and bitter orange, and some of its isolated components, was researched. However, rare studies have been made correlating these data with the psycho-emotional symptoms (54). Despite the existence of a relationship between psycho-emotional states and parameters of immune system, this correlation is not fully understood (55). It is very important to deepen the studies that correlate cytokines fluctuations with psychometrics protocols, which are usually neglected by researchers in the field of mental health (56).

This study was designed to investigate if inhalation of a mixture of *citrus* EO rich in VTs alters some parameters in humans. The primary aim was to evaluate effects of this inhalation on psycho-emotional parameters: anxiety, depression, stress, positive and negative affection, and well-being. A secondary aim was to test hypothesis that this practice influences the change in the salivary levels of biochemical parameters: IL-2, IL-6, TNF-alpha, IL-10, Leptin, Dopamine, and Cortisol.

The choice of these inflammatory markers aimed to obtain a broad overview of both the pro-inflammatory and anti-inflammatory systems, based on other research (17-21). The results of this study will help the scientists to deepen their understanding of diseases related to the parameters studied here, as well as will serve as a basis for the development of natural therapies.

2. MATERIALS AND METHODS

2.1. Population

The population of this study was composed by Brazilian volunteers, residents in Brazil, recruited with the support of Cognitive Behavioral Institute of Therapy (InTCC) and Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil over a period of six months. The inclusion criteria of volunteers were adult people, in physical and psychological health (Table 1). The physical and psychological health, criteria of not being a user of illicit drugs, and not being addicted to alcohol were freely declared by the volunteers.

The study volunteers were not selected on any specific condition, therefore, prior tests were not performed for the psychological and biochemical parameters evaluated in this research. Some volunteers reported being occasional nicotine smokers, not addicts.

The exclusion criteria were people with pathologies (cancer, diabetes, degenerative illnesses, chronic periodontitis), having suffered serious traumatic events (personal experiences), or medication (psychotropic, melatonin, and anti-inflammatory).

All volunteers were informed about the conditions of research, and each volunteer read and signed the Informed Consent Form (ICF). Besides, all the volunteers filled out the Confidential Questionnaire each month in order to obtain clinical and psychological data to assist in the inclusion-exclusion criteria in this study.

This study was approval and published in network International Clinical Trials Registry Platform (ICTRP), RBR-6ktp6t9 - UTN: U1111-1332-7952. Ethical approval for this study was obtained from the ethical committee Certificate of Presentation of Ethical Appreciation (number CAAE: 46098615.5.0000.5347, Federal University of Rio Grande do Sul, UFRGS, Brazil, August 24, 2015).

2.2. Study design

This is a parallel, double-blind, randomized controlled trial, and design of temporal dynamics of research procedure is shown in Figure 1. The volunteers stayed in a scented room for one hour, once a week, for 2.5 months, followed by a 1-month break, and repeated the process once more until it completed the 6-month study. Once our sampling was by convenience (57), multiple variables were tested, and there was no previous study that allowed us to estimate neither the effect size, nor sample size previously determined. However, a sample size of 25 would be sufficient for a t-test with alpha = 0.05 and power = 0.80, considering a Standardized Effect Size of 0.8 (58). Ultimately, due to the diverse nature of studied variables, they did not yield the same variability, jeopardizing the power of test. Such drawbacks are debated in the results section.

After applying the exclusion criteria, we randomized the volunteers (n= 75) and separated them into three groups: Experimental Group (n=25), Placebo Group (n=25), and Control Group (n=25). The randomization method was to separate the volunteers into the respective groups - Experimental, Placebo, and Control - according to the volunteers' availability on the day of week they could join the study. The double-blind method was designed so that the scales applicator and each volunteer did not know which group they were in the Experimental, Placebo, or Control group. The double-blind study could be carried out because the volunteers were not aware of the dilution of EO that would be used. The volunteers in the Placebo and Control groups thought it was a very diluted solution in which the *citrus* aroma was imperceptible. During the test period, the volunteers remained seated, reading or studying neutral subjects. In addition, conditions of room environment were the same for the three groups,

which helps controlling the interference of potential influence of room environment.

This study did not apply treatment to volunteers, so the patient compliance to the treatment's criterion is not applicable. Therefore, there was no intention-to-treat analysis in this study.

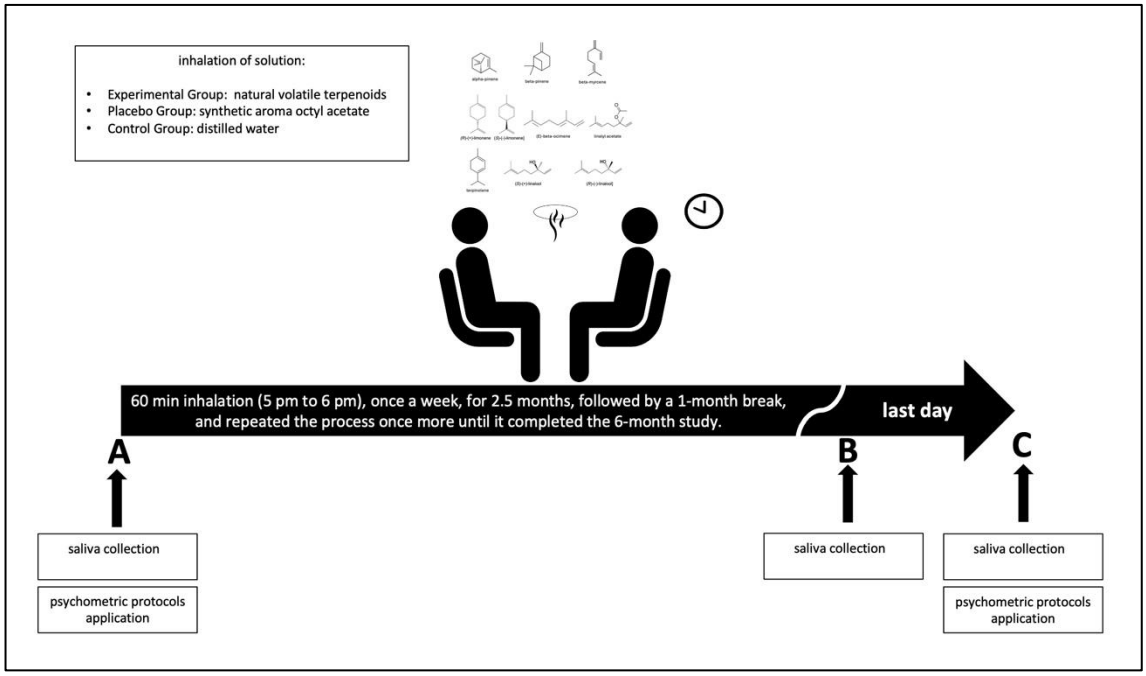


Figure 1 - Design of temporal dynamics of research procedure: A-moment, B-moment and C-moment.

2.3. Intervention

Brazilian EOs of *Citrus sinensis* (L.) Osbeck obtained from the peel, by cold pressing extraction, and of *Citrus* x *aurantium* L. obtained from leaves, by steam distillation extraction (brand Laszlo Aromatherapy Brazil, conventional cultivation, location for growing in south-central Brazil: latitude 22° 21' 58" S.; longitude 48º 22' 48" W.; altitude 669 m) were used (49, 59). The methodology used to analyze the chemical composition of EO blend is in Supplementary Material.

The Experimental Group inhaled the vaporization of EO blend (50% *Citrus sinensis* EO and 50% *Citrus aurantium* EO, each EO pure without dilution). The Placebo Group inhaled the vaporization of 10% Octyl Acetate solution in saline (0.9% NaCl solution). Octyl Acetate (Flavor and Extract Manufacturers Association of the United States, FEMA number 2806; Chemical Abstract Service, CAS number 112-14-1) have a similar smell of orange fruit, it is regulated for safe use as a flavoring additive in human foods (60). The Control Group inhaled vaporized distilled water.

2.4. Vaporizer apparatus

The apparatus used to vaporize the solutions was an electric ceramic vaporizer (Via Aroma Company Brazil). Following the guidance of ceramic manufacturer, one apparatus was installed for every 30 m^2 of room and it turned on during the test time. The temperature that the liquid reaches inside the electric ceramic vaporizer was considered constant at 40 ºC, sufficient to vaporize the EO blend, the placebo solution, and control water. The volume of 7 mL of each solution was added inside of apparatus, according to each group. It was determined that this volume was enough for the entire room to be vaporized during the 60 minutes.

2.5. Measures of Psychometric protocols

The psychometric scales were chosen considering they are well established instruments in the scientific psychological literature for measuring symptoms of depression, anxiety, stress, as well as affect and well-being. In addition, they have adequate psychometric properties and they have been validated for the Brazilian population. Psychometric protocols were applied on different days of intervention

in the afternoon (Figure 1). Six psychometric protocols were used in this research: Beck Anxiety Inventory (BAI) (61), Beck Depression Inventory (BDI) (57), State-Trait Anxiety Inventory (IDATE) (62), Lipp's Adult Stress Symptoms Inventory (ISSL) (63), Positive and Negative Affect Schedule (PANAS) (64) and Goldberg's General Health Questionnaire (GHQ-12) (65). All these psychometric protocols were validated in the Portuguese language for Brazilian population.

BAI is a self-report questionnaire with 21 multiple-choice questions of four points, used to measure the severity of an individual's anxiety. Anxiety levels are evaluated based on physiological, cognitive, and affective symptomatology. A good internal consistency of BAI was observed with Brazilian population (Cronbach's alpha=0.92) (66). BDI measures the symptoms of depression through 21 self-report multiple-choice questions used to measure the severity of an individual's clinical depressive symptomatology. This instrument investigates how the individuals have been feeling in the last week regarding cognitive, affective, and somatic symptoms and sensations. In Brazil, BDI showed adequate internal consistency with Cronbach's alpha of 0.88 (66). IDATE is an inventory that consists of two scales, each with 20 questions that separately measure state anxiety (IDATE-S) and trait anxiety (IDATE-T), totaling 40 questions. In IDATE-S, the questions refer to how the person feels at the time the assessment is being carried out; IDATE-T questions represent how the person normally feels. The responses indicate levels of anxiety in these two contexts using a four-point Likert scale ranging from little to very much. The IDATE Brazilian version presents Cronbach alphas of 0.89 for the scale of state and 0.88 for trait anxiety (67).

ISSL is an inventory that detects the presence of stress and phase of stress in which the person is, as well as the predominance of physical and/or

psychological symptoms in each phase. It consists of three assessments: the first concerns the symptoms presented in the last 24 hours - alert phase; the second is related to the symptoms experienced in the last week - phases of resistance and near-exhaustion; and third refers to the symptoms presented in the last month - exhaustion phase. It is a self-report questionnaire with 53 items, 34 of which assess somatic symptoms and 19 assess psychological symptoms. ISSL is an instrument validated for the Brazilian population with an alpha of 0.91 (63). PANAS is a scale, adapted for the Brazilian population, containing 20 evaluative items for each category of affect: positive (alpha = .88, PANAS-P) and negative (alpha = .86, PANAS-N) (68). Using a five-point Likert scale ranging from very little or nothing to extremely, participants should assess the degree to which they have experienced these affects lately. The scores for positive affect and negative affect are generated separately, and higher the score, the greater the report of affect. GHQ is a 12-item scale, which assesses the mental health of non-clinical adults using a four-point Likert scale to investigate psychological well-being indicators. GHQ was validated for the Brazilian population. The instrument showed adequate factorial validity and reliability, with a Cronbach's alpha of 0.85 for depression and 0.82 for social dysfunction (69).

2.6. Saliva samples

Saliva samples were collected at different moments on the last day of intervention (Figure 1). Prior 2 hours of collection of saliva, the volunteers must not have ingested any solid or liquid food. The Salivette™ device was used for the collection of saliva (code blue, ref 51.1534.500, Sarstedt). After the collection, the samples were centrifuged by Centrifuge 5430R Eppendorf at 2000 rpm for 2

minutes, at a temperature of 4 °C. The centrifuged saliva was transferred to a cryogenic tube with a screw cap followed by the addition of Sigma Protease Inhibitor Cocktail code P8340 (2 µL inhibitor / 1 mL centrifuged saliva). The tubes were then kept refrigerated and protected from light by using aluminum foil and placed in a freezer under -80 ºC.

It is important to note that Dopamine oxidizes very quickly and addition of an antioxidant right after sample collection is necessary. In this study, where multi-analyses were performed on the same sample collected, it was added the Protease Inhibitor, previously specified.

2.7. Biochemical analysis

Salivary levels of interleukin-2 (IL-2), interleukin-6 (IL-6), TNF-alpha, interleukin-10 (IL-10), Leptin, and Cortisol were measured in a multianalyte profile using the Luminex 200IS™ immunoassay system (Luminex Corporation, Osterhout, The Netherlands), the Milliplex MAP™ commercial kit Millipore Corporation, Billérica, MA 01821, USA (calibration microspheres for classification and report readings as well as sheath fluid were also purchased from Luminex Corporation and fluorescence data obtained were analyzed by the Xponent software), specifications HCYTOMAG-60K (IL-2, IL-6, TNF-alpha, IL-10), HMHEMAG-34K (Leptin) and HNCSMAG-35K (Cortisol).

The salivary level of Dopamine was measured by an ELISA immunoassay system (Enzyme-Linked Immune Sorbent Assay, manufacturer LDN Labor Diagnostika Nord, reference specification BA E-5300, kit number LOT 151038-1), the optical density of samples was measured in a spectrophotometer at 450 nm. All analyses were performed according to the manufacturers.

The analysis of salivary levels of IL-6, IL-10, and TNF-alpha cytokines using the Milliplex MAP and Luminex™ immunoassay methodology has already been used by researchers (39, 70-76). The assessment of hormones and interleukins in saliva has gained wide acceptance in clinical endocrinology (77). The relationship between salivary levels and levels in other biological matrices such as plasma has been established for the analytes in many studies (30, 39, 78).

2.8. Procedure

Each group of volunteers remained together seated for 60 minutes inside the bright room, with a total area of 30 m^2 x 2 m of height. Each group used their respective room throughout the entire study, being room prepared specifically for each test group. The three rooms used in this study were similar in size. Each group of volunteers occupied an exclusive and specific room throughout the study, on different days, to ensure that there was no contamination of aromas between the study rooms.

Vaporization started 15 min before volunteers entered the room, to homogenize the environment with inhalation solution. The volunteers started inhalation at 5 pm and ended inhalation at 6 pm. This method of environmental vaporization and inhalation of scented solution is one of ways of applying aromatherapy. The selection of 60 minutes as the duration of inhalation was based on other studies that used a similar methodology (79, 80).

Psychometric protocols and saliva samples were collected at three different moments of experiment: A-moment (saliva collection and psychometric protocols application, before any experimental procedure, at 5 pm), B-moment

348 (saliva collection, before starting the experiment on the last day inhalation, at 5
349 pm) and C-moment (saliva collection and psychometric protocols application,
350 after finishing the experiment on the last day inhalation, at 6 pm) (Figure 1).
351 The volunteers themselves answered the psychometric protocols, no
352 interview was carried out. This study was dedicated to comparing the results of
353 psychometric protocols within a six-month interval. It is recommended that the
354 evaluation of psycho-emotional parameters occurs with a time interval between
355 3 to 6 months (56, 81, 82). Before answering the psychometric protocols, the
356 volunteers declared that they were not subjected to situations of extreme stress
357 or extreme anxiety in the 3 months that preceded the moment of self-report of
358 these protocols.
359 This study collected saliva to determine baseline levels of biological
360 parameters and then compared the levels over a six-month interval of inhalation
361 of the essential oil mixture; data not presented here. However, many authors have
362 performed the methodology of comparing salivary levels obtained from
363 collections at short time intervals, such as 60 minutes to one week (34, 38, 41,
364 76, 83). due to their extremely fluctuating over extended periods of time.
365 Therefore, this study also collected saliva before and after the last inhalation
366 intervention of EO blend, on the last day of the study, six months after its start.
367
368 **2.9. Data analysis**
369 Three statistical tests were employed, depending on the characteristic of data:
370 for the ordinal qualitative data, the Friedmann Test was performed to test the
371 difference between medians; to compare the mean difference within samples
372 among time, a Paired Student's t-Test was employed (two-sided for the

Psychological Variables and one-sided for the Biochemical Parameters); and Fischer's Exact Test was employed to verify the difference between two distributions of some characteristic. All analyses considered an alpha = 0.05. The software R (package stats) was used to perform the analysis.

3. RESULTS AND DISCUSSION

We considered the Consolidated Standards of Reporting Trials (CONSORT) checklist statement to report Randomized Controlled Trial (RCT) of aromatic interventions and used them in conjunction with the existing CONSORT Flow Diagram (84, 85). Eighty-nine volunteers were recruited for the study at the beginning. Seventy-five (84%) volunteers were randomly selected and allocated to the Experimental Group (n=25, 28%) the Placebo Group (n=25, 28%), and Control Group (n=25, 28%). Forty-six volunteer data were analyzed in the Experimental Group (n=17), Placebo Group (n=14), and Control Group (n=15). Twenty-nine volunteers did not complete the study. The description and reasons for volunteers' withdrawal were moving to another city and tiredness of being part of this study for many months. The exclusion criteria were starting medication use, or starting psychotherapeutic treatment, or illness listed in item Population. Figure 2 shows the flow diagram of this study. Men and women with ages from 20 to 40 years completed the study and their sociodemographic characteristics are demonstrated in Table 1.

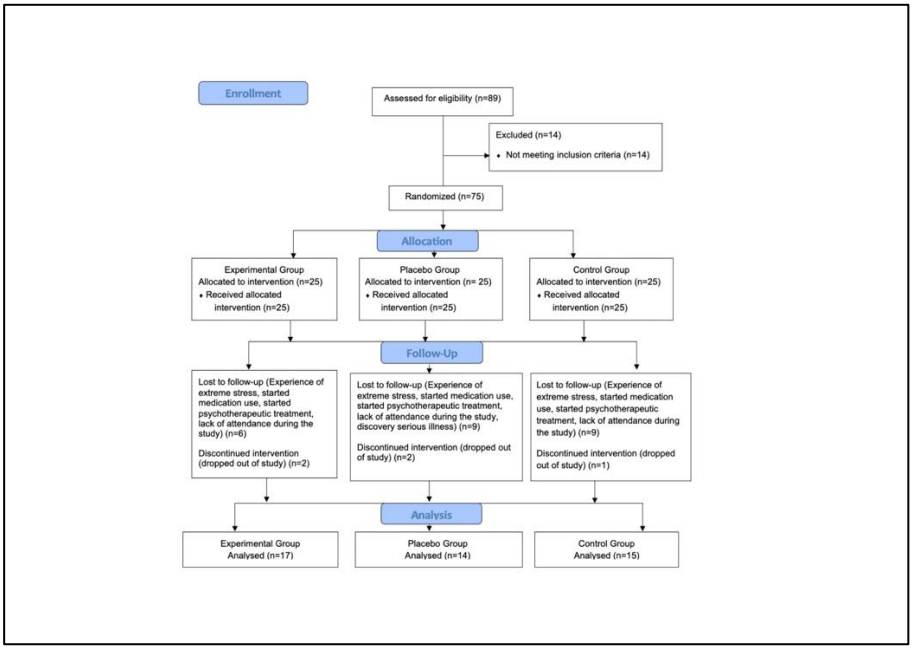


Figure 2 - Study randomization process CONSORT flowchart.

Table 1. Sociodemographic characteristics of volunteers.

	Experimental Group (n=17)	**Placebo Group (n=14)**	**Control Group (n=15)**
	n (%)	**n (%)**	**n (%)**
Gender:			
Female	15 (88 %)	11 (79 %)	12 (80 %)
Male	2 (12 %)	3 (21 %)	3 (20 %)
Education Level	100% undergraduate degrees	100% undergraduate degrees	100% undergraduate degrees
	Mean (SD)	**Mean (SD)**	**Mean (SD)**
Age	29.1 (8.5)	36.3 (13.5)	31.5 (12.0)

Note: “n” refers to absolute sample number, “%” refers to relative frequency, “SD” refers to Standard Deviation.

3.1. Psychological Variables

The variables of Beck Anxiety’s scores (BAI), Beck Depression scores (BDI), and Goldberg`s General Health scores (GHQ) showed non-parametric distributions. The variables IDADE-S, IDATE-T, PANAS-P, and PANAS-N showed normal distribution. The groups did not differ in the psychological outcomes at baseline. The summary results of psychological outcomes are presented at Table 2.

Table 2. Summary results of Psychological Outcomes.

Variable	Experimental Group (n=17)			Placebo Group (n=14)			Control Group (n=15)		
	A-moment	C-moment		A-moment	C-moment		A-moment	C-moment	
	Median (IQR)	**Median (IQR)**	***p-value*[a]**	**Median (IQR)**	**Median (IQR)**	***p-value*[a]**	**Median (IQR)**	**Median (IQR)**	***p- value*[a]**
Beck Anxiety Inventory (BAI)	5.50 (2.00;12.00)	5.00 (1.00; 9.00)	.139	4.00 (3.00;6.00)	6.50 (2.00; 9.00)	.109	6.00 (3.00;9.00)	6.00 (3.00;13.00)	.101
Beck Depression Inventory (BDI)	3.00 (1.00; 5.00)	3.5 (1.00; 7.00)	.065	3.50 (1.00; 9.00)	3.50 (2.00; 5.00)	.069	7.00 (3.00; 10.00)	4.00 (1.00; 10.00)	.094
Goldberg's General Health Questionnaire (GHQ)	1.00 (0.00; 4.00)	1.00 (0.00;4.00)	.098	1.00 (0.00;4.00)	1.00 (1.00;3.00)	.369	2.00 (0.00;4.00)	3.00 (1.00;6.00)	.056
	Mean (SD) (SE)	**Mean (SD) (SE)**	***p-value*[b]**	**Mean (SD) (SE)**	**Mean (SD) (SE)**	***p-value*[b]**	**Mean (SD) (SE)**	**Mean (SD) (SE)**	***p-value*[b]**
State Anxiety Inventory (IDATE-S)	40.33 (11.87) (1.73)	32.88 (11.83) (1.72)	<.001	37.57 (8.38) (1.22)	43.64 (12.41) (1.80)	.016	37.47 (6.29) (0.92)	43.00 (6.79) (0.99)	<.001
Trait Anxiety Inventory (IDATE-T)	39.06 (9.64) (1.40)	35.50 (9.56) (1.39)	.160	34.86 (5.58) (0.81)	39.85 (8.76) (1.27)	.044	35.60 (7.17) (1.04)	41.67 (7.48) (1.09)	.012
Positive Affect Schedule (PANAS-P)	60.72 (11.01) (1.60)	61.50 (8.76) (1.27)	.617	58.28 (7.12) (1.04)	52.57 (10.57) (1.54)	.058	56.13 (8.87) (1.29)	48.93 (11.83) (1.72)	<.001
Negative Affect Schedule (PANAS-N)	35.83 (9.44) (1.38)	34.27 (13.97) (2.03)	.545	37.21 (12.42) (1.81)	44.05 (14.04) (2.05)	.002	37.47 (7.36) (1.07)	42.92 (9.09) (1.33)	.011
	n%	**n%**	***p-value*[c]**	**n%**	**n%**	***p-value*[c]**	**n%**	**n%**	***p-value*[c]**
Lipp's Adult Stress Symptoms Inventory (ISSL)	38.88%	11.11%	.641	35.71%	57.14%	.028	6.67%	33.33%	.333

Note: description and comparison of medians and interquartile range (IQR), means, standard deviation (SD) and standard error (SE), and percentages of the participants of the Experimental, Placebo, and Control groups at baseline A-moment and C-moment. The Groups did not differ at baseline in any variable. The medians are followed by the Interquartile Range (IQR). [a] refers to Friedmann Test to compare differences between the medians of A-moment and C-moment of each group. [b] refers to Paired Student's t-Test for compare the differences between the mean scores at A-moment and C-moment of each Group. [c] refers to Fisher's Exact Test was used to compare the frequency of stress symptoms between the A-moment and C-moment for each group. Comparing groups at C-moment, we observed statistical difference only for the prevalence of stress symptoms, which was lower in the Experimental Group (p=.016). Highlight gray cells have data with statistical significance.

The results obtained from the mean data of each group, for each psychological variable at A-moment and C-moment are shown in the following.

The BAI scores showed a trend towards significance as the Experimental Group had decreased mild anxiety symptoms. The opposite occurred in the Placebo and Control Groups. The BDI scores showed a trend towards significance as the Experimental Group eliminated the mild depression symptoms. The opposite happened in the other groups, since ‘the moderate level depression’ increased in the Placebo Group, and ‘the mild level depression’ increased in the Control Group.

We observed a decrease in IDATE-S scores between A-moment and C-moment for the Experimental Group (Figure 3), with statistically significant. The opposite behavior occurred with the scores of the Placebo and Control groups. About IDATE-T scores, the results of decrease in C-moment showed a trend towards significance for Experimental Group. In IDATE-T scores, Placebo and Control groups increase scores at C-moment, with statistically significant.

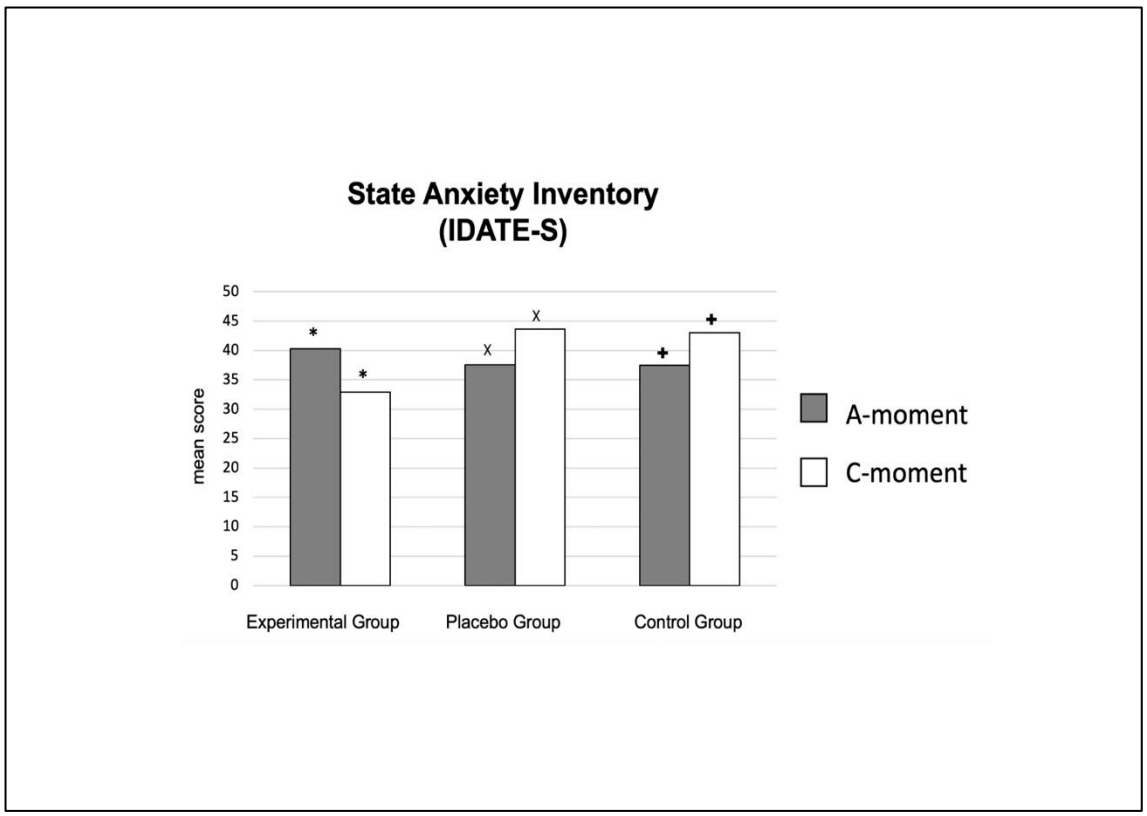


Figure 3 - Compilation of responses, at A-moment and C-moment, from Experimental (EG), Placebo (PG), and Control (CG) groups to questions of State Anxiety Inventory (IDATE-S). The statistical significance in the data of each group is represented by the symbols: * , ˣ and ✚.

In Figure 4, there are the ISSL comparing A-moment and C-moment for all groups. It can be observed that the stress reduction in the C-moment occurred exclusively for the Experimental Group. As well as there was statistical significance at C-moment between Experimental and Placebo groups.

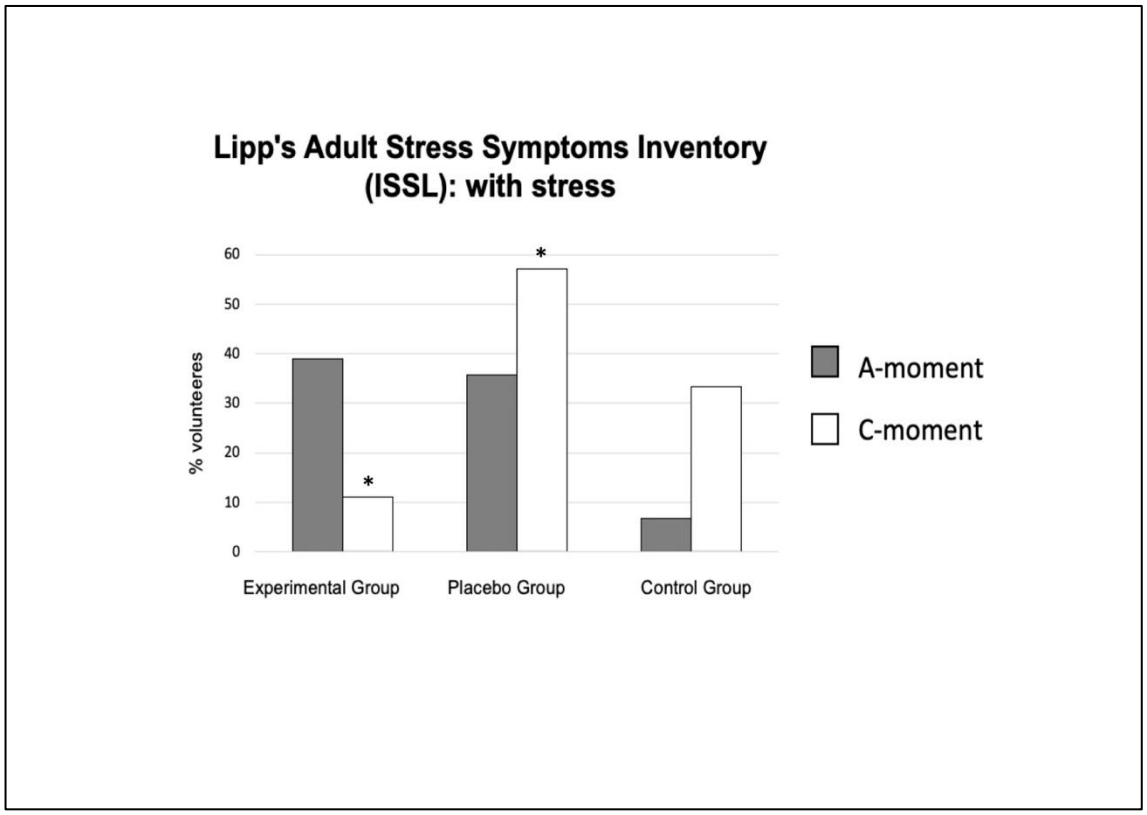


Figure 4 - Compilation of responses, at A-moment and C-moment, from Experimental (EG), Placebo (PG), and Control (CG) groups to questions of Lipp's Adult Stress Symptoms Inventory (ISSL) with stress. The statistical significance in the data of each group is represented by the symbol *.

Results of PANAS score in the Experimental Group showed a trend towards significance of increase the Positive Affect and decrease the Negative Affect. Inverse effect occurred with scores of Placebo and Control groups. The Placebo and the Control groups showed of increased Negative Affect in the scores C-moment, with statistical significance.

About the GHQ scores, the results showed a trend towards significance as the Experimental Group decreased the presence of “psychiatric disorders with suffering”, whereas the volunteers of Placebo and Control groups did not show the same health-improving behavior at C-moment.

3.2. Biochemical Parameters

Normal distribution is assumed for the quantitative difference of paired samples for biochemical parameters. Table 3 shows the summary statistic results of biochemical parameters.

Table 3. Summary statistic results of Biochemical Parameters.

Biochemical parameter	Experimental Group (n=17)	Placebo Group (n=14)	Control Group (n=15)
IL-2	.039	.931	.774
IL-6	.007	.958	.965
TNF-alpha	.009	.918	.497
IL-10	.062	.984	.988
Leptin	.025	.846	.802
Dopamine	.437	.801	.839
Cortisol	.519	.729	.791

Note: highlight gray cells show statistical significance by Paired Student’s t-Test. Test compared the differences between the mean data at B-moment and C-moment of each Group.

The results were obtained by analyzing the variation of salivary levels collected at the B-moment and C-moment. Thus, the mean values of each group, for each biochemical parameter are shown in the following.

According to Figure 5, it was observed that volunteers of Experimental Group had a significant reduction of pro-inflammatory salivary levels of IL-2 (Figure 5A), IL-6 (Figure 5B), and TNF-alpha (Figure 5C), while in the Placebo and Control groups, we observed that this reduction did not occur.

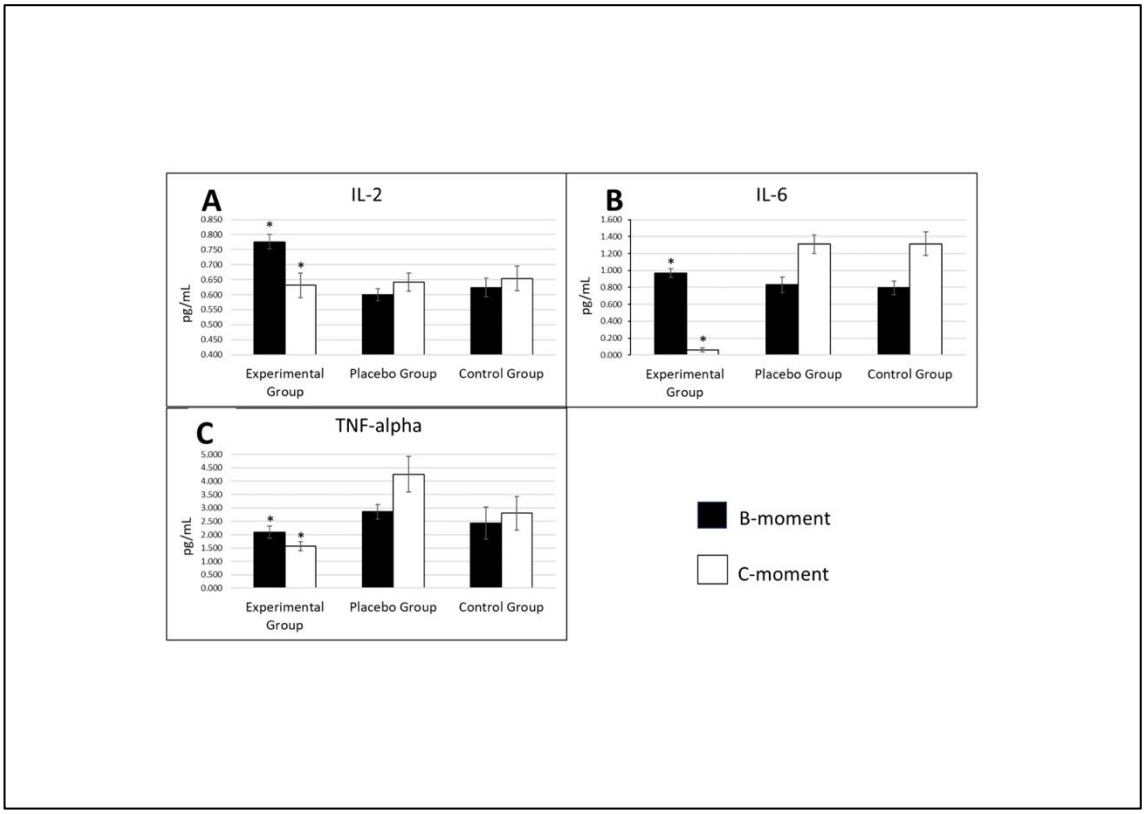


Figure 5 - Salivary level biochemical parameter of Experimental (EG), Placebo (PG) and Control (CG) groups volunteers, at B-moment and C-moment collections: IL-2, IL-6 and TNF-alpha. The error bar is represented by the parallel line to axis (pg/mL), which represents the uncertainty or variation of corresponding coordinate of point. The statistical significance in the group data is represented by the symbol: * .

The results of anti-inflammatory cytokine salivary level IL-10 showed a trend towards significance as the Experimental Group increased this parameter, between B-moment and C-moment. It decreased in the Placebo and Control groups.

The Figure 6 show that the salivary level of protein marker of food satiety sensation Leptin. Salivary level Leptin increased in volunteers of Experimental Group while decreasing in the Placebo and Control groups.

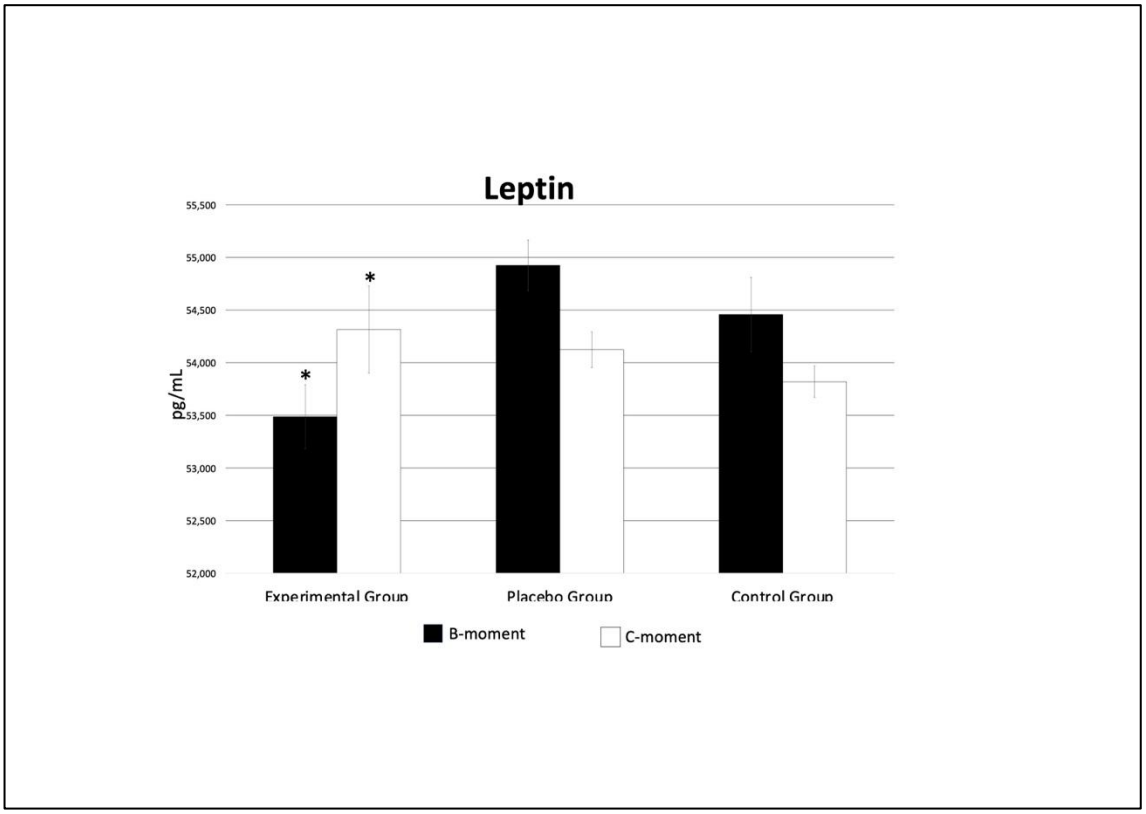


Figure 6 - Salivary level Leptin biochemical parameter of Experimental (EG), Placebo (PG), and Control (CG) groups volunteers, at B-moment and C-moment collections. The error bar is represented by the parallel line to axis (pg/mL), which represents the uncertainty or variation of corresponding coordinate of point. The statistical significance in the group data is represented by the symbol: ***** .

About salivary level Dopamine, the results showed decrease in this parameter at the C-moment in Placebo and Control groups. Experimental Group showed a trend towards significance with the increase of this parameter, between B-moment and C-moment.

Finally, about hormone marker of stress Cortisol, the results showed a trend towards significance as the Experimental Group decreased salivary level Cortisol, while it increased in Placebo and Control groups volunteers, between B-moment and C-moment.

Table 4 presents an overview of results for the Experimental, Placebo, and Control Groups in relation to psychometric protocols and biochemical parameters with statistical significance.

Table 4. Resume results of Psychometric Protocols and Biochemical Parameters analysis in the Experimental, Placebo and Control groups with statistical significance.

Essay	Variable	Characteristic	Analytical method	Figure number
Psychometric Protocols	IDATE-S	anxiety	State Anxiety Inventory	3
	ISSL	stress	LIPP'S Adults Stress Symptoms Inventory with stress	4
Biochemical Parameters	IL-2	pro-inflammatory	Luminex 200IS™ immunoassay system	5A
	IL-6	pro-inflammatory	Luminex 200IS™ immunoassay system	5B
	TNF-alpha	pro-inflammatory	Luminex 200IS™ immunoassay system	5C
	Leptin	marker of food satiety sensation	Luminex 200IS™ immunoassay system	6

3.3. Decreased parameters in Experimental Group

The inhalation methodology of *Citrus sinensis* and *Citrus aurantium* EO blend rich in VT reduced the anxiety state IDATE-S (Figure 3), stress symptoms ISSL (Figure 4), as well as the salivary IL-2 (Figure 5A), IL-6 (Figure 5B) and TNF-alpha (Figure 5C). Our results agree with the other studies. The effects on the psycho-emotional state in humans after inhaling aromas and EOs were observed, when *Rosa damascena* EO and orange *Citrus sinensis* EO from Brazil were used (3, 86). These researchers showed an improvement in the emotional state through the reduction of IDATE data. The anxiolytic effect of inhalation of *Matricaria chamomile* EO on patients before esophagogastroduodenoscopy was confirmed through the IDATE (87). The extensive review, containing 191 studies, shows EOs and their major components being administered by inhalation and other methods, in humans and animals, to assess systems for anxiety. This review concluded that monoamine neurotransmitters, amino acid neurotransmitters, and hypothalamic−pituitary−adrenal (HPA) axis are thought to play important roles in the anxiolytic effects of EOs (88). Our study observed that there was a simultaneous decrease, together with the anxiety state, of

proinflammatory interleukins. This result may indicate that there is a correlation between the psycho-emotional state and inflammation.

There is evidence consistent of possibility that HPA axis activity may mediate associations between psychosocial stressors and inflammatory processes through the investigation of salivary levels of Cortisol, and inflammatory markers IL-6, IL-10, and TNF-alpha in a population of 869 adults (73). The connection between anxiety and depression is known in the field of mental health (33). The reduction of depression levels, when observing the reduction of pro-inflammatory cytokine IL-6 and TNF-alpha, in human serum, during an intervention for 12 weeks was studied was studied (56), as well as the immunological changes in depression (22). The results of this work are in line with these previous studies. It can be assumed that hectic and very busy urban life naturally causes anxiety in volunteers of all groups, and there is a tendency to increase the pro-inflammatory parameters. The Experimental Group inhaled the blend EO, had a decrease in anxiety state, and pro-inflammatory parameters.

About linalool enantiomers, the inhaling of (S)-(+)-linalool proved to be an activating schedule when evaluated against the electrodermal activity and increasing alertness. The inhaling of (*R*)-(-)-linalool proved to be a relaxing agent when evaluated against heart rate, causing stress relief (15, 89). When humans inhaled enantiomers (*R*)-(+)-limonene and (*S*)-(-)-limonene it was a slight increase in systolic blood pressure, however only (*R*)-(+)-limonene changed the state of alertness and mood, which was also attributed to its pleasant aroma (90). The inhalation of enantiomer (*R*)-(+)-limonene was an anxiolytic agent in mice (91). The intervention with *Citrus aurantium* EO, rich in limonene, orally and inhaled, in mice, showed anxiolytic-like activity (92). The *citrus* EO and its components are indicated for applications in the therapy of diseases related to inflammatory processes because they are potent anti-inflammatory and anti-stress agents (5, 14, 54, 93-100). Our study used EO blend rich in bioactive monoterpenes like (*S*)-(+)-linalool, (*R*)-(-)-linalool, (*R*)-(+)-limonene, (*S*)-(-)-

limonene, linalyl acetate, alpha-pinene, alpha-terpineol, beta-myrcene and others (Table 5). We can suppose that the composition of inhaled *citrus* EO blend rich in VT influenced the results of this study.

3.4. Increased parameters in Experimental Group

The inhalation methodology of *Citrus sinensis* and *Citrus aurantium* EO blend rich in VT increased the salivary Leptin in the Experimental Group (Figure 6). It is in line with results obtained by other researchers. The influence of inhaling aromas and EO on hunger was also investigated and results were a reduction in the number of neuropeptides in the hypothalamus, as well as a reduction in appetite (101-104). The inhalation of EO containing geraniol, for 30 minutes, showed an appetite reduction effect attributing this effect to the possible action of activation of pro-opiomelanocortin (POMC) neurons in the hypothalamus. We emphasize that the amount of geraniol in the EO blend used in the present study is lower than that used in Choi *et al*. (102) research, thus, a hypothesis is that its action has been potentiated through the synergistic effect. Salivary levels Leptin are found to be lower than plasma levels (105). There is also a link between Leptin and immune system since Leptin is considered an integral part of immune response, as shown in the study that correlates the elevation of salivary levels Leptin with the reduction of some inflammatory states (78, 106), agreeing with the results shown in the present study.

A negative association between changes in Leptin and Cortisol levels is well documented in the literature which may indicate a suppressive effect of Leptin on the HPA axis, and disorders associated with stress and eating disorders (107, 108). The present study also found an increase in Leptin (Figure 6) and a decrease in Cortisol (trend towards significance of the results).

The correlation between the anxiety, depression symptoms, mood and anti-inflammatory interleukin IL-10, has been studied (109,110). These studies conclude that the improvement of psycho-emotional health is related to the

increase of endogenous anti-inflammatory interleukins. This occurs through an intimate and delicate mechanism of influence between the immune system and CNS (111-114). Researchers seek to elucidate the correlation mechanism between the immune system and CNS in the HPA axis, but there is still no clarity about this regulation (115, 116). About Dopamine, studies reported an increase in Dopamine levels after use of some *Citrus* ssp. EO (117-121). The argument would be the action on anxiolytic-like effect, possibly through the modulation of complex neurotransmitter system GABAergic, serotonergic, and dopaminergic in prefrontal cortex. Our results corroborate the previously mentioned studies, since they showed decrease in anxiety and stress symptoms (Figures 3 and 4), increase in the salivary levels of IL-10 and Dopamine (trend towards significance of the results). As far as to the authors' knowledge, this is one of first works to present salivary level Dopamine in humans.

3.5. *Citrus* EO blend rich in VTs

In Table 5, it was observed a change in the chemical constitution of *citrus* EO blend rich in VT that remained inside the electrical ceramic apparatus (49).

614 Table 5. Identification of chemical compounds of liquid EO blend, before and
615 after 60 minutes by heating in the electrical ceramic apparatus.

616

Chemical compounds	Peak number	EO blend before the experiment (% of compounds)	EO blend after the experiment (% of compounds)
alpha-pinene	1	2.49	1.24
sabinene	2	0.25	nd
beta-pinene	3	1.01	0.66
beta-myrcene	4	5.78	3.26
octanal	5	2.03	1.87
alpha-phellandrene	6	0.31	nd
delta-3-carene	7	0.43	0.64
limonene*	8	38.63	21.94
(Z)-beta-ocimene	9	0.29	0.34
(E)-beta-ocimene	10	4.14	4.48
terpinolene	11	1.54	1.84
linalool**	12	10.66	5.05
nonanal	13	0.01	0.27
cis-limonene oxide	14	0.41	0.55
trans-limonene oxide	15	0.50	0.67
isopulegol	16	0.01	0.24
alpha terpineol	17	0.43	0.10
nerol	18	0.24	0.06
linalyl acetate	19	27.39	41.63
geranial (Z-citral)	20	0.44	0.64
isopulegol acetate	21	0.01	0.25
neryl acetate	22	0.38	0.55
geranyl acetate	23	0.64	0.89
(E)-caryophyllene	24	0.21	1.29
alpha-bergamotene	25	0.01	0.26
valencene	26	0.22	1.92
beta- bisabolene	27	0.26	1.82
% Sum of compounds total identificated		98.69	92.46

617 Notes:

618 “nd” refers to no detected compound.

619 * Bioactive enantiomers limonene: *Citrus aurantium* EO [71% (*R*)-(+)-limonene and 29% (*S*)-(-)-limonene] and *Citrus*
620 *sinensis* EO [95% (*R*)-(+)-limonene and 5% (*S*)-(-)-limonene] (49).

621 ** Bioactive enantiomers linalool: *Citrus aurantium* EO [36% (*S*)-(+)-linalool and 64% (*R*)-(-)-linalool] and *Citrus sinensis*
622 EO [93% (*S*)-(+)-linalool and 7% (*R*)-(-)-linalool] (49).

623

624

Figure 7 shows the main components, which were in the highest percentage in the *citrus* EO blend rich in VT and their chemical structures.

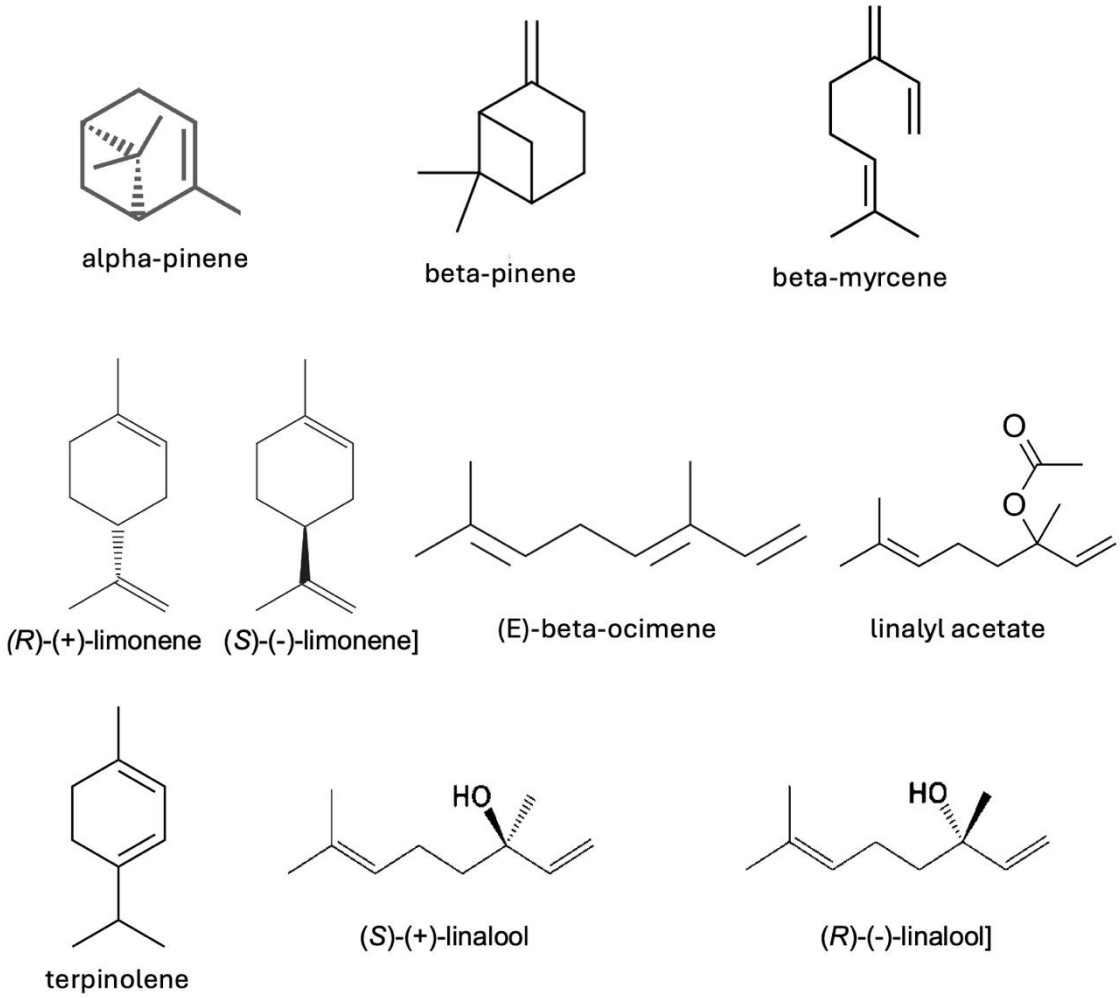


Figure 7 - Some of volatile terpenoids (VTs) presents in *citrus* blend

The combined composition of *Citrus* spp. EO was shown in Table 5, before and after vaporization. This table shows that after 60 minutes of mild heating for vaporization in an electric ceramic device, the liquid EO blend had a decrease in percentages of monoterpene components. Monoterpenes have greater volatility, such as alpha-pinene and beta-pinene, sabinene, beta-myrcene, limonene, linalool. Consequently, the percentage of other components with higher molecular weight, such as linalyl acetate, oxides, valencene, and beta-bisabolene sesquiterpenes, increased in relation to the total sum of components identified in the analytical conditions. Our results agree with the study that observed the evaporation of *Citrus sinensis* EO, where there was a rapid evaporation of

monoterpene hydrocarbons and a gradual increase in the percentage of oxygenated components and sesquiterpenes (122). The ceramic electric device proved to be suitable for the volatilization of EOs in aromatherapy procedures.

Although we have discussed the results based on the highest percentage of VT components in the *citrus* EO blend, we cannot fail to mention the synergistic or antagonistic effects in aromatherapy. To understand the effects of EO action on the immune system and mental health it is more important to study the integral composition of EO, than some of its components. The synergism theory seems to be more significant, that is, each component does not act in isolation, being influenced by the others that integrate the composition of EO (123, 124). Researchers investigated how VT from EOs influence human relaxation through specific neurological mechanisms, such as the interactions between terpene components and neurotransmitter receptors in the brain and their impact on rhythms detected by electroencephalography. Thus, the study sought to establish how aromatic compounds alter brain activity to promote a state of calm (125). Recent research has demonstrated that VT (limonene, linalool, myrcene and other) from *Citrus sinensis* oil interact with six human olfactory receptors (OR1A1, OR1D2, OR2W1, OR5AC2, OR1A2, and OR5M3). These interactions are through hydrogen bond, and they would be responsible both olfactory perception of aroma and its bioactivity (12, 126). The authors of present study have a hypothesis that VT in blend of *Citrus sinensis* and *Citrus aurantium* EOs, and not their isolated components, has influenced the psycho-emotional and biochemical parameters studied.

4. LIMITATIONS, STRENGTHS AND ADVERSE EVENTS

This study had some limitations such as controlling the study room to prevent tension-causing situations or preventing the volunteers' conversation with each other about topics that cause anxiety or depression. Some volunteers left the study because they fitted the exclusion criteria, such as the beginning of

medication use, discovery of pathology, and report of severe stress such as the death of a close family member. We did not use a screening test or interview for psychiatric diagnostic. Nevertheless, the psychological scales did not suggest severe clinical symptoms. In addition, there were no outliers in the sample who might suggest severe conditions. It can be considered a bias that the volunteers in all groups were mostly female, also that 100% of them had undergraduate degrees, and with a good socioeconomic condition. The limitations, potential biases, and weaknesses of present study were volunteers from each group start a friendship with each other and begin to express themselves emotionally in a similar way, due to the long duration of this study. The analytical methodology Luminex 200IS™ immunoassay system was efficient in relation to small amount of saliva used, precise and selective, however it has a high cost. This limitation was also reported by another group of researchers regarding the high cost of biochemical analysis kits (22). A bias is observed at the level of some biological parameters – IL-2 and Leptin - in the Experimental Group at B-moment, which differ from the other groups, Placebo and Control. According to Boutron *et al*. (127), these biases do not mean a problem with the saliva collected, trends or biases may occur in long-term clinical trial groups. Other bias is observed at the level of ISSL psychological variable in Control Group at A-moment, which is lower than other groups, Experimental and Placebo. Some volunteers expressed that they were tired of participating in the study because it was for a long period of 6 months, which we consider an adverse effect.

The authors of this research recommend an in-depth study in relation to parameters that did not obtain statistical significance, both psycho-emotional and biochemical parameters. The lack of statistical significance could be attributed to bias, a reduced number of volunteers, or other reasons. We also recommend further studies to elucidate the correlation between salivary and plasma Dopamine levels, as well as with the states of mental disorders such as depression and anxiety. Even so, it is suggested to carry out research with a

larger number of volunteers, and daily interventions with a weekly measurement of biochemical parameters, aiming to elucidate the cumulative effect of each intervention.

5. CONCLUSION AND FUTURE OUTLOOK

The healthy state in human life is a delicate balance between many biochemical and psycho-emotional parameters, always combined with adaptation to the external environment and lifestyle. The research work is a cut in this broad vision. Upon observing the set of results and the discussion surrounding them, it becomes clear that this study did not establish a diagnosis for psychopathologies such as anxiety, depression, stress, and physiological diseases. This study supports the hypothesis that inhalation of *citrus* EO blend rich in VT can help in human health, it comes in line with traditional usage and the empirical knowledge of ancestral cultures Brazilian population. The inhalation methodology of *Citrus sinensis* and *Citrus aurantium* EO blend induced the reduction of anxiety state, stress symptoms, salivary IL-2, IL-6, TNF-alpha and an increase in salivary Leptin. To the best of our knowledge, there are few studies published that correlate the biochemical parameters using saliva as a biological matrix and psychometric protocols of psychology, with a long-term intervention of 6 months. It is assumed that there is an intimate correlation between the psycho-emotional alteration and change of salivary biochemical parameters, not identifying which one determines the alteration of other. It may contribute to supporting the idea that the inhalation of natural products - such as *citrus* VT - can improve psycho-emotional states with positive repercussions on the immune system. Perhaps it can be used as a complementary therapy to the conventional methods employed.

ABBREVIATIONS

BAI: Beck Anxiety Inventory

BDI: Beck Depression Inventory

CAPES: Coordination for the Improvement of Higher Education Personnel

CAS: Chemical Abstract Service

CNPq: National Council for Scientific and Technological Development

CNS: Central Nervous System

CONSORT: Consolidated Standards of Reporting Trials

EO: Essential oil

EOR: Ectopic olfactory receptors

FAPERGS: Research Support Foundation of the State of Rio Grande do Sul

FEMA: Flavor and Extract Manufacturers Association of the United States

GABA: gamma-aminobutyric acid

GHQ-12: Goldberg's General Health Questionnaire, 12 item scale

ICF: Informed Consent Form

ICTRP: International Clinical Trials Registry Platform

IDATE-S: State Anxiety Inventory

IDATE-T: Trait Anxiety Inventory

IDATE: State-Trait Anxiety Inventory

IL-10: interleukin-10

IL-2: interleukin-2

IL-6: interleukin-6

OR: olfactory receptors

PANAS-N: Negative Affect Schedule

PANAS-P: Positive Affect Schedule

POMC: pro-opiomelanocortin

RCTs: Randomized Controlled Trial

ISSL: Lipp's Adult Stress Symptoms Inventory

TNF-alpha: tumor necrosis factor alpha

VT: volatile terpenoid

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AUTHOR CONTRIBUTION STATEMENT

ANW: Writing the original draft; ANW, RPL, CM: Conceptualization; LNW, PB, PT, AK, MKS, LV: Data curation; ANW, LNW, PB, PT, AK, MKS: Formal analysis; ANW, RPL: Funding acquisition; ANW, MKS, AZ, MPC, VJO, PFM, LV: Investigation; ANW, RPL, CM, AIE, AZanonato: Methodology; ANW, RPL, AIE, AZanonato: Project administration; PT, AK, LV: Software; RPL, AIE: Supervision; ANW, RPL, CM, MKS, MPC, VJO, PFM, LV, AIE: Validation; ANW, RPL, CM, MKS, MPC, VJO, PFM, AIE: Visualization. All the authors have read, approved, and made substantial contributions for the manuscript. All the authors have read, approved, and made substantial contributions for the manuscript. We also confirm that there are no known conflicts of interest associated with this publication and the manuscript has been read and approved by all named authors. We declare that no type of artificial intelligence (AI) was used at any stage of this manuscript. None of the original material contained in this manuscript has been previously published or is currently under review for publication elsewhere.

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