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"EFFICACY OF NUTRACEUTICAL GUARANA (PAULLINIA CUPANA) ON ERECTIVE FUNCTION AND CARDIOMETABOLIC STATUS, IN PATIENTS WITH MILD-MODERATE ERECTILE DYSFUNCTION, AND IN VITRO STUDY OF EFFECTS ON ENDOTHELIAL FUNCTION"

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INTRODUCTION

1. ERECTILE DYSFUNCTION

Erectile dysfunction (ED) is a n is a multidimensional but common male sexual dysfunction. It is defined by the Fourth International Consultation on Sexual Medicine as the persistent or recurrent inability, for at least three months, to reach and/or maintain an erection sufficient to conduct a satisfactory sexual intercourse[1].

The mechanism of erection of the penis is a complex process that involves the relaxation of the smooth arterial and trabecular muscles of the cavernous body, allowing a greater inflow of arterial blood to penis[2]. This process is mainly regulated by nitric oxide (NO), produced by the neuronal NO synthase (nNOS) of the NANC fibers of the penis and the endothelial NOS (Enos) of the endothelial cells of the arteries of the penis. This causes the relaxation of smooth muscle cells, allowing the blood to fill the gaps of the cavernous bodies and blocking venous outflow (veno-occlusion)[2]. Subsequently, the process is reverted by the action of phosphodiesterase-5 (PDE-5), which hydrolyses the cGMP, produced under the stimulus of NO, direct effector of the stimulus of smooth muscle cell release[2] (Figure 1).

Erectile dysfunction occurs when any of these processes is compromised, making it difficult to obtain or maintain an erection sufficient for satisfactory sexual intercourse[1].

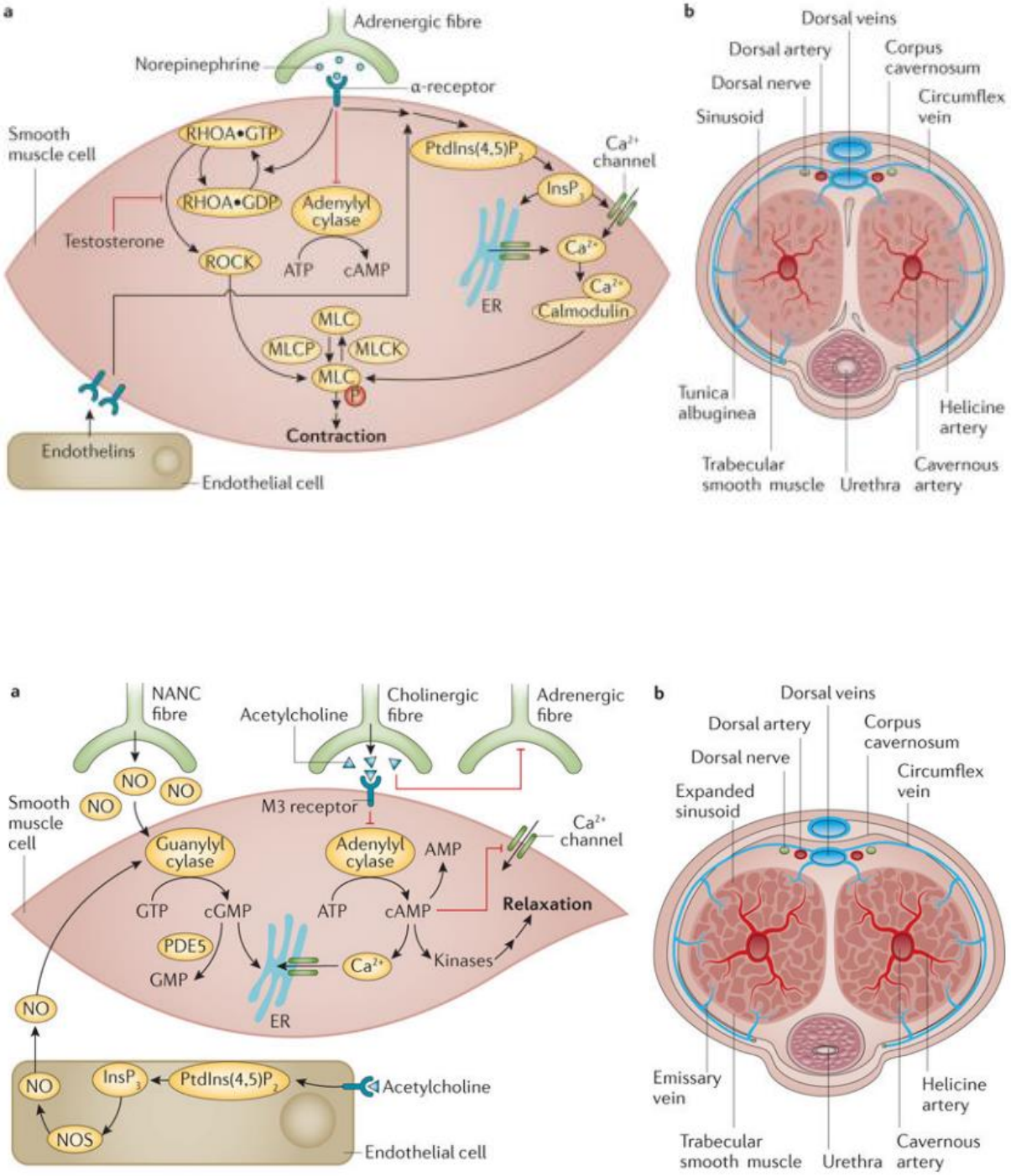


Fig.1 Physiology of the process of erection[2]

1.1 EPIDEMIOLOGY OF ERECTILE DYSFUNCTION

Several studies have explored the epidemiology of ED considering different contexts and populations. Since ED is considered a more common condition in older men, two fundamental studies have provided valuable results in this context: the Massachusetts Male Aging Study (MMAS)[3] and the European Male Aging Study (EMAS)[4]. MMAS showed a combined prevalence of mild to moderate ED of 52% in men aged 40-70; ED was strongly correlated with age, health, and emotional function[3]. By contrast, EMAS, the largest multicentric European study based on the population aged 40-79, reported a prevalence of ED ranging from 6% to 64% depending on the different age groups and increasing with age, with an average prevalence of 30%[4] (Figure 2). Few studies have evaluated the prevalence of ED worldwide; in particular, what emerges from these studies is a systematically higher prevalence of ED in the United States and East and South Asiathan Europe or South America.

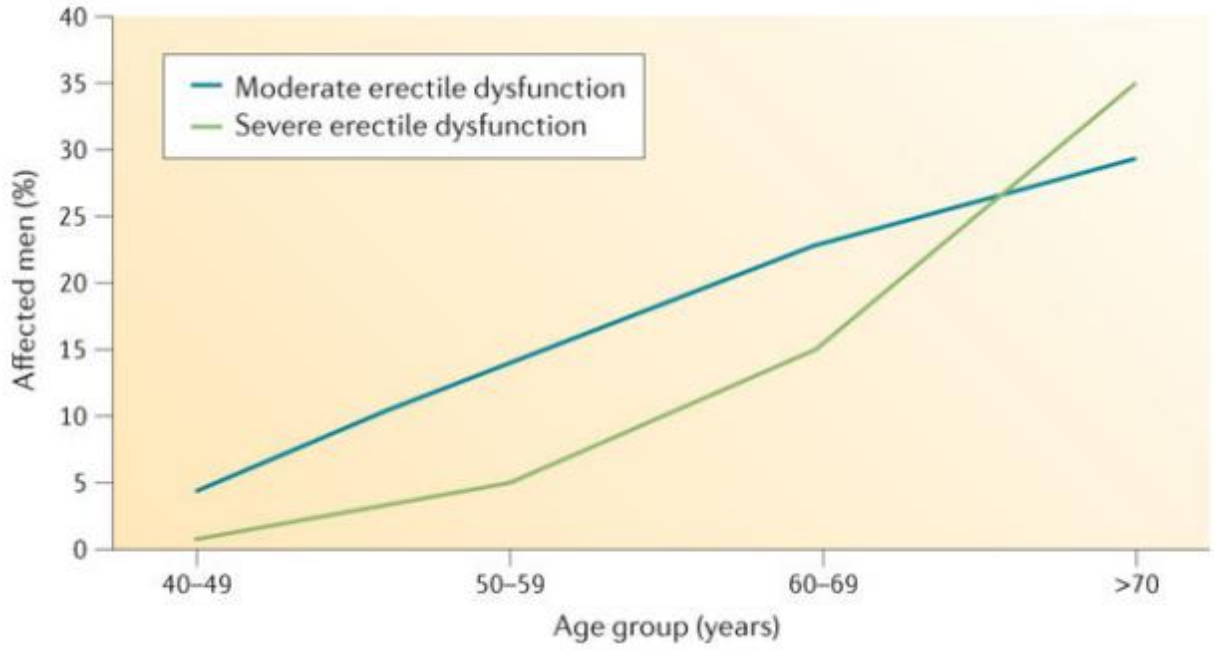


Fig.2 Prevalence of ED across age groups[2]

1.2 ETIOPATHOGENESIS OF ERECTILE DYSFUNCTION

1.2.1 PSYCHOGENIC ERECTILE DYSFUNCTION

Non-organic ED is also known as psychogenic or adrenaline-mediated erectile dysfunction (noradrenaline-mediated or sympathetic system-mediated erectile dysfunction). Stress, depression and anxiety are generally the etiological factors of psychogenic ED, which contribute to the inability to achieve and maintain an erection before or during sexual intercourse[5].

1.2.2 NEUROGENIC ERECTILE DYSFUNCTION

Neurogenic ED is caused by a deficiency in nerve transmission to the corpora cavernosa. Such deficits may be secondary, for example, to spinal cord injury, multiple sclerosis, Parkinson's disease, lumbar disc disease, traumatic brain injury, radical pelvic surgery (radical prostatectomy, radical cystectomy, abdominoperineal resection) and diabetes. Lesions of the upper motor neurons (above the T10 spinal level) do not cause local changes in the penis but may inhibit central nervous system (CNS) mediated erection control[6].

On the other hand, any sacral lesions (S2-S4 are typically responsible for reflex erections) cause functional and structural alterations due to reduced penile innervation[7-8]. In particular, the functional change resulting from such lesions is the reduction of the NO load available for cavernosa smooth musculature[7]; In addition, structural changes result in apoptosis of smooth muscle cells and endothelium of the cavernous arteries, as well as upregulation of cytokines that stimulate fibrosis of the smooth musculature[8].

1.2.3 VASCULOGENIC ERECTILE DYSFUNCTION

In vasculogenic ED, the most common form of ED, the reduced supply of cavernous blood is induced by vascular disease and concomitant endothelial dysfunction, resulting in a reduction in the fundamental contribution of the endothelium (NO release and response to shear stress) in the process of erection[9]. About the frequent association of ED with a vasculogenic etiology, it is important to note that ED can be an early and independent predictor of cardiovascular events (CVs) [9]. The risk of developing vasculogenic ED is increased in men with

hypertension, diabetes and dyslipidemia[9-10]. Cigarette smoking has also been shown to increase the risk of vasculogenic ED, although quitting smoking may reduce the risk[10]. From a physiopathological point of view, vasculogenic ED does not develop from hypertension in itself, but is secondary to changes in the arterial wall, in particular reduction of parietal elasticity, in response to increased blood pressure[11]; Moreover, atherosclerosis related to diabetes, dyslipidemia and/or cigarette smoking can lead to arterial stenosis and aggravate vascular damage[11-12].

1.2.4 IATROGENIC ERECTILE DYSFUNCTION

The most common iatrogenic cause of ED is radical pelvic surgery. Generally, the damage that occurs during these procedures is mainly of a neurogenic nature but can also contribute the accessory damage of the pudenda artery[12]. Pelvic fractures can also cause ED in a similar way, due to nerve distraction injuries and arterial trauma. It has also been shown that various drugs are associated with the development of DE[11]. The drugs used to treat arterial hypertension, especially thiazide and β-blocker diuretics, are those most commonly associated with ED (Table 1), but also others, including psychodrugs, antiandrogens, opiates and digoxin, have been associated with this condition[11].

DRUGS	MECHANISMS OF INDUCED ED	NOTES
Non-cardioselective β-blockers	- Inhibition of β2 adrenergic receptors-mediated vasodilation, with contextual unopposed α-adrenergic stimulation within cavernous arteries.	Cardioselective β-blocker Nebivolol might improve ED through a NO-mediated vasodilatory effect.
Thiazide diuretics	- Direct effect on cavernous vascular smooth muscle cells; - Indirect increase of α-adrenergic activity, through lowering of serum sodium levels and blood pressure; - Decrease of serum zinc levels.	
Potassium-sparing diuretics	- Crossover inhibition of androgen receptor (higher for spironolactone than eplerenone).	

Table 1: Antihypertensive drugs associated with ED and related mechanisms.

1.2.5 ERECTILE DYSFUNCTION, MALE HYPOGONADISM AND OTHER ENDOCRINE CAUSES.

Endocrinological disorders may also contribute to the etiopathogenesis of ED and, in particular, hypogonadism and hyperprolactinemia are often associated with ED. Male hypogonadism is defined as a clinical syndrome in which the presence of reduced total (TT) or free testosterone values (calculated using the Vermeulen formula that takes into account the values of TT, SHBG and albuminemia) is associated with signs and/or symptoms such as decreased mood, difficulty concentrating, reduced physical strength and muscle mass, anemia, gynecomastia, decreased libido, reduced spontaneous erections and DE[12]; the association between male hypogonadism and ED has been widely described and finds its physiopathological foundation in pre-clinical evidence that a condition of androgenic deprivation is associated with atrophy of penile tissues, degeneration of penile innervation and significant reduction of endothelial progenitor cell recruitment, expression/activity of fundamental molecules in the erection process such as PDE-5, nNOS, Enos, and increased levels of Dimethyl-Arginine-Asymmetric (ADMA), a selective nitric oxide synthase inhibitor, resulting in a reduction in the production of NO, and in clinical evidence whereby in subjects with hypogonadism, regardless of age, reduced response to penile vasodilator drugs with reduced relaxation of the cavernous bodies and smooth muscle cells of the cavernous arteries[13]. From a physiological point of view, moreover, the mechanism of control of testosterone on erectile function is due not only to the aforementioned effects of the same hormone at the penile level, but also to positive actions at the level of the brain areas involved in sexual appetite and at the level of the pelvic spinal areas involved in the nervous impulse of erection[2] (Figure 3).

In addition, evidence from a recent meta-analysis including randomized clinical trials that address the role of testosterone treatment in ED confirms significant beneficial effects of this treatment on various areas of erectile function only in males hypogonadic with starting testosterone levels below 12 nmol/L (345 ng/dl)[14]. Another relevant emerging aspect is the time course of the effects of testosterone, or the duration of treatment required to achieve the maximum result); in this regard, a recent systematic review revealed that although the effects on libido, on ejaculation and sexual activity are noticeable within only 2-3 weeks after the start of treatment, the effects on erectile function may take up to 6-12 months to be noticeable[15]. After testosterone, prolactin is the most commonly altered hormone in men with

sexual dysfunction; in this context, the main pathophysiological effect of hyperprolactinemia is to inhibit the secretion of gonadotropins to induce hypogonadismo[2]. Therefore, prolactin should be considered for screening, along with testosterone and luteinizing hormone (LH) in men suffering from ED[2].

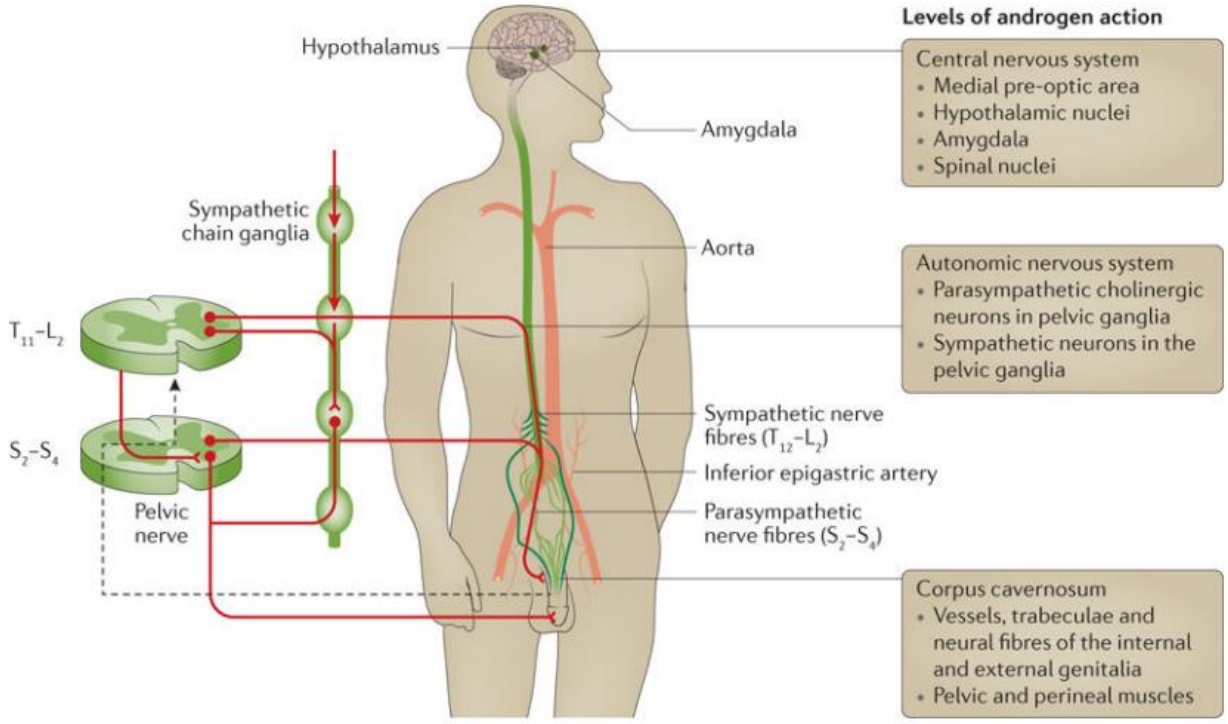


Figure 3: Physiological effects of testosterone in the control of erection

2. DIAGNOSTIC WORK UP OF ERECTILE DYSFUNCTION

An appropriate diagnostic work-up requires integrated multidimensional type assessment of anamnestic, clinical, laboratory and instrumental types (Figure 4).

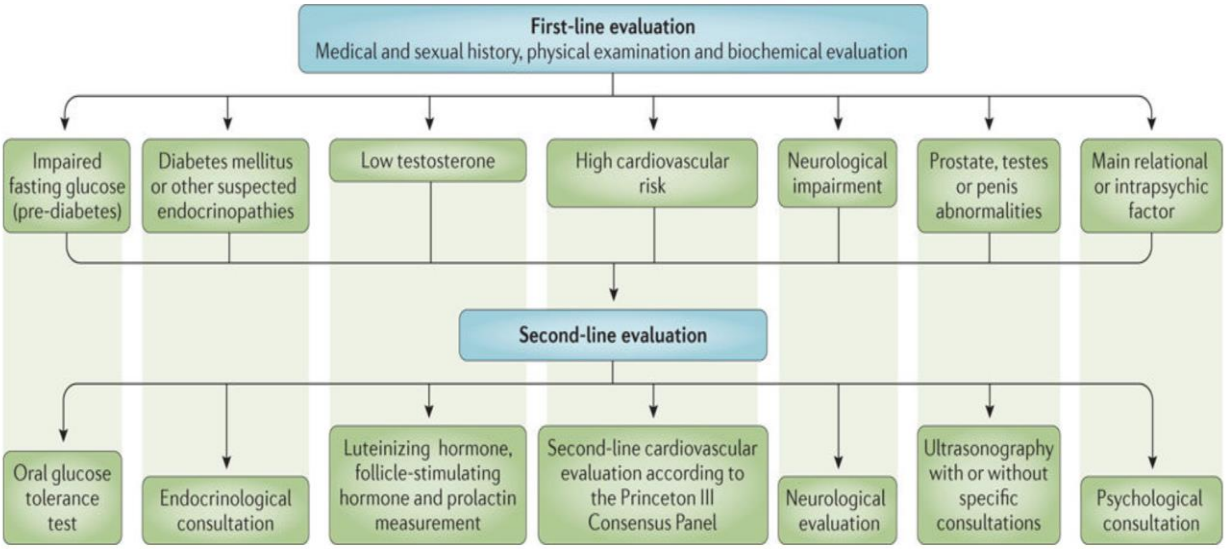


Figure 4: ED diagnostic workup

2.1 HISTORY AND ASSESSMENT OF RISK FACTORS

2.1.1 RISK FACTORS

The identification of risk and pathogenetic factors involved in ED is crucial for an accurate diagnosis and effective treatment.

The identification of risk and pathogenetic factors involved in ED is essential for accurate diagnosis and effective treatment. The smoking and alcohol habits have been demonstrated as a potential risk factors contributing to ED. In particular, observational studies suggest a linear dose-response association between the amount and duration of smoking and the risk of DE[16] and similar findings have been documented for alcohol abuse[17]. In addition, lifestyle, in terms of diet and physical activity, can also contribute to the appearance of ED; specifically, low-grain dietary patterns, legumes, vegetables and fruits and high in red meat, Dairy products and sugary foods and beverages are all associated with an increased risk of ED[18]. On the other hand, the available evidences support the reduction of the ED risk associated to a moderate and more frequent physical activity[19], while an increased ED risk associated to a condition of

obesity and metabolic syndrome[20]; in this regard, it is conceivable that obesity could induce a significant increasing risk of developing hypogonadism associated with ED, although recent evidence supports the hypothesis of a physiopathological correlation between obesity and ED independent of hypogonadism and potentially mediated by low-grade systemic inflammation associated with obesity with a prominent role of inflammatory cytokines such as TNFα[20].

There is also a well-known association between diabetes mellitus and ED within metabolic risk factors; in particular, this condition could induce alterations in penile erection through different physiopathological mechanisms such as hypogonadism secondary to metabolic disorders, peripheral neuropathy and atherosclerosis of large blood vessels with a subsequential endothelial dysfunction of the cavernous arteries[21-23]. As part of the association with these metabolic disorders, it is important to highlight that vasculogenic ED and cardiovascular diseases are considered to be different manifestations of a common underlying vascular disorder and that ED represents an early and silent predictor of potential subsequent cardiovascular events, especially in men under 55 years and also in those who have not already developed other comorbidity[24]; in this sense the recommendations of the Consensus Princeton III for the management of ED and cardiovascular disease indicate that ED has a similar, or even greater, predictive value for cardiovascular disease than traditional risk factors such as diabetes, hypertension, dyslipidemia or smoke[24].

In addition to organic factors, psychogenic and relational aspects must also be evaluated in men with ED symptoms as these can play a dominant role or coexistent with organic factors in promoting the appearance of this sexual dysfunction; in fact, it is important to highlight that anxiety related to the sexual performance is a common problem in men with sexual dysfunctions, both organic and psychogenic, resulting in avoidance of sexual intercourse, loss of self-esteem and depression[25].

2.1.2 MEDICAL HISTORY

Considering the personal, interpersonal and social implications of sexuality, the evaluation of sexual history is not an easy task. From this point of view, in the anamnestic approach to the patient it is important to find the correct way to investigate his history by initially avoiding direct questions and "decoding" his answers on health and sexual diseases[26].

It is therefore essential to use structured and standardised questionnaires to estimate the presence and severity of ED, such as the International Index of Erectile Function (IIEF),

which allows to investigate erectile function and different domains related to sexuality such as desire, orgasm and satisfaction in relationships; in addition, an additional structured interview DE is represented by SIEDY, composed of 13 items composed of three scales that identify and quantify important domains in men affected by ED (organic, scale 1; relational, scale 2; and intrapsychic, scale 3)[27].

2.2 PHYSICAL EXAMINATION

Physical examination of patients includes: thoracic examination with cardiac evaluation, rhythm, respiratory function and any signs of gynecomastia; andrological examination with clinical evaluation of penis, prostate and testicles, and the distribution of body hair: in particular, the detection of testicular and/or prostatic hypotrophy, depending on the age of the patient, could imply a picture of underlying hypogonadism; other possible signs of hypogonadism include gynecomastia and decreased beard and reduced body hair distribution[28]. Evaluation of the flaccid penis may instead show the presence of Peyronie's disease (which results in the presence of palpable fibrous penile plaques), phimosis (congenital narrowing of the opening of the foreskin) or short frenulum (whereby the tissue under the glans that connects to the the foreskin is too short and limits the movements of the foreskin), all factors that could contribute to the appearance of ED[28].

Finally, very important are also the detection of blood pressure, waist circumference and BMI in order to estimate and evaluate the presence of cardio-metabolic comorbidities potentially predisposing to ED[28].

2.3 BIOCHEMICAL AND HORMONAL EVALUATIONS

The evaluation of biochemical and hormonal parameters to detect ED is more important. As regards biochemical evaluation, the evaluation of cholesterol, triglycerides, fasting blood sugar and glycated hemoglobin (HbA1c) levels are important determinants of cardiovascular and metabolic risk in order to determine the presence of cardio-metabolic comorbidities associated with DE[28].

As regards hormonal component, implicated in ED, the dosage of total testosterone is essential but not sufficient to discover a hypogonadism condition, because much more reliable is the calculation of free testosterone, through the formula of Vermeulen, which requires also the Sex Hormone Binding Globulin (SHBG) value, a globulin that binds sexual hormones

including testosterone; in presence of reduced levels of total testosterone and/or free testosterone calculated, a more extensive hormonal evaluation is necessary with the assessment of prolactin and gonadotropin levels (LH and FSH) helpful to estimate the primary (testicular) or secondary (hypothalamic-pituitary) origin of hypogonadismo[28]. Therefore, the need to perform further and specific cardiovascular examination should finally be based on the risk criteria established by the Consensus of Princeton[25] (Table 2).

Profile	Description	Sexual activity and PDE5 inhibitor use
Low	• Fewer than three risk factors for coronary artery disease * (excluding sex) • Controlled hypertension • Class I or II stable angina ‡ • Successful coronary revascularization • History of uncomplicated myocardial infarction • Mild valvular disease, congestive heart failure without left ventricular dysfunction and/or New York Heart Association class I heart failure	• Cleared to resume sexual activity • Cleared to take PDE5 inhibitors
Intermediate	• At least three risk factors for coronary artery disease * (excluding sex) • Class I or II stable angina ‡ • Recent myocardial infarction (within 2–6 weeks) • Left ventricular dysfunction and/or New York Heart Association class II congestive heart failure • Noncardiac sequela from atherosclerotic disease (stroke and/or peripheral vascular disease)	• Cardiac evaluation necessary prior to resuming sexual activity • No contraindication to PDE5 inhibitor use
High	• Unstable or refractory angina • Uncontrolled hypertension • New York Heart Association class III–IV congestive heart failure • Recent myocardial infarction (within 2 weeks) • High-risk arrhythmias • Severe cardiomyopathy • Moderate to severe vascular disease	Sexual activity delayed until cardiac condition stabilized

Table 2: Recommendations of Consensus Princeton III

2.4 BASAL AND DYNAMIC PENILE ECOCOLORDOPPLER

Penile colordoppler ultrasound (CDU) can be performed in basal conditions with flaccid penis and in dynamic conditions after intracevernous injection of Prostaglandin-1 (PGE-1) with an erect penis and represents the functional diagnostic method to determine the vasculogenic etiology of ED[29]. Performing penile CDU under basal conditions allows identifying subjects with pathological flow indices with more than 80% accuracy and allows you to identify arterial indices, such as basal acceleration, able to act as independent predictors of cardiovascular risk[30].

However, dynamic penile ECD is still the gold standard for determining the presence of arteriogenic alterations underlying ED; this investigation involves evaluation by penile CDU 10, 20 and 30 minutes after intracavernous injection of 10 mcg of PGE-1; the index for detecting the presence of a cavernous arterial vascular abnormality is the peak systolic velocity (PSV) which is diagnostic for vasculogenic ED for values < 35 cm/s and for severe vasculogenic ED has been defined as PSV < 25 cm/s[31].

3. TREATMENT OF ERECTILE DYSFUNCTION

In the absence of a specific modifiable etiology, the therapeutic management of ED is based in the first instance on the modification of the lifestyle followed by the first line pharmacological therapy represented by PDE5-inhibitors and therefore by other second line strategies, such as Vacuum devices, intracavernous E1 prostaglandin injection (PGE1) and finally surgery.

3.1 LIFESTYLE CHANGE

Lifestyle modification can play an important role in managing ED especially in younger patients. The available datas recommend an aerobic physical activity of moderate intensity of almost 30 minutes every day of week[32]; moreover, promoting weight loss in men with obesity and the transition from a Western diet to a Mediterranean diet, combined with physical activity, can help improve the DE[33-35]. On the other hand, a critical review of the patient's pharmacological history may also be useful in improving an ED condition, since there are drugs that have been associated with the worsening/appearance of ED (Table 1). It has also been shown that there is a linear dose-response relationship between cigarette smoke and ED, with a higher risk for men with more packets of cigarettes smoked or more years of

smoking[36,37], with a consequent possible improvement of ED following the cessation of the habit of smoking[38]. It has instead been suggested that moderate alcohol consumption could improve erectile function by reducing anxiety, but on the other hand, chronic use or alcohol abuse can have a detrimental effect on liver function contributing to the reduction of serum testosterone levels and the increase of serum estrogen levels that can both contribute to the appearance of DE[39,40]. Finally, patients with psychological stress factors (such as performance anxiety, relationship problems and everyday life stress) could benefit from the restoration of confidence by counseling with a psycho-sexologist[41,42]. In conclusion, given the above evidence, the European Association of Urology argues that "lifestyle changes and changing risk factors must precede or accompany any treatment of ED[43].

3.2 DRUG THERAPY WITH PDE5-INHIBITORS

Oral phosphodiesterase inhibitors (sildenafil, vardenafil, tadalafil, avanafil) have completely revolutionized the therapeutic management of DE[44].

From a pharmacodynamic point of view, these drugs competitively inhibit PDE-5, leading to a persistent accumulation of cGMP, resulting in increased release of NO, initiating a cascade of events that lead to relaxation of smooth cavernous muscles and promotion of erection[45]. Sildenafil and vardenafil reach their median Cmax (maximum observed plasma concentration) about 1 hour after oral administration and have a half-life of about 3-5 hours[46]. Due to these pharmacokinetic aspects, these drugs should be administered with adequate time before sexual intercourse to allow for maximum absorption and effect of the drug, and patients should be instructed on the optimal conditions for the drugs to work effectively; it is also important to point out that Sildenafil and Vardenafil should always be taken on an empty stomach because lipids in food can decrease and delay absorbing, while this type of precautions do not concern Avanafil and Tadalafil that are not so strongly influenced by the possible meal that precedes the intake and, in addition, Tadalafil is characterized by a pharmacokinetic profile that guarantees a median Cmax 3-5 hours after oral intake of the drug and a half-life of 16-18 hours[46,47].

It is essential to remind patients, taking PDE-5 inhibitors, that these drugs do not have a mechanical action, but their effectiveness always depends on the simultaneous presence of adequate physical and mental sexual stimulation, in order to create excitement and initially

increase the levels available at the cavernous level of NO for an adequate release of cavernous smooth muscle functional to the process of erection[48].

Beyond the general efficacy widely demonstrated for the use of PDE5 inhibitors as ED therapy, there are specific patient settings that deserve targeted considerations regarding the use of these drugs. In particular, in patients with ED and hypogonadism it has been shown that only therapy with PDE-5 inhibitors may be partially or totally ineffective if not combined with a simultaneous hormone replacement therapy with testosterone aimed at restoring a condition of eugonadism, resulting evidence of ED improvement in these patients only in combination therapy with PDE5 inhibitors and testosterone[48]. In men, who undergoing radical prostatectomy, with a technique aimed at saving the nervous component, for prostate cancer, there may be a reduction in erectile function in the immediately post-surgical period until a functional recovery of the nervous component is achieved after the surgical trauma; moreover, no study has clearly defined the exact role PDE5-inhibitors in penile rehabilitation in these patients[48], although a randomized placebo-controlled trial in men undergoing prostate cancer radiotherapy has shown greater preservation of erectile function in subjects treated with PDE5 inhibitors than a placebo[49].

Despite the evidence of widespread use of PDE5 inhibitors in ED, patients should still be informed about possible adverse events of these drugs, which may include headaches, heartburn, facial flushing, nasal congestion and visual disturbances (due to cross reactivity with PDE6); myalgia (muscle pain) is more common with tadalafil than other PDE5 inhibitors[44]. In addition, the use of a PDE5-inhibitor in association with nitrate-containing drugs (for example, those used to treat angina) may cause a significant decrease in arterial pressure, so it is contraindicated, while PDE-5 inhibitors and α-adrenergic receptor blockers, often used for treatment of benign prostatic hypertrophy, should be taken at least 4 hours apart in order to avoid a significant combined hypotensive effect resulting from the close use of these two categories of pharmacies[50].

Very rare among the side effects is priapism (prolonged erection >4hours) which can be worrying, but rarely occurs with PDE5-inhibitors therapy (about 0.1% of patients)[51]; moreover, some pathological ophthalmological conditions require more precautions, as macular degeneration, retinitis pigmentosa, while the optic neuropathy linked to anterior non-iarterial ischemia there is a contraindication to the use of PDE-5 inhibitors for their mild

inhibitory action against PDE6, which is present exclusively in the receptors of rods and cones of retina[49].

3.3 THERAPY WITH VACUUM ERECTION DEVICES (VED)

A VED is a cylindrical mechanical device that is placed over the penis and then pumped creating a vacuum resulting in negative pressure to attract blood into the penis, resulting in filling the gaps within the corpora cavernosa, causing tumescence; specifically, these devices are often used together with a constriction band positioned on the base of the penis after reaching tumescence, to prevent blood reflux [52].

Patients may benefit from a training session to help them optimise their understanding of the correct use of VED; the use of lubricants and/or cutting of pubic hair may be important for the effective use of VED, however, the presence of adipose tissue at the pubic level and/or a penis partially sunken in the overpubic fat can make the correct positioning of the device difficult[53]. There are, however, possible adverse effects associated with the use of VED such as petechiae (capillary bleeding) and hematomas (swelling related to clotted blood); the use of the constriction band to maintain penile tumescence may give the penis a gray-blue color and the penis may become cold to the touch, due to obstructed venous outflow, resulting in a risk of penile ischemic diseases[54].

3.4 INTRACAVERNOSAL INJECTION DRUG THERAPY

The intracavernosal injection (ICI) drug therapy provides for the use of vasoactive substances injected directly into the corpora cavernosa through a small needle. These vasoactive agents mainly include prostaglandin E1, and to a lesser extent papaverine and phentolamine and sometimes atropine, which act alone or in combination to elicit an erection. Prostaglandin E1 has been approved by the FDA therapy with ICI for ED for its ability to increase intracavernous levels of cAMP, papaverine is instead a non-selective inhibitor of phosphodiesterase leading to increased levels of cAMP and cGMP, while phentolamine is a α1-adrenergic receptor inhibitor that helps prevent vasoconstriction maintain tumescence[55].

The therapy approved for ICI with PGE-1 involves dosages ranging from 10 to 40 mcg. ICI is characterized by high initial satisfaction rates, but at the same time also abandonment rates are high (46-80% in the first year) for pain due to the injection, the lack of partner, high costs, problems with the concept of injection into the penis and desire for a permanent solution; it

should also be considered that one of the main concerns related to the use of ICI is priapism for which urgent medical care would be needed, such as the aspiration of blood trapped inside the cavernous bodies or the use of phenylephrine to cause cavernous vasoconstriction and promote the outflow of blood from the corpora cavernosa[56].

3.5 SURGICAL THERAPIES

Although therapies with PDE-5 inhibitors, VED and ICI are effective as first- and second-line management options for most men with ED, surgery remains a useful and important therapeutic strategy in certain situations. Surgery may be indicated especially in patients who have contraindications, adverse effects or are refractory to medical therapy, penile fibrosis secondary to La Peyronie disease, prolonged priapism or severe infections, structural and/or vascular pathologies, penis defects due to genital or pelvic trauma[57]. Surgical treatment includes insertion of a penile prosthesis, (malleable or inflatable) and vascular reconstructive surgery. Malleable penile prostheses consist of semi-rigid cylinders that can be bent upwards for intercourse and downwards to hide the penis when not in use; these devices are easy to handle, have a low risk of mechanical failure and are optimal for patients with reduced manual dexterity, however, due to their constant stiffness, they can be uncomfortable and cause social problems of embarrassment and have an increased risk of erosion[57]. Inflatable penile prostheses consist of two cylinders with a scrotal pump, which allows the transfer of fluid to the cylinder chambers when an erection is desired; although it provides a stiffness similar to a malleable device, also allows better flaccidity when deflated and consequently causes less social discomfort[58].

Surgical techniques of revascularization of the penis involve anastomosis of the lower epigastric artery with the dorsal artery or with the deep dorsal vein (arterialization), with or without venous ligature, to improve the vascular inflow of the penis with simultaneous reduction of venous outflow[59]. However, vasculogenic ED is often associated with a multifactorial systemic vascular disease, potentially caused by a combination of endothelial cellular dysfunction and vascular smooth muscle which results in a reduced response to penile revascularization procedures performed with a reduced sexual satisfaction by patients[59]. For these reasons, penile revascularization is currently recommended for younger men (<55 years) who are not diabetic, non-smoking and have a documented isolated stenotic segment of the internal pudenda artery without concomitant veno-mechanism deficiency[59-60].

However, it should be noted that these interventions used as a third line of treatment in patients with arteriogenic ED can be burdened by complications such as glans hyperemia, shunt thrombosis and inguinal hernias[61].

4. NUTRACEUTICALS AND ERECTILE DYSFUNCTION: EVIDENCE AND RESEARCH PERSPECTIVES

PDE5 inhibitors (currently the first-line treatment for erectile dysfunction (ED); in particular, Sildenafil, Vardenafil, Tadalafil and Avanafil are used in clinical practice with variable dosages and formulations that allow them to be used individually according to the needs of patients. In any case, despite the consolidated effectiveness, there are some limitations related to the use of these drugs mainly due to contraindications, potential adverse effects and the potential interruption of treatment. In fact, current guidelines do not recommend the administration of PDE-5 inhibitors to patients with unstable angina, severe congestive heart failure, uncontrolled arterial hypertension, severe arrhythmias or in therapy with nitrates and in addition the 7-25% of patients experience adverse events after treatment with PDE-5 inhibitors, which results in a discontinuation of treatment in 3-5% of patients[45]. Overall, these limitations encourage the search for different approaches that could combine more favourable compliance and security with acceptable efficacy and more affordable treatment, potentially more suitable in the categories of patients experiencing one or more impediments to treatment with PDE5-inhibitors[45].

Among the most investigated nutraceuticals as single agents, yohimbine has been used for several decades as a therapy for ED on the basis of its mechanism of action on the central nervous system as a sympatholytic and has proven effective particularly in patients with non-organic forms of DE[65]. Some evidence, however, also support the possibility of a beneficial role of vitamin D supplementation on erectile function; in particular, it was showed that ergocalciferol supplementation in male patients with hypovitaminosis D produced a significant improvement in erectile function likely to be mediated by a beneficial effect on testosterone levels and metabolic changes, playing a crucial role in the initial ED[66]. More and more promising evidence is available about the use of L-Arginine (L-Arg) in the treatment of ED; in particular, it was demonstrated different degrees of effectiveness of L-Arg in the improvement of erectile function evaluated both through standardized questionnaires and in

terms of cavernous vascular indices to the penile ecodolorDoppler in patients suffering from DE[67].

Paullinia cupana (Guarana) is an evergreen climbing shrub, native to Brazil, Guyana, Venezuela, Ecuador, and Peru and cultivated extensively in Brazil[68, 69]. The major compound present in guarana seedpowder is cafeine and they have long been used in traditional folk medicine due to its several properties, bu also flavanols represented by catechin and epicatechin, monomeric flavan-3-ols, proanthocyanidins B1 and B2.[69, 70] Guaraná had a wide variety of pharmacological properties, including anticarcinogenic, antiproliferative, antimicrobial, antioxidant, cytoprotective, energetic, thermogenic, antidepressant and anxiolytic qualities[71]. Some evidence suggested its potential role in reducing weight, in particular it was demonstrated its involvement in brown adipose tissue expansion, mitochondrial biogenesis, uncoupling protein-1 overexpression, AMPK activation[72]. Guarana exerts an anti-adipogenic potential due to its ability to modulate miRNAs related to adipogenesis in 3T3L1 cells[73]. Paullinia cupana had demonstrated notable anti-inflammatory and antioxidant effects, which may prevent chronic diseases caused by changes in lipid profile. In fact, a recent study demonstrated that guarana powder and caffeine promoted changes in the activity of E-NTPDase and E-ADA enzymes of lymphocytes, lead to a shift from a proinflammatory to an anti-inflammatory profile in rats with induced hyperlipidemia[74]. These anti-inflammatory and antioxidant effects were demonstrated also in testicular. In fact, the pre-administration of guarana extract induced a protection towards testicular damage in adult Wistar rats., induced by Methotrexate and cadmium, through the regulation of inflammation and oxidative stress associated pathways, the decreased DNA damage and the normalization of histopathological changes[75,76]. Moreover, an emerging effect of Paullinia cupana was to inhibit the histological changes of the penis corpora, typical of aging associated to ED, showing a similar role as PDE5 inhibitors in enhancing the effects of NO on the progressive apoptosis of the smooth muscle cells of the corpora and NO from iNOS. In particular, the association of Paullinia cupana, to other similar nutraceutics, demonstrated to be effective as daily PDE5 inhibitor therapy in either delaying or reversing the onset of the histological and functional characteristics of aging related erectile dysfunction, through the stimulation of iNOS production, soluble guanylate cyclase (sGC), and cGMP as well as a reduction in the content of the PDE5 enzyme[77,78].

Another important effect of guarana consumption on LDL-cholesterol and serum oxidation was associated with its bioactive compounds, such as catechins and methylxanthines, even if, the underlying mechanisms of action remain unclear. It was reported that polyphenols from guarana may incorporate into LDL-cholesterol, rendering the serum of individuals less susceptible to oxidation[79,80]. Based on this evidence, guarana powder could be a promising supplement for patients with hyperlipidemia.

AIM:

The study is focused on the impact of Guaranà (Paullinia Cupana) on erectile function, estimated by questionnaires and basal and dynamic penile colordoppler ultrasound, in subjects with mild-moderate grade of erectile dysfunction and on the effects of Guarana on cardiometabolic status.

Moreover, the study evaluated the potential effects of Paullinia Cupana extract on endothelial function through in vitro studies on endothelial cell lines. In particular, to evaluate the transcriptional activity potentially modulated by Paullinia Cupana, mRNA expression level experiments were performed with culture medium, replaced with oxidized low-density lipoprotein (OX-LDL) for the assessment of endothelin-1 (ET-1) mRNA expression, or with High Glucose (HG) medium for the assessment of endothelial nitric oxide synthase (eNOS) mRNA expression.

METHODS:

The study was a double-blind cross-over interventional study, treatment vs placebo, lasting 3 weeks. 52 consecutive male patients with mild to moderate erectile dysfunction were recruited for the study, after signing informed consent, and randomized in treatment group with a pharmaceutical form of Paullinia cupana at the dose of 2 g (compound A) → placebo group (compound B) or placebo group (compound B) → treatment group (compound A).

Were excluded patients with psychiatric and/or neurological disorders, drug and alcohol abuse disorders, trauma or previous surgical interventions to the genitourinary system, damage to the spinal cord, vascular pathologies in the aortic district-iliac-femoral, thyroid disorders, hypogonadism and not in treatment with other drugs known to interfere with sexual function (e.g. hormonal therapy in patients suffering from prostate cancer).

Overall the duration of the study was 34 months, with a total enrollment time of 26 months. Each patient was underwent to 3 visits: basal examination (V1), 1 week after starting treatment with nutraceutical or placebo (V2) and after 2 additional weeks (including an intermediate week for wash out and one week for the therapeutic switch) (V3).

At any visit, each patients was subjected to a collection of physiological and pathological anamnestic data, andrological and general objective examination, evaluation of anthropometric parameters, measurement of blood pressure and heart rate, collection of venous blood for blood chemistry routine including glycolipid profile (glycemia, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol) and hormones (follicle stimulating hormone (FSH), luteinizing hormone (LH), total testosterone (TT). It was performed basal and dynamic (performed with intracavernous injection of 10 micrograms of prostaglandin-E1) penile colordoppler ultrasound, carried out according to standard procedures using an ultrasound equipped with a high frequency linear probe (7.5 to 14 MHz), with the color Doppler method to detect low intensity flows; the flow-metric wave was then sampled by an angle of inclination of 60° in both cavernous arteries, considering as flow indexes the average acceleration (mean ACC), average basal peak systolic velocity (mean bPSV), average dinamic systolic velocity (mean dPSV).

Moreover, patients had filled out questionnaires for erectile dysfunction assessment and psycho-sexological questionnaires: (IIEF-15, BDI-II, STAIY-1, STAIY-2).

- International Index of Erectile Function (IIEF), consisting of 15 questions concerning sexual domains: sexual desire, erectile function, orgasmic function, satisfaction of the relationship and general satisfaction. Erectile function is explored with questions 1, 2, 3, 4, 5 and 15 with a total score ranging from 1 to 30. Based on the score, erectile dysfunction could be classified in severe (score 6-10), moderate (score 11-16), mild (score 17-25) and absent (score 26-30)[62].
- The Beck Depression Inventory II (BDI-II) is a widely used 21-item self-report inventory measuring the severity of depression in adolescents and adults. Each of the 21 items corresponding to a symptom of depression is summed to give a single score for the BDI-II. There is a four-point scale for each item ranging from 0 to 3. On two items (16 and 18) there are seven options to indicate either an increase or decrease of appetite and sleep. Total score of 0-13 is considered minimal range, 14-19 is mild, 20-28 is moderate, and 29-63 is severe[63].
- The STAI-Y is a self-report questionnaire consisting of two 20-item scales providing separate measures of state and trait anxiety (S-Anxiety and T-Anxiety, respectively). S-Anxiety is a transitory response to an event perceived as adverse, characterized by

feelings of tension, apprehension, nervousness, and worry. T-Anxiety is, instead, a more stable predisposition to perceive stressful situations as dangerous or threatening On a 4-point Likert scale (1–4), a score equal to 4 indicates the presence of a higher level of anxiety for ten S-Anxiety (3, 4, 6, 7, 9, 12, 13, 14, 17, 18) and eleven T-Anxiety items (2, 4, 5, 8, 9, 11, 12, 15, 17, 18, 20). As for the remaining items, the scoring weights are reversed. The total score for both scales ranges from 20 to 80, with higher scores indicating more severe anxiety[64].

The 52 enrolled patients were randomized in 4 groups of treatment to complete the 3-week study: 13 in group A1, 13 in group A2, 13 in group B1 and 13 in group B2.

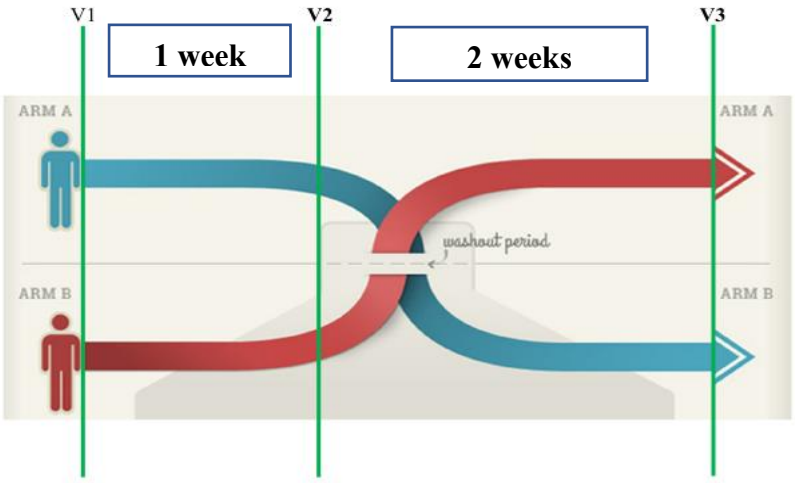
The treatment arms were as follows:

- Group A1) treatment with compound A (2 pills in the morning/day) for 1 week, followed by 1 week of wash-out and 1 week of compound B (2 pills in the morning/day);

- Group B1) treatment with compound B (2 pills in the morning/day) for 1 week, followed by 1 week of wash-out and 1 week of compound A (2 pills in the morning/day);

- Group A2) treatment with compound A (1 pill in the morning + 1 pill in the evening/day) for 1 week, followed by 1 week of wash-out and 1 week of compound B (1 pill in the morning + 1 pill in the evening/day);

- Group B2) treatment with compound B (1 pill in the morning + 1 pill in the evening/day) for 1 week, followed by 1 week of wash-out and 1 week of compound A (1 pill in the morning + 1 pill in the evening/day).



The second part of the study was performed in vitro to evaluate the effect of Paullinia on endothelial cell lines.

Cell culture and treatments

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Endothelial Cell Growth Medium-2 (EGM-2) with 20% fetal bovine serum at 37 °C in a 5% CO2 incubator. All the experiments were performed between the second and fifth passage. After HUVEC reached confluence, culture medium was replaced with high-glucose (HG) medium (28.5 mM) for 24 hrs after a pre-treatment for 24 hrs with 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml Paullinia Cupana extract dissolved in 70% ethanol (final concentration of ethanol 0.07%), for reactive oxygen species quantification experiments. For mRNA expression level experiments, culture medium was replaced with oxidized low-density lipoprotein (OX-LDL) medium (100 µg/ml) for 12 hrs, for the assessment of endothelin-1 (ET-1) mRNA expression, or with HG medium (28.5 mM) for 24 hrs for the assessment of endothelial nitric oxide synthase (eNOS) mRNA expression, in each case after a pre-treatment for 24, 48 and 72 hrs with 1 mg/ml Paullinia Cupana extract dissolved in 70% ethanol (final concentration of ethanol 0.07%).

Determination of reactive oxygen species production.

The production of intracellular ROS induced by HG challenge and the protective effect of pre-treatment for 24 and 48 hrs with Paullinia Cupana extract at concentration of 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml, were detected by the 2′,7′-Dichlorofluorescein diacetate (DCFH DA) fluorescence probe, according to the manufacturer's protocol. DCFH-DA is a sensitive cell-permeable redox probe. The lipophilic non-fluorescent DCFH-DA readily crosses the cell membrane through passive diffusion followed by deacetylation. The deacylated product is an oxidant sensitive to DCHF. DCHF is oxidized later to form highly fluorescent DCF, which is measured. In brief, HG- and HG+Paullinia Cupana-treated HUVEC were washed twice with 1XPBS and then incubated with DCFH DA (10 µM) for 20 min in the dark at 37˚C. Nuclei were counterstained with DAPI. After washing again with 1XPBS, images were captured under a fluorescence microscope. 100 cells were evaluated in 5 different representative microscope fields, and % of DCF+ cells was calculated in HG-treated and HG+Paullinia Cupana-treated cells relative to control cells. Experiments were performed in quadruplicate.

Real-time quantitative PCR

Total RNA was extracted using TrIzol Reagent (Invitrogen, Carlsbad, CA) and equal amounts (1 μg) of RNA were reverse transcribed using High Capacity RNA-to-cDNA PCR kit (Applied Biosystems, Foster City, CA). Human gene PCR primers for ET-1 and eNOS were used for mRNA expression analysis in HUVEC challenged by OX-LDL/HG treatment, and the effects on gene expression of pre-treatment for 24, 48 and 72 hrs with Paullinia Cupana extract at concentration of 1 mg/ml were evaluated. SYBR green PCR Master Mix (Applied Biosystems) was used with Step-One-Plus real-time PCR System (Applied Biosystems). The protocol included melting for 15 min at 95 °C, 40 cycles of three-step PCR including melting for 15 s at 95 °C, annealing for 30 s at 58 °C, elongation for 30 s at 72 °C with an additional detection step of 15 s at 81 °C, followed by a melting curve from 55 to 95 °C at the rate of 0.5 °C per 10 s. The samples of 25 ng cDNA were analyzed in quadruplicate. Data were normalized against the expression of the housekeeping gene beta-actin. The relative expression of target genes was calculated using the comparative threshold method $2^{-\Delta Ct}$. To exclude contamination of the PCR mixtures, in parallel with cDNA samples, reactions were also performed in the absence of cDNA template. mRNA expression level was calculated in OX-LDL-treated, HG-treated and OX-LDL/HG+Paullinia Cupana-treated cells relative to control cells.

STATISTICAL ANALYSIS

Statistical analysis was performed with GraphPad Prism and IBM SPSS Statistics softwares. Normality of distribution was assessed by D'Agostino & Pearson normality test. Continuous variables with normal distribution were reported as mean ± standard deviation (SD). Within-group comparison of continuous outcome measures was analyzed by paired Student's t-test for normally distributed variables or Wilcoxon test for variables following non-normal distribution. Between-groups comparison of continuous outcome measures was analyzed by unpaired Student's t-test for normally distributed variables or Mann-Whitney test for variables following non-normal distribution. Categorical variables were reported as absolute numbers and percentages. Within-group comparison of categorical outcome measures was analyzed by Chi-squared test and Fisher's exact test, when appropriate. A two-tailed p value < 0.05 was considered significant.

RESULTS

52 patients with mild to moderate ED, aged 22 to 65, were consecutively enrolled at the Centre and were randomized in 4 groups of treatment to complete the 3-week study: 13 in group A1, 13 in group A2, 13 in group B1 and 13 in group B2.

The psycho-sexological interview, during enrolment visit, allowed to obtain the following descriptive information related to the sexual function of the cohort of patients: all patients were heterosexual, of which 41 were in a stable couple (78.8%) and 11 single (21.1%), all patients were affected by secondary ED, generalized in 40 patients out of 52 (76.9%).

40 patients (76.9%) reported being able to achieve an erection, although of suboptimal quality, with an average duration of 1 to 10 minutes (average 5 minutes), while 12 patients (23.07%) reported to get an erection satisfying for penetration, but quickly lost during intercourse. Patients reported a reduction in desire in 12 of 52 cases (23.07%), to get spontaneous morning erections with a weekly frequency between 0 and 5 times (average 2 times) and to have sex with a weekly frequency between 0 and 7 times (average 2 times). In the cohort of patients, the IIEF-15 Questionnaire, for the assessment of male sexual function, showed a prevalence of mild ED in 55% and moderate ED in 45% of patients (Table 3).

At T0, patients did not shown difference in ultrasound parameters and IIEF-15 score.

	Prevalence
Heterosexual	52 (100)
Stable couple status	41 (78.8)
Single status	11 (21.1)
Secondary erectile dysfunction	52 (100)
Generalized erectile dysfunction	40 (76.9)
Mild erectile dysfunction	29 (55)
Moderate erectile dysfunction	23 (45)
Able to achieve a suboptimal quality erection	40 (76.9)
Erection quickly lost during intercourse	12 (23.07)
Reduced desire	12 (23.07)

Table 3: Psycho-sexological characteristics of the cohort

1. Effect of treatment with compound A vs B on erectile function

After 1 week of treatment with compound A, the flux indices to the dynamic penile doppler were significant increased, compared to basal, in particular in mean bPSV (16.3 cm/sec vs 14.7 cm/sec, p=0.011) and mean dPSV (41.1 cm/sec, vs 31.5 cm/sec, p=0.001); the mean ACC and IIEF-EF scores increased but not significantly (Table 4, Figure 5). After 1 week of treatment with compound B, there were no significant changes compared to baseline in any of the endpoints studied (Table 4, Figure 5). The comparison, by statistical testing of deltas, before and after 1 week of treatment for compound A vs B showed that treatment with compound A induced significantly greater improvements than treatment with compound B in the mean bPSV parameters (14.5 vs -3.1, p=0.021) and in mean dPSV (44.2 vs -3.7, p=0.001) (Table 5).

	Compound A Treatment			***Compound B Treatment***		
	Pre	***Post***	***p-value***	***Pre***	***Post***	***p-value***
mean ACC (m/s^2)	2.5 ± 0.6	2.7 ± 0.8	0.115	2.6 ± 0.8	2.6 ± 0.6	0.784
mean bPSV (cm/sec)	14.7 ± 3.7	16.3 ± 4.1	0.011	15.1 ± 3.8	14.1 ± 3.4	0.088
mean dPSV (cm/sec)	31.5 ± 11.4	41.1 ± 10.2	0.001	39.3 ± 13.4	36 ± 10.4	0.150
IIEF-EF	15.7 ± 11.4	18.6 ± 8.6	0.218	17.4 ± 8.4	16.7 ± 8.1	0.698

Table 4: Comparison Pre e Post treatment with compound A and B

	Δ% Compound A Treatment	*Δ% Compound B Treatment*	*p-value*
Mean ACC (m/s²)	11.7 ± 33.5	2.9 ± 19.6	0.667
mean bPSV (cm/sec)	14.4 ± 25.7	-3.1 ± 27.2	0.021
mean dPSV (cm/sec)	31.5 ± 11.4	-3.7 ± 29.1	0.001
IIEF-EF	99.8 ± 252.3	24.1 ± 125.1	0.006

Table 5: Δ% Comparison treatment with compound A and B

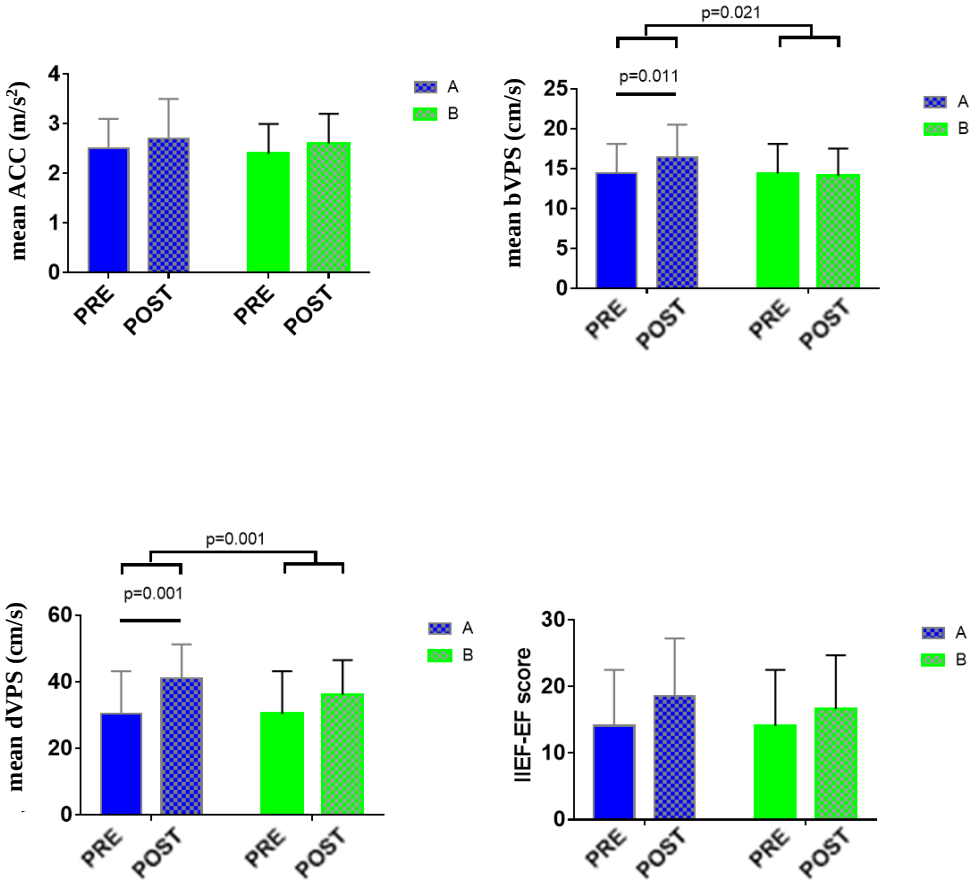


Figure 5: Comparison Pre e Post treatment with compound A and B

2. Effect of treatment with compound A vs B on erectile function according to different intake mode

In order to determine whether the method of taking the compound could have a differential impact on the endpoints studied, in a first sub-analysis the pre-post variation in the endpoints after a week of treatment with compound A or B was compared, depending on the method of taking the compound, over the entire cohort of 52 patients; the comparison groups are therefore made up of 26 subjects analyzed pre-post relative to 1-week treatment with compound A or B who had taken the compound in dosage of 2 pills in the morning vs 26 subjects analyzed pre-post concerning the no significant differences were found on the other parameters. who had taken the compound in dosage of 1 pill in the morning and 1 pill in the evening.

After 1 week of treatment with compound A, in patients taking 2 pills in the morning, the flow indices to the dynamic penile colordoppler ultrasound were significant increased, compared to basal one, in particular in mean bPSV (16.4 ± 3.5 cm/sec vs 14.6± 4.3 cm/sec, p=0.03), mean dPSV (39.3 ± 10.9 cm/sec vs 30.9 ± 11.4 cm/sec, p=0.032) and mean ACC (2.8 ± 0.3 m/s2 vs 2.4± 0.6 m/s2, p=0.06). After 1 week of treatment with compound B, in patients taking 2 pills in the morning, there were no significant changes compared to basal in any of the endpoints studied (Table 6).

	Compound A Treatment 2 pills in morning			***Compound B Treatment 2 pills in morning***		
	Pre	***Post***	***p-value***	***Pre***	***Post***	***p-value***
mean ACC (m/s²)	2.8 ± 0.3	2.4 ± 0.7	0.06	2.7 ± 0.9	2.6 ± 0.7	NS
mean bPSV (cm/sec)	16.4 ± 3.5	14.6 ± 4.3	0.03	15.3 ± 3.9	14.2 ± 3.6	NS
mean dPSV (cm/sec)	39.3 ± 10.9	30.9 ± 11.4	0.032	38.9 ± 12.3	35.9 ± 10.6	NS
IIEF-EF	15.6 ± 11.3	17.7 ± 8.8	NS	17.4 ± 8.4	17.9 ± 8.4	NS

Table 6: Comparison effect of intake 2 pills/morning mode compound A vs B

The comparison by statistical testing of deltas before and after 1 week of treatment, for compound A vs B, showed that the group of patients, who taken 2 pills in the morning of compound A, had a significantly greater improvements, compared to treatment with compound B, in mean dPSV (50.8 vs -4.5, p=0.011) (Table 7).

	Compound A Treatment ***2 pills in morning***	***Compound B Treatment*** ***2 pills in morning***	
	Δ%	**Δ%**	**p-value**
mean dPSV (cm/sec)	50.8	- 4.5	0.011

Table 7: Δ% Comparison effect of intake 2 pills/morning mode compound A vs B

Similarly, after 1 week of treatment with compound A, in patients taking 1 pill in the morning and 1 pill in the evening, the flow indices to the dynamic penile doppler, were significant increased, compared to basal, in particular mean dPSV (42.8 cm/sec vs 32.1 cm/sec, p<0.001) and in IIEF-EF score (21.7± 9.4 vs 14.1 ± 6.8, p=0.006). After 1 week of treatment with compound B, in patients taking 1 pill in the morning and 1 pill in the evening, no significant changes compared to basal were found in any of the endpoints studied.

	Compound A Treatment ***1 pill in morning + 1 pill in evening***			***Compound B Treatment*** ***1 pill in morning + 1 pill in evening***		
	Pre	***Post***	***p-value***	***Pre***	***Post***	***p-value***
mean ACC (m/s^2)	2.5 ± 0.4	2.4 ± 0.8	NS	2.7 ± 0.9	2.6 ± 0.7	NS
mean bPSV (cm/sec)	16.4 ± 4.5	14.6 ± 6.3	NS	16.5 ± 6.9	18.2 ± 9.6	NS
mean dPSV (cm/sec)	42.8 ± 10.7	32.1 ± 8.6	0.001	38.9 ± 12.3	35.9 ± 10.6	NS
IIEF-EF	21.7 ± 9.4	14.1 ± 6.8	0.006	18.4 ± 9.8	20.9 ± 11.8	NS

Table 8: Comparison effect of intake 1 pill in morning and 1 pill in evening mode compound A vs B

Comparison by statistical testing of deltas before and after 1 week of treatment, between compound A vs B, both taken 1 pill in the morning and 1 pill in the evening, the group treated with compound A induced significantly greater improvements than treatment with compound B, in the parameters mean dPSV (50.8 vs -4.5, p=0.011), and IIEF-EF (211.4 vs -8.4, p=0.015) (Table 9).

	Compound A Treatment ***2 pills in morning***	***Compound B Treatment*** ***1 pill in morning + 1 pill in evening***	
	Δ%	**Δ%**	**p-value**
mean dPSV (cm/sec)	50.8	- 4.5	0.011
IIEF-EF	211.4	-8.4	0.015

Table 9: Δ% Comparison effect of intake 2 pills in morning vs 1 pill in morning and 1 pill in evening mode of compound A

These results showed that 1-week treatment with compound A proved to be significantly effective in improving flow parameters and score to questionnaires, compared to compound B, which shows no effect, regardless of the mode of intake.

In addition, the comparison by statistical testing of deltas before and after 1 week of treatment with compound A, the mode of taking 2 pills together vs 1 pill + 1 pill taken in different moment, showed that the mode of taking 1 pill + 1 pill in different moment induced significantly greater improvements, compared to the mode of taking 2 pills together, on the scores IIEF-EF (147.3 ± 2.5 vs 11.8 ± 3.2, $p<0.001$), but not in the flow index.

3. Subanalysis on the effect of timing treatment compound A vs B

In order to determine whether treatment timing could have a differential impact on the endpoints studied, a comparison of pre-post variation in endpoints after one week of treatment A or B was performed subanalysis, depending on the timing of taking the compound, over the entire series of 52 patients; the comparison groups are therefore made up of 26 subjects analyzed pre-A or B treatment week that had taken the single compound as treatment 1 vs treatment 2.

After 1 week of treatment with compound A, taken as treatment 1, the mean dPSV (42.1 ± 11.7 cm/sec vs 24.2 ± 6.3 cm/sec, p<0.001) and IIEF-EF score (17.5 vs 10.9, p=0.021) were significant increased compared to basal. After 1 week of treatment with compound B, taken as treatment 1, no significant changes, compared to basal, were discovered in any of the endpoints. Comparison by statistical testing of deltas before and after 1 week of treatment, for compound A vs B, in groups of patients who had taken these compounds as treatment 1, treatment with compound A had shown to induce significantly greater improvements than treatment with compound B in mean dPSV (80.4 vs 11.6, p=0.003) parameter.
(Table 10).

	Compound A as Treatment 1			***Compound B as Treatment 1***			***Δ% A vs B***
	Pre	***Post***	***p-value***	***Pre***	***Post***	***p-value***	***p-value***
mean ACC (m/s²)	2.7 ± 0.5	2.4 ± 0.4	NS	2.9 ± 0.7	2.7 ± 0.6	NS	NS
mean bPSV (cm/sec)	14.6 ± 4.5	14.6 ± 5.4	NS	16.5 ± 9.8	18.2 ± 6.1	NS	NS
mean dPSV (cm/sec)	42.1 ± 11.7	24.1 ± 6.3	0.001	38.9 ± 12.3	35.9 ± 10.6	NS	0.003
IIEF-EF	21.7 ± 9.4	14.1 ± 6.8	0.006	21.4 ± 11.8	20.9 ± 15.8	NS	NS

Table 10: Comparison effect of intake compound A vs B as treatment 1

After 1 week of treatment with compound A, taken as treatment 2, there were no significant changes, compared to basal, in flow indices and scores to questionnaires. After 1 week of treatment with compound B, taken as treatment 2, the mean bPSV (13.1 ± 9.8 cm/sec vs 15.9 ± 6.1 cm/sec, p=0.004) and mean dPSV (33.3 ± 12.3 cm/sec vs 42.1 ± 10.6 cm/sec, p=0.011) were significantly reduced compared to basal.
These results showed that, depending on the timing of treatment, the 1week-treatment with compound A, taken as first, was effective in improving flow parameters and scores to questionnaires, compared to compound B, taken as first, while compound B, taken as second treatment, was associated with a worsening of the outcomes. In addition, comparison by

statistical testing of deltas before and after 1 week of treatment, for compound A taken as treatment 1 vs A taken as treatment 2, showed that compound A taken as treatment 1 induced significantly greater improvements, compared to taking as treatment 2 in mean dPSV (80.4 vs 7.9, p<0.001) and IIEF-EF (202.8 vs -2.2, p=0.002) (Table 11).

	Compound A as Treatment 2			***Compound B as Treatment 2***			***Δ% A vs B***
	Pre	***Post***	***p-value***	***Pre***	***Post***	***p-value***	***p-value***
mean ACC (m/s^2)	2.9 ± 0.3	2.7 ± 0.7	NS	2.9 ± 0.7	2.7 ± 0.6	NS	NS
mean bPSV (cm/sec)	14.8 ± 4.3	14.6 ± 5.4	NS	13.1 ± 9.8	15.9 ± 6.1	0.004	NS
mean dPSV (cm/sec)	39.1 ± 10.1	24.1 ± 6.3	NS	33.3 ± 12.3	42.1 ± 10.6	0.011	0.001
IIEF-EF	21.7 ± 9.4	14.1 ± 6.8	NS	21.4 ± 11.8	20.9 ± 15.8	NS	0.002

Table 11: Comparison effect of intake compound A vs B as treatment 2

4. Subanalysis on effect of wash-out and therapeutic switch treatment A vs B

In order to determine whether the effect of 1 week - treatment with compound A or B persisted after 1 week of wash-out and 1 week of therapeutic switch, the variation of endpoints after 1 week of treatment and after 3 weeks including wash-out was analysed out and therapeutic switch.

Group A1 results (A->B; 2 pills together): At T0, the following parameters were detected: mean ACC 2.1 ± 0.6 m/s2, mean bPSV 15 ± 3.7 cm/sec, mean dPSV 22 ± 11.4 cm/sec, score IIEF-EF 15.7 ± 11.4. At T1, after 1 week of taking compound A, there was a statistically significant increase of mean dPSV 41.4 ± 10.2 cm/sec (p<0.001) compared to the T0. At T2, after the wash-out and switch period to compound B, a worsening, although not statistically significant, of all parameters compared to T1 was found; however, the mean dPSV values remained significantly higher than T0 (38 ± 13.4 cm/sec vs 22 cm/sec; p=0.011) (Table 12).

Group A1 Treatment

	T0	***T1***	***p-value***	***T2***	***p-value***
mean ACC (m/s²)	2.1 ± 0.6	2.6 ± 0.7	NS	2.6 ± 0.6	NS
mean bPSV (cm/sec)	15.0 ± 3.7	16.3 ± 6.1	NS	15.8 ± 3.8	NS
mean dPSV (cm/sec)	22.0 ± 11.4	41.4 ± 10.2	0.001	38.0 ± 13.4	0.011
IIEF-EF	15.7 ± 11.4	16.8 ± 8.7	NS	17.4 ± 8.4	NS

Table 12: Comparison effect of wash-out and therapeutic switch of Group A1 treatment

Group A2 results (A->B; 1 pill+1 pill): At T0, the following parameters were detected: mean ACC 2.6 ± 0.3 m/s^2, mean bPSV 13.0 ± 3.4 cm/sec, mean dPSV 26.4 ± 11.4 cm/sec, IIEF-EF 6.0 ± 3.4. At T1, after 1 week of taking compound A, there was a statistically significant increase of: mean dPSV 42.8 ± 7.2 cm/sec ($p<0.001$), IIEF-EF 18.7 ± 3.4 ($p=0.006$) compared to T0. At T2, after the wash-out and switch to compound B, a statistically significant reduction of the mean dPSV 28.6 ± 11.4 cm/sec ($p=0.03$) compared to T1; the values at T2 compared to T0 did not differ statistically significant (Table 13).

Group A2 Treatment

	T0	***T1***	***p-value***	***T2***	***p-value***
mean ACC (m/s²)	2.6 ± 0.3	2.6 ± 0.4	NS	2.5 ± 0.4	NS
mean bPSV (cm/sec)	13.0 ± 3.4	16.3 ± 6.1	NS	15.3 ± 3.7	NS
mean dPSV (cm/sec)	26.4 ± 11.4	42.8 ± 7.2	0.001	28.6 ± 11.4	0.03
IIEF-EF	6.0 ± 3.4	18.7 ± 4.3	0.006	17.5 ± 8.7	NS

Table 13: Comparison effect of wash-out and therapeutic switch of Group A2 treatment

Group B1 results (B->A; 2 pills together): At T0, the following parameters were detected: mean ACC 2.5 ± 0.4 m/s2, mean bPSV 14.4 ± 4.5 cm/sec, mean dPSV 37.3 ± 10.7 cm/sec, IIEF-EF 14.7 ± 9.4. At T1, after 1 week of taking compound B, the parameters were not significantly changed, compared to T0. At T2, after the wash-out and switch to compound A, all parameters were increased, compared to T1 and T0, although not statistically significant (Table 14).

Group B1 Treatment

	T0	***T1***	***p-value***	***T2***	***p-value***
mean ACC (m/s^2)	2.5 ± 0.4	2.7 ± 0.6	NS	2.8 ± 0.5	NS
mean bPSV (cm/sec)	14.4 ± 4.5	16.3 ± 6.1	NS	18.2 ± 6.1	NS
mean dPSV (cm/sec)	37.3 ± 10.7	41.1 ± 10.6	NS	39.5 ± 10.1	NS
IIEF-EF	14.7 ± 9.4	18.6 ± 13.8	NS	20.9 ± 11.8	NS

Table 14: Comparison effect of wash-out and therapeutic switch of Group B1 treatment

Group B2 results (B->A; 1 pill +1 pill): At T0 the following parameters were detected: mean ACC 2.3 ± 0.5 m/s2, mean bPSV 14.2 ± 4.4 cm/sec, mean dPSV 30.5 ± 10.6 cm/sec, IIEF-EF 19.7 ± 9.3. At T1, after 1 week of taking compound B, the parameters were not significantly changed, compared to T0. At T2, after the wash-out and switch to compound A, all parameters were increased, compared to T1 and T0, although not statistically significant (Table 15).

Group B2 Treatment

	T0	***T1***	***p-value***	***T2***	***p-value***
mean ACC (m/s^2)	2.3 ± 0.5	2.4 ± 0.5	NS	2.7 ± 0.3	NS
mean bPSV (cm/sec)	14.2 ± 4.4	14.6 ± 4.1	NS	17.2 ± 4.6	NS
mean dPSV (cm/sec)	30.5 ± 10.6	31.1 ± 11.6	NS	33.9 ± 7.6	NS
IIEF-EF	19.7 ± 9.3	18.7 ± 13.6	NS	21.7 ± 12.3	NS

Table 15: Comparison effect of wash-out and therapeutic switch of Group B2 treatment

As regards the cardiometabolic status, it was found only a mild significant reduction of total- (227.8 ± 60.9 mg/dl vs 207.6 ± 48.3 mg/dl, p=0.045) and LDL-cholesterol (155.9 ± 57.2 mg/dl vs 140.5 ± 45.0, p=0.045) associated to 1 week - treatment with compound A, while no significant differences were found on weight, waist circumference, blood pressure and hormonal parameters.

RESULTS IN VITRO

1. Determination of reactive oxygen species production.

Treatment with 28.5 mm glucose for 24 hrs significantly increased intracellular ROS concentration in HUVEC, as highlighted by a 2.58 fold increase ($p<0.01$) in DCF fluorescent staining, compared to untreated cells. Pre-treatment of HUVEC for 24 hrs with Paullinia Cupana extract at concentrations of 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml, exerted a dose-dependent protective effect from the HG-induced increase in intracellular ROS concentration, with a significant effect reached with concentrations of 0.8 mg/ml and 1 mg/ml of extracts. Specifically, in the 0.8 mg/ml and 1 mg/ml Paullinia Cupana treatment groups, DCF fluorescent staining was increased only by 2 fold ($p<0.01$) and 1.4 fold ($p<0.05$), compared to untreated cells, respectively, and DFC fluorescent staining was significantly lower ($p<0.05$ and $p<0.01$, respectively), compared to HG group (Figure 6).

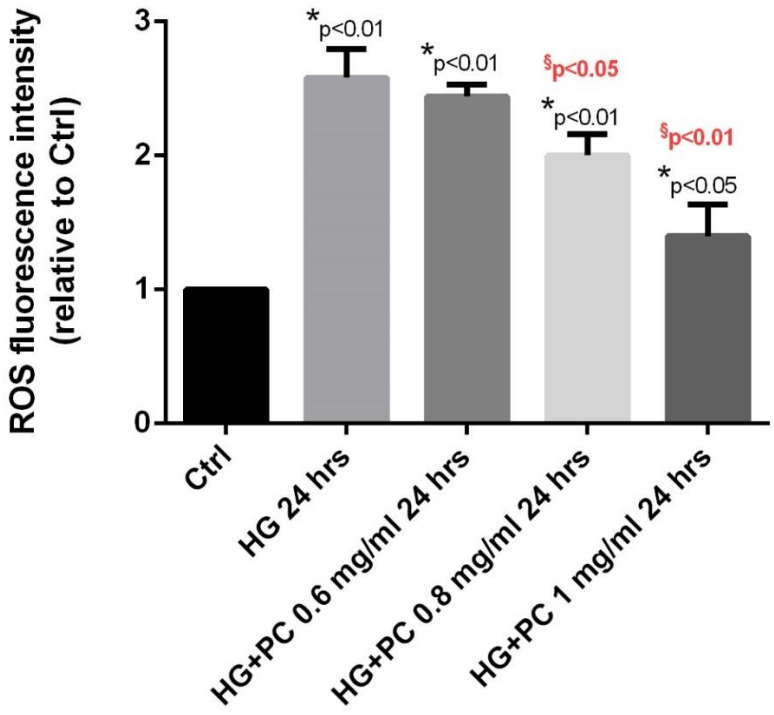


Figure 6: Reactive oxygen species quantification by DCFH DA fluorescence assay in HUVEC subjected to high-glucose (HG) challenge for 24 hrs after a pre-treatment for 24 and 48 hrs with Paullinia Cupana extract at concentration of 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml. Data expressed as fold change relative to Ctrl. *significantly different from Ctrl; §significantly different from HG.

2. **Determination of endothelin-1 and endothelial nitric oxide synthase mRNA expression level.**

Treatment with 100 µg/ml OX-LDL for 12 hrs significantly increased ET-1 mRNA expression level in HUVEC, as highlighted by a 2.9 fold increase ($p<0.01$), compared to untreated cells. Pre-treatment of HUVEC for 24, 48 and 72 hrs with Paullinia Cupana extract at concentration of 1 mg/ml, exerted a time-dependent protective effect from the OX-LDL-induced increase in ET-1, with a significant effect reached after 48 and 72 hrs. Specifically, in the 48 and 72 hrs treatment groups, ET-1 mRNA expression level was increased only by 2.38 fold ($p<0.01$) and 2.32 fold ($p<0.01$), compared to untreated cells, respectively, and the mRNA expression level was significantly lower compared to OX-LDL group (both $p<0.05$)(Figure 7).

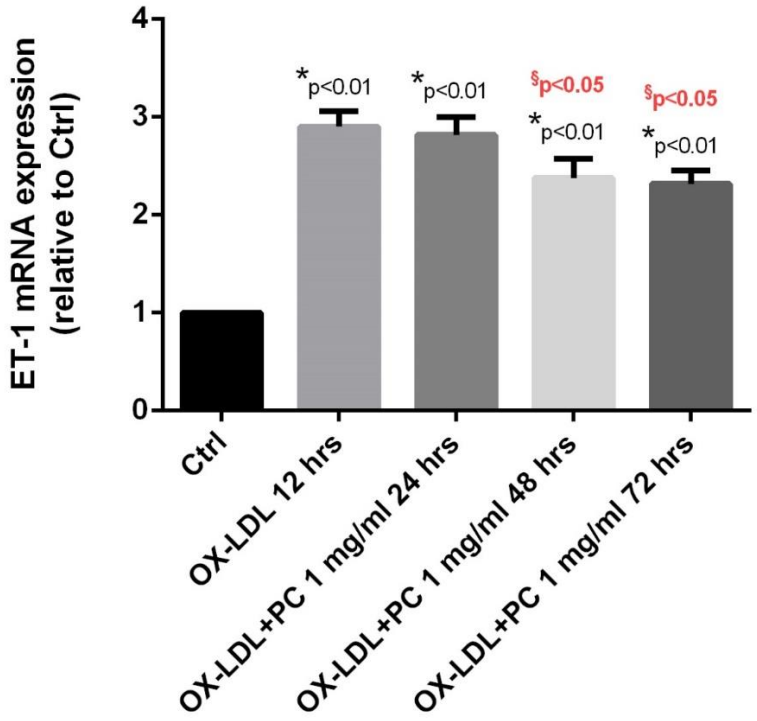


Figure 7: Endothelin 1 (ET-1) mRNA expression level quantification by qRT-PCR in HUVEC subjected to oxidized low-density lipoprotein (OX-LDL) challenge for 12 hrs after a pre-treatment for 24, 48 and 72 hrs with Paullinia Cupana extract at concentration of 1 mg/ml. Data expressed as fold change relative to Ctrl. *significantly different from Ctrl; §significantly different from OX-LDL.

Treatment with 28.5 mm glucose for 24 hrs significantly increased eNOS mRNA expression level in HUVEC, as highlighted by a 3.28 fold increase ($p<0.01$), compared to untreated cells. Pre-treatment of HUVEC for 24, 48 and 72 hrs with Paullinia Cupana extract at concentration of 1 mg/ml, exerted a time-dependent protective effect from the HG-induced increase in eNOS, with a significant effect reached after 24, 48 and 72 hrs. Specifically, in the 24, 48 and 72 hrs treatment groups, eNOS mRNA expression level was increased only by 2.58 fold ($p<0.01$), 2.24 fold ($p<0.01$) and 2.32 fold ($p<0.01$), compared to untreated cells, respectively, and the mRNA expression level was significantly lower compared to HG group (all $p<0.05$) (Figure8).

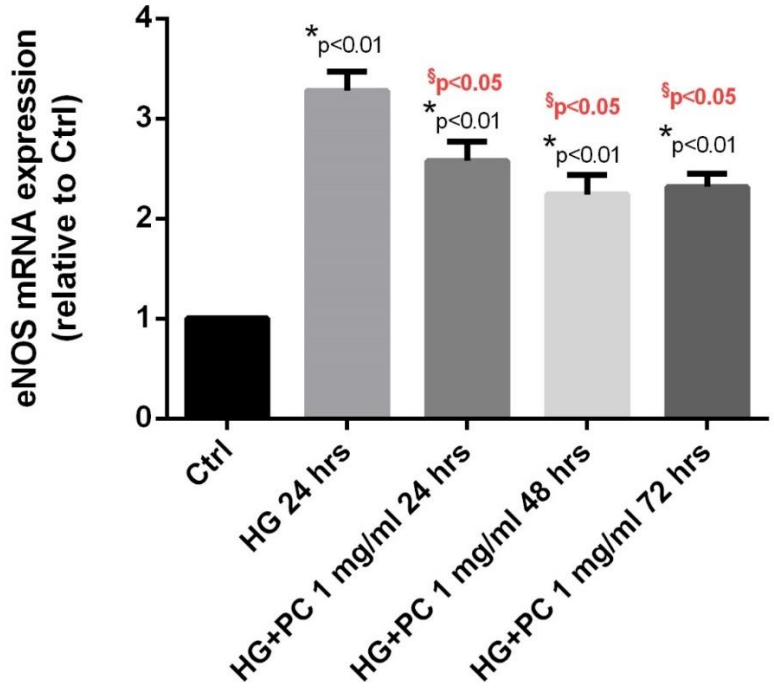


Figure 8: Endothelial nitric oxide synthase (eNOS) mRNA expression level quantification by qRT-PCR in HUVEC subjected to high-glucose (HG) challenge for 24 hrs after a pre-treatment for 24, 48 and 72 hrs with Paullinia Cupana extract at concentration of 1 mg/ml. Data expressed as fold change relative to Ctrl. *significantly different from Ctrl; §significantly different from HG.

DISCUSSION

The results of this randomized triple-blind crossover study, conducted on 52 patients, suggested that the administration of Paullina Cupana (compound A) is able to improve the quality of erection in patients with mild- moderate grade ED, compared to placebo (compound B). This improvement has been found both in the objective measurement of cavernous fluxes during dynamic colordoppler ultrasound of penis and through IIEF-EF score.

In addition, the study showed that the method of administration 1+1 pill of Paullina Cupana is associated with a more marked improvement, compared to the method of administration 2 pills together, in the sexual parameters evaluated through the IIEF questionnaires, although there are no differences in flow indices objectively evaluated through the dynamic penile colordoppler ultrasound. Moreover, the administration of Paullina Cupana as the first compound is associated with a more marked improvement of the analysed parameters than when it is administered as the second compound during the study; while the administration of placebo as the first compound had neutral effects on different outcomes while as the second compound was associated to a worsening effect of the parameters.

In addition, Paullina Cupana treatment, regardless of the mode of intake, seemed to be affected by the effect of the wash-out and therapeutic switch to placebo, with reduction of all endpoints and the return to the starting values with the exception of mean dPSV, whereas in patients receiving 2 pills together of Paullina Cupana, persisted significantly higher than at baseline, suggesting a persistence of the effect even at 3 weeks.

On the other hand, treatment with placebo, regardless of the mode of intake seemed to benefit from the therapeutic switch to Paullina Cupana, with an increase of all endpoints compared to the baseline values. These results are in line with the emerging effect of Paullinia cupana, similar to PDE-5 inhibitors, to stimulate the iNOS production, the soluble guanylate cyclase (sGC), and cGMP as well as a reduction in the content of the PDE5 enzyme, necessary to ameliorate not only the vascularization of corpora cavernosa of the penis, but also, probably, to inhibit the histological changes of the penis corpora, typical of aging associated to ED[77,78].

The direct antioxidant action was correlated to the increased regulation of antioxidant/detoxifying enzymes (e.g., superoxide dismutase, catalase, and glutathione per-oxidase), which also an acute and cumulative effects on the activity of glutathione peroxidase and catalase, which are phase II antioxidant enzymes that reduce peroxides to water molecules. In contrast, antioxidant status markers, such as reduction in LDL-c oxidation ex

vivo and in lymphocytes DNA damage induced by hydrogen peroxide, only improved temporarily[81].

After 1 week- treatment of Paullina Cupana, it was found also a mild significant decrease of total and LDL cholesterol as demonstrated in a study focused on people with habitual intake in guarana[79]. Another study showed that the consumption of 3 g of guarana powder (90 mg of catechin and 60 mg of epicatechin) by healthy individuals increased plasma oxygen radical absorbance capacity, indicating a reduction in damage caused by peroxyl radicals, while reducing ex vivo oxidation of LDL cholesterol[81]. Further studies are needed on widely cohort to demonstrate the effect of Paullina Cupana on the vascular and cardiometabolic endothelial effect.

In the current study, an in vitro assay was undertaken to investigate the protective effect of Paullinia Cupana extract against OX LDL induced HUVEC injury, a typical in vitro model of atherosclerosis. The results of the current study demonstrated that a pre-treatment of HUVEC for 24, 48 and 72 hrs with Paullinia Cupana extract at concentration of 1 mg/ml time-dependently prevented OX-LDL-induced increase in ET-1 mRNA expression level, therefore suggesting that supplementation with Paullinia Cupana might represent a beneficial adjuvant treatment in the prevention of endothelial dysfunction and, therefore, ED.

Indeed , ET-1, a 21-amino acid peptide mostly expressed in endothelial cells, is a well known vasoconstrictor[82] involved in a range of pathological stresses, including hypoxia, angiotensin II, thrombin and inflammatory cytokines signaling activation[83], and, importantly, in vascular ROS production, by acting per sè as a pro-inflammatory cytokine[84]. All of these mechanisms underline the key role of ET-1 in atherosclerosis[85].

Moreover, the protective effect of Paullinia Cupana extract against HG induced HUVEC injury, a typical in vitro model of endothelial oxidative stress due to uncontrolled hyperglycemia, i.e. as in diabetes, was also evaluated. The results of the current study demonstrated that a pre-treatment of HUVEC for 24 hrs with Paullinia Cupana extract at various concentration dose-dependently prevented HG-induced increase in ROS, whereas a pre-treatment of HUVEC for 24, 48 and 72 hrs with Paullinia Cupana extract at concentration of 1 mg/ml time-dependently prevented HG-induced increase in eNOS mRNA expression level at all assessed timepoints, therefore suggesting that supplementation with Paullinia Cupana might also contribute to prevent endothelial dysfunction and, therefore, ED, by an additional mechanism of action.

Indeed , several evidences demonstrated that oxidative stress is a major driver of endothelial dysfunction, and, therefore might severely impact on penile erection[67]. Moreover , within oxidative stress processes, excessively increased eNOS expression and activity might determine a consequential excessive production of peroxynitrite radicals derived from NO in endothelial cells exposed to HG, therefore contributing to oxidative stress ; this evidence might represent one of the underlying mechanisms associated to premature vascular disease observed in uncontrolled hyperglycemia, the main risk factor in the development of diabetic vascular complications, such as ED[86].

CONCLUSION

Collectively, the in vitro results of the current study suggested that supplementation with Paullinia Cupana might be beneficial, in a preventative prospective, in at least two different settings of endothelial damage, potentially associated to an increased risk of ED, namely, metabolic derangements of glycol-lipid profile, by potentially impacting on three different mechanisms with differential timing of action. Moreover, the results of this randomized triple-blind crossover study suggest that Paullina Cupana may be effective in treating mild to moderate ED even in a short term setting of 1 week, preferably 1+1 pills; further studies of longer duration may be useful in further confirming the data in this pilot study and determining whether these beneficial effects are also found in patients with severe ED.

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