The following excerpts from a thesis titled **"Antioxidant and antitumor effect in vitro of the ethanolic extract of the root of Waltheria ovata Cav. Lucraco"** by Oscar Herrera Calderón, written in Lima, Peru, in 2014, are offered for research purposes only. Whole World botanicals offers Royal Lucraco Plus™ not for prostate cancer but for optimal function of the prostate gland through the reduction of ROS (reactive oxidative stress), or free radicals, described in this thesis. If you have prostate cancer, do not attempt to self-treat; it could be dangerous. Get a diagnosis and treatment from a qualified healthcare practitioner.

2.4. THEORETICAL BASES

2.4.1. Antioxidant defense systems

In a biological system, an antioxidant is able to react with free radicals becoming an oxidative chain terminator. The antioxidant capacity of a substance or molecule is defined as the ability to inhibit oxidative degradation (eg, lipoperoxidation). The antioxidant capacity is determined in turn by: a) chemical reactivity of the antioxidant associated with antiradical or free radical stabilizing activity; b) ability to access the reaction site and; c) stability of the products formed after the free radical stabilization process **(Craig, 2009).**

Given the incessant damage that free radicals exert in living systems, nature has provided different sources of protection that allow this damage to be mediated.

These systems, according to their origin, can be classified as endogenous and exogenous **(See figure 01).**

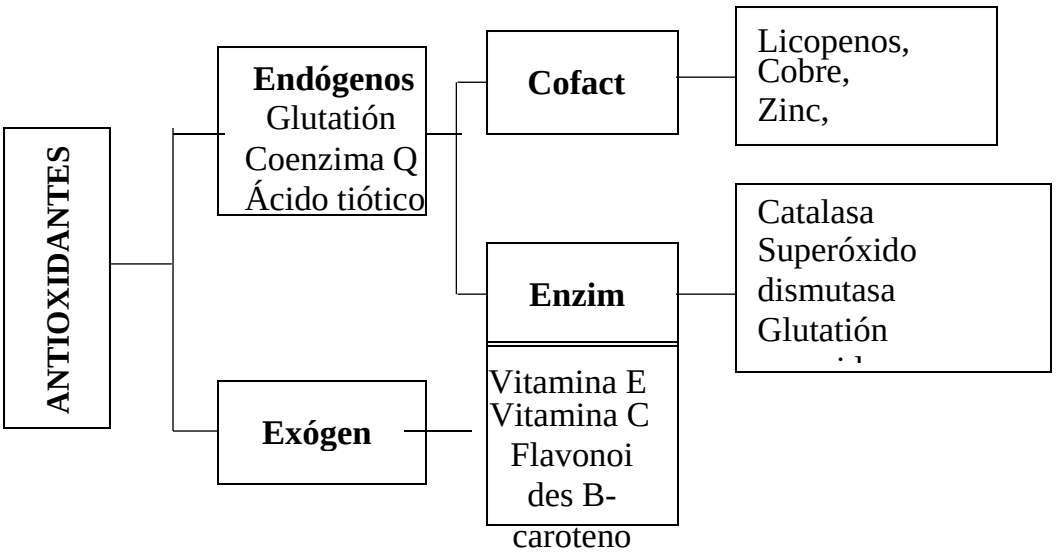


Figure 1. Classification of antioxidants (Craig, 2009)

Endogenous antioxidant defense systems consist of a series of biological elements that provide protection against the continuous production of free radicals generated by cellular functions such as respiration and synthesis of ATP at the mitochondrial level; the endogenous antioxidant system, in turn, is divided into enzymatic and

non-enzymatic. The first is the antioxidant enzymes: Superoxide dismutase, Catalase, Glutathione peroxidase, glutathione reductase, glutathione S-transferase, among the most important. The second is made up of glutathione, coenzyme Q and thioctic or lipoic acid. The exogenous antioxidant defense system is formed by all elements provided with the environment (food, plants, etc.) that have been subjected to scientific studies and have been identified antioxidant activity. Among the most studied are vitamin E, vitamin C, þ-carotenes and polyphenols **(Martínez-Flores, et al., 2002).**

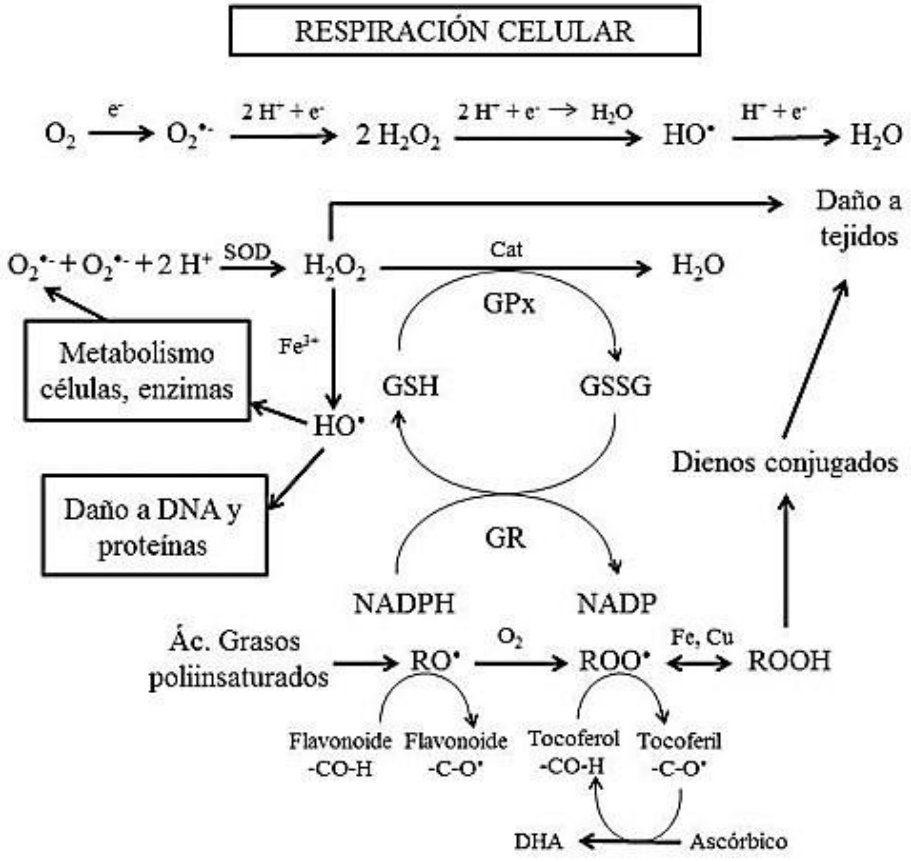


Figure 2. Representation of the main metabolic pathways producing free radicals.

(Martínez-Flores at al., 2002)

2.4.1. Endogenous antioxidant defense systems.

- **Antioxidant Enzymes**

Antioxidant enzymes are part of the endogenous antioxidant defense system of organisms and has emerged throughout the evolution of species as a mechanism of protection against damage caused by ROS. Antioxidant defense enzyme systems consist of a series of coordinated enzymes: copper and zinc dependent

superoxide dismutases (CuZnSOD) and manganese (MnSOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme (ME). **Figure 03** shows the action and coordination of these systems and their relationship with the redox cycle of glutathione **(Pérez, 2003).**

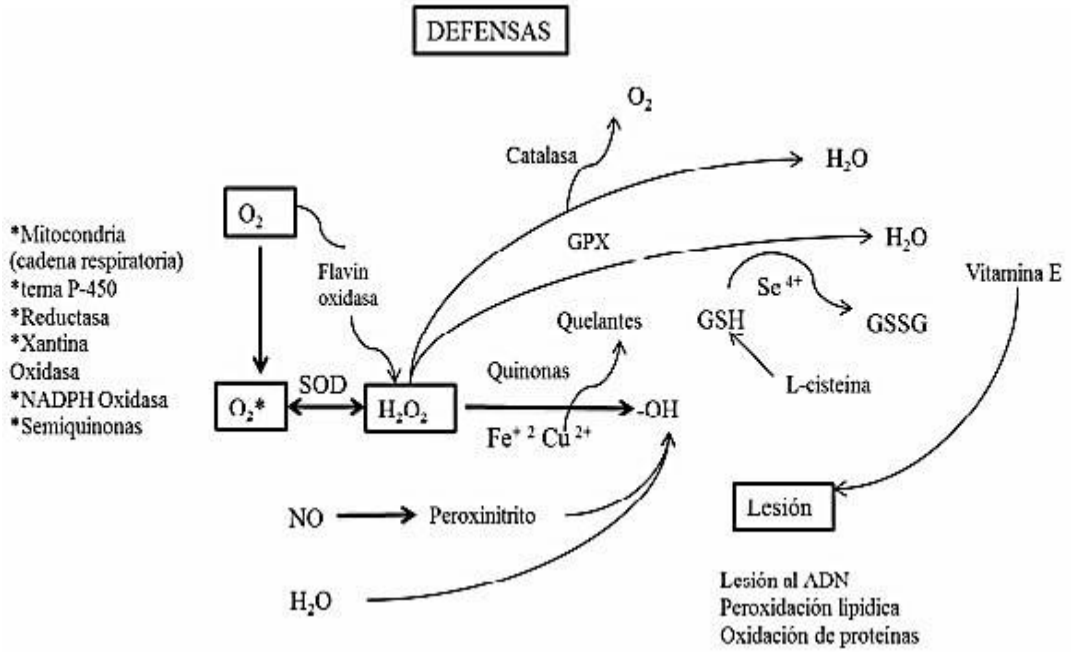


Figure 3. Detoxification of reactive oxygen species by enzymatic antioxidant defense systems (SOD, catalase and GPx) (Pérez, 2003)

- **Superoxide dismutase (SOD).**

SOD is a very important component of the antioxidant system; its function is to catalyze the mutation of O_2 a H_2O_2 in the following reaction:

$$2O_2^{\bullet} + 2H^{+} \longrightarrow O_2 + H_2O_2$$

The SOD family is formed by the Cu and Zn-dependent SOD (CuZnSOD) found in the cytosol of eukaryotic cells, the Mn-dependent SOD (MnSOD) located in the mitochondrial matrix in eukaryotes and the Fe-dependent SOD (SOD) in aerobic bacteria (Madhu, et al., 2012).

O2 is formed as a product of the electronic transport chain in complexes I and III; it is known that under normal conditions there is ROS production well tolerated by the cellular economy but the excessive increase of these radical species generates oxidative stress causing damage in various cellular structures, membrane

lipoperoxidation and DNA damage leading to premature aging and associated diseases such as cardiovascular diseases, neurodegenerative diseases, diabetes and carcinogenesis (Alimirah, et al., 2006).

- **Catalase (CAT)**.

It is one of the enzymes involved in the protection and maintenance of the oxidant / antioxidant balance. Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase) is one of the most abundant enzymes in nature and is widely distributed in the human organism, although its activity varies depending on the tissue; it is higher in the liver and kidneys, lower in connective tissue and epithelia, and almost nil in nerve tissue. At the cellular level, it is located in mitochondria and peroxisomes, except in erythrocytes, where it is found in the cytosol. This enzyme is a tetrameric metalloprotein whose molecular weight is in the range of 210-280 kD.

It catalyzes the decomposition of hydrogen peroxide into water and oxygen. In the absence of this enzyme, the accumulated H_2O_2 reacts with metals such as Fe^{2+} and Cu^{2+} found in the medium to produce the radical • OH, such reactions are known as Fenton.

$$H_2O_2 \longrightarrow 2H_2O + O_2$$

$$O^{2\bullet-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

This function is shared with the enzyme glutathione peroxidase that does not require cofactors. In general, low concentrations of hydrogen peroxide stimulate peroxidase activity, while high peroxide concentrations are preferably catalase catalyzed (Hari, et al., 2013).

- **Glutathione Peroxidase (GPx**).

It was discovered by Mills in 1957. It is a selenoprotein that, in animal cells, is located in the mitochondrial matrix and in the cytosol. In the presence of GSH as a reducing agent, it catalyzes the reduction of hydrogen peroxide and other organic hydroperoxides in water and alcohol respectively, catalyzes the oxidation reaction of GSH to GSSG at the expense of H_2O_2 or organic peroxides. The reactions are as follows:

$$ROOH + 2\ GSH \longrightarrow GSSG + H_2O + ROH$$

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + H_2O$$

Selenium-dependent GPx acts on lipid hydroperoxides and Selenium-dependent GPx which is dimeric of lower molecular weight and relates to Glutathione S-transferases because it participates in the elimination of xenobiotics (Webster, et al., 1996).

- **Glutathione Reductase (GR).**

GR was first observed by Hopkins and Elliot in 1931, subsequently isolated from rabbit liver in 1932. It catalyzes the restoration reaction of glutathione in its reduced form with the presence of reductive equivalents of NADPH. The reaction in which it participates is as follows:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

- **Glutathione.**

Glutathione (ç-L-glutamyl-L-cysteinylglycine) is the most important non-protein thiol compound in cells. The liver is the organ most abundant (5-10 mM). It exists in reduced form (GSH) and oxidized (GSSG) in a proportion of 100 to 1. In the cells, they are in three reservoirs: 90% in the cytosol, 10% in the mitochondria, and a small amount in the endoplasmic reticulum. It is basically formed in two steps, first from glutamate and cysteine, ç-glutamylcysteine is produced by the action of ç-glutamylcysteine synthetase, then the enzyme GSH synthetase uses ç-glutamylcysteine and glycine to form GSH; these reactions are carried out in the presence of ATP. This compound has previously been described as a potent antioxidant and reducing agent as it can react with various electrophilic and oxidizing molecules such as H_2O_2, $O_2^{\bullet}$ and $^{\bullet}OH$. It has also been associated with physiological processes including the metabolism of xenobiotics, thiol group exchange reactions and signaling processes involving the cell cycle, proliferation and apoptosis. Due to its reactive power the GSH is easily reduced due to the presence of hepatotoxic chemical substances inducing lipid peroxidation, destabilization of structures and cellular damage (Naz et al., 2012).

2.4.2. Exogenous antioxidant defense systems.

Exogenous antioxidants are present in drugs and in the diet including fruits, vegetables, seeds, grains, spices, etc. The term Phytochemical is the generic name received by organic compounds in foods and plants, many of them have a high antioxidant potential. These antioxidant compounds form a heterogeneous group of molecules capable of capturing free radicals that produce less harmful species

reducing the level of oxidative stress. The most studied compounds are vitamin E, vitamin C, some minerals such as selenium, copper, zinc and magnesium, þ -carotenes and polyphenols including tannins and flavonoids (Céspedes & Sanchez, 2000).

- **Vitamin C (Ascorbic Acid).**

It is in the form of ascorbate, distributed intra- and extracellularly. It reacts directly with free radicals (RL) superoxide, hydroxyl and various lipid hydroperoxides. This process transforms ascorbate into RL dehydroascorbate. The return to its native form is by enzymatic action or by thiol cellular substrates. Despite its apparent antioxidant property, ascorbate can play a potent pro-oxidant in the presence of excessive concentrations of Fe^{+3} and Cu^{+2} ions. There are studies suggesting that antioxidant action against the oxidative stress of the gastric mucosa may be due to vitamin C, being a potent antioxidant soluble in water traps and neutralizes a variety of reactive oxygen species such as hydroxyl, alkoxy, peroxyl, superoxide anion, hydroperoxyl radicals and radicals nitrogen reactants at very low concentrations; it can also regenerate other antioxidants such as alpha-tocopheroxy and beta-carotene from their radical species. Other studies have shown that ascorbic acid is also a nitrosation inhibitor with potential importance as a nitrite scavenger in vivo, decreases the risk of cancer of the stomach and esophagus, increases immune function by increasing natural Killer cells and the function of T and B lymphocytes, inhibits the growth of different human melanoma cells and induces apoptosis in HL-60 promyelocytic leukemic cells and in human fibroblasts, combats cancer by promoting collagen synthesis and preventing tumors from invading other tissues; and it has been suggested that a daily supplement of 1 g of vitamin C could protect people against chemotherapy-induced mutagenesis (Céspedes & Sanchez, 2000).

2.4.3. Prostate cancer and oxidative stress

Prostate carcinoma is a disease associated with age (Malins et al., 2001). This state along with genetic predisposition, race, environmental factors, diet, infectious agents, exposure to androgens and other hormones produce a redox imbalance leading to a greater oxidative state of the tissue.

The role of oxidative stress in prostate cancer has been widely recognized (De Marzo et al., 2003). Such stress causes tissue remodeling and proliferation. Acute and chronic inflammation generated by reactive oxygen species (ROS) at the site of inflammation, leads to damage to cellular structures. Exposure to chronic oxidative

stress should be one of the possible etiological factors in the development of cancer (Jiang et al., 2008).

Inflammation can lead to the destruction of epithelial cells of the prostate and this may lead to increased proliferation as a compensatory response to cell death. Such proliferation may be mechanistically related to a decrease in the inhibitor of the cellular cycle p27Kip1 as was observed in proliferative inflammatory atrophy (PIA: foci of proliferative glandular epithelium with the morphology of simple atrophy or prostatic hyperplasia, both in relation to chronic inflammation). The decrease in apoptosis associated with these events may also be related to an increase in the expression of the anti-apoptotic gene Bcl-2 (De Marzo et al., 1999).

An increase in oxidative and electrophilic stress in a proliferation environment associated with these events, could lead to elevation of GST-P1 isoform expression (GST-P1) as a protective measure of the genome. However, aberrant methylation of the GST-P1 gene promoter CpG islands silences the expression and protein levels of this gene, causing additional genetic damage, and accelerating the progression towards prostatic intraepithelial neoplasia (PIN) and carcinoma (De March et al., 2003). In patients with prostate cancer, alterations in lipid peroxidation have been detected with concomitant changes in the antioxidant defense system. This leads to an altered pro-oxidant-oxidant balance that can lead to increased oxidative damage and consequently play an important role in prostate carcinogenesis (Aydin et al., 2006).

4.1.1. PRELIMINARY PHYTOCHEMICAL ANALYSIS OF THE ETHANOLIC EXTRACT.

The results of the qualitative phytochemical analysis are shown in Table 3.

Table 3. Qualitative preliminary test of the secondary metabolites of the ethanolic extract of the root of Waltheria ovata Cav. "lucraco".

Characterization test	Result	Secondary metabolite
Reaction of the	(+)	Free amine groups
Reaction of the GELATIN	(-)	Tannins
Reaction with FERRIC CHLORIDE ($FeCl_3$)	(+)	Phenolic Compounds
Reaction of DRAGENDÖRFF	(-)	Alkaloids
Reaction MAYER	(-)	Alkaloids
Reaction of SHINODA	(+)	Flavonoids
Reaction of BORNTRAGER	(+)	Quinones
Reaction from LIEBERMAN-BURCHARD	(+)	Triterpene and / or steroids
Test of the FOAM	(+)	Saponins

(-) = Absence; (+) = Presence

4.1.2. QUANTIFICATION OF TOTAL POLYPHENOLS PRESENT IN THE ETHANOLIC ROOT EXTRACT OF Waltheria ovata Cav.

The content of total polyphenols present in the ethanolic extract of Waltheria ovata Cav. is expressed in mg equivalents to gallic acid (mgEAG / g).

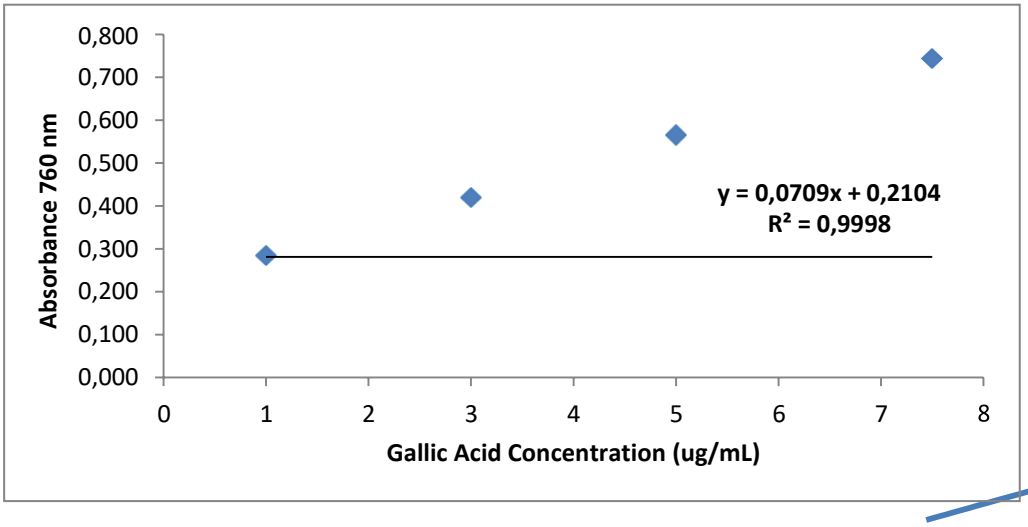


Figure 24. Gallic acid calibration curve

Table 4. Quantification of total polyphenols of the ethanolic extract of Waltheria ovata Cav.

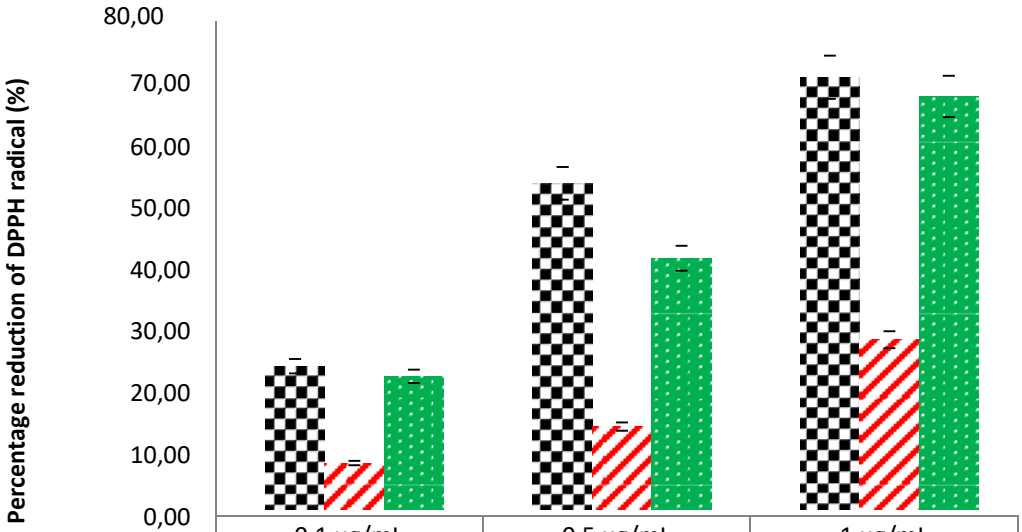
Sample	mgEAG/g extract	D.E.
Ethanolic extract of Waltheria ovata root Cav. "lucraco"	**2250**	**23**

D.E.: standard deviation

4.1.3. ANTIOXIDANT EFFECT IN VITRO OF THE ETHANOLIC EXTRACT OF THE ROOT OF Waltheria ovata Cav. "lucraco"

Table 5. Determination of the antioxidant activity by the uptake of 1,1-diphenyl-2-picrihydrazyl (DPPH) radical from the ethanolic root extract of Waltheria ovata Cav.

Substance tested	Concentration ug/mL	Percentage of inhibition (%)	CI_{50} ± DE (ug/mL)
Trolox	0,1	21,57	0.89 ± 0.04
	0,5	40,72	
	1	66,93	
	2	99,14	
Vitamin C	0,1	7,54	1,90 ± 0,04
	0,5	13,45	
	1	27,53	
	2	51,63	
	3	96,58	
Waltheria ovata	0,1	23,21	0,48 ± 0,02
	0,5	52,85	
	1	70,04	
	2	98,96	
	3	99,56	



	0,1 ug/mL	0,5 ug/mL	1 ug/mL
Waltheria ovata	23,21	52,85	70,04
Vitamin C	7,54	13,45	27,53
TROLOX	21,57	40,72	66,93

Figure 25. Comparison of the antioxidant activity by the DPPH radical uptake of the ethanolic root extract of Waltheria ovata Cav.