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Redox properties of a standardized extract of *Chenopodium quinoa*Willdfruit pericarp modify rat liver GST activities

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Abstract

Research of antioxidant properties of herbal thiol compounds is scarce. The main non-enzymatic antioxidant compounds in animal cell are GSH and cysteine. Therefore, in this work, we studied the redox effects of a *Chenopodium quinoa* Willd (Quinoa) coats extract previously titrated in its polyphenol and thiol compounds. The effects of quinoa extract on rat liver cytosolic and microsomal GSH-transferase was tested. The catalytic active form of this enzyme is its disulphide dimer, and then its reduction provokes its inactivation. Quinoa extract inhibited both enzymatic activities in a concentration-dependent manner. The reducing power of this extract was significantly higher than *N*-acetyl-cysteine and dithiothreitol. Low concentrations of quinoa extract (without surfactant properties) decrease the apparent V_{max} of both cytosolic and microsomal GST, increase their apparent GSH K_m and not modify their apparent K_m for substrate 1-chloro-2,4-dinitrobenzene. Redox effects of Quinoa extract would be the main cause involved in the inhibition of GSH-transferase activities. Moreover, thiol compounds present in this extract and not polyphenols seem to be the most important reducing agents acting on disulphide bond of GST active dimer. New pharmacological experiments are being carried out in order to evaluate the redox importance of thiol compounds present in this Quinoa extract.

Keywords: Quinoa; GST; Thiol reducing compounds; Herbal thiols.

Introduction

All living organisms have antioxidant mechanisms mainly based on thiol compounds in order to prevent oxidative damage [1]. Un like animals, plants are constantly exposed to solar UV radiation; therefore, they require an antioxidant capacity higher than that of animals. Mechanisms through thiol and polyphenolic compounds develop their redox activity include their ability to trap oxygen free radicals and to chelate transition metals in their free ionic state[2], [3].This latter mechanism prevents the generation of oxygen free radical through Haber-Weiss and/or Fenton reactions[4], [5].Nevertheless, there are differences between the antioxidant properties of thiol and polyphenolic compounds. The most important difference relates to the ability to reverse thiol oxidation by thiol antioxidants, preserving the thiol cellular reducing equivalents[1]. In all cell types, this reversibility is the result of concerted enzymatic mechanisms that involve the use of the tripeptide glutathione (GSH), the most abundant non-enzymatic antioxidant in the animal cell [1].

A complementary hypothesis has been proposed to explain the mechanisms involved in oxidative stress. It postulates that this phenomenon can also occur without the presence of reactive oxygen species (ROS) [6]. Thus, besides participating in redox reactions, polyphenol and thiol compounds may act as nucleophilic agents [1], [3]. As such, they form covalent adducts with electrophilic metabolites, which are highly reactive and toxic compounds for cell. These electrophilic compounds can be generated as products of xenobiotic biotransformation, including drugs and components of environmental pollutants [7].When thiol and polyphenolic compounds react as nucleophiles forming adducts, they are consumed irreversibly, causing a decrease in cellular antioxidant capacity [3]. In this regard, thiol compounds appear to be more efficient nucleophilic agents than polyphenols. In animal cells, the formation of S-adducts between GSH and electrophilic compounds is catalysed by Glutathione S-transferase (GST), but it can also occur non-enzymatically[8]. Therefore, the role of GSTs is critical in eliminating highly toxic electrophilic compounds from cells by initiating the pathway leading to their excretion as mercapturic acid derivatives [1].

GST is represented by a family of iso-enzymes widely distributed in the body[1]. This family of iso-enzymes is localized in different organs and subcellular organelles[9]. It has been shown that the active form of GST corresponds to a disulphide dimer; the monomeric reduced forms of cytosolic and microsomal GST are fully inactive[10]. Since cytosolic GST isoforms contain 3-4 cysteine residues, only one specific disulphide linked dimer is the active form, while non-specific dimers are inactive [11]. On the other hand, the microsomal GST isoform contains only one cysteine residue and thus, the only possible disulphide-linked dimer is the active form of the enzyme [12]. Consequently, when liver cytosol and microsomes are exposed to oxidizing agents, the GST activity of the GST isoforms present in these cellular organelles corresponds to the type and quantity of formed dimers. Hence, it is wide spread that cytosolic GST activity is decreased by ROS since more non-specific than specific disulphide-linked dimers can be formed through oxidation; instead of this, microsomal GST activity is increased by ROS since through oxidation only the active dimer can be formed [12]. High concentrations of ROS however, decrease microsomal GST activity by altering the conformation of the microsomal membrane through lipid peroxidation [13]. In addition, both cytosolic and microsomal GST activities are decreased by copper ions due to their chelation by GST cysteine residues, which presumably hinder formation of any dimeric form of this enzyme[14], [15].

Abundant data exist in the literature regarding antioxidant capacity of herbal polyphenols. There are few studies on the composition of sulphur compounds and their antioxidant properties, especially regarding thiol compounds in herbal preparations. The characteristics of the GST iso forms led us to use the activity of these iso-enzymes as a model to study the redox activity of Quinoa fruit pericarp extract. The presence of thiol compounds, in addition to polyphenols, in this herbal extract could prevent (and possibly reverse) redox modifications in the cysteine residues occurring in GST isoforms with consequent changes in their enzymatic activities.

Herbal thiol compounds. Extensive research has been developed regarding the antioxidant mechanisms of polyphenols compounds from plant sources [16], but not for the antioxidant action of herbal thiol compounds. Plants, like other aerobic organisms, require oxygen for efficient energy production. To accomplish this, photorespiration is performed in plant cells. In this process, $NADP^+$ is regenerated through the electron transport chain, in order to reduce $O_2$to H_2O, necessary conditions for CO_2 fixation. In the same way as in animal organisms, photorespiration also generates oxygen free radicals and H_2O_2; the latter can also be formed during the catabolism of lipids as a by-product ofβ-oxidation of fatty acids. A stressful environment for the plant, such as drought, salt stress, ozone and high or low temperatures, reduce the defences, including the antioxidant capacity [17].Therefore, it is reasonable to expect that plants growing in high stress environments should have greater antioxidant capacity than those growing in wet meadows and low solar radiation. One example is *Chenopodium quinoa* Willd (Quinoa), dicotyledonous plant belonging to the family Chenopodiaceae growing in the region of the Andes between 2500to 4000 meters of altitude [18].

The grain of the Quinoa is one of the main foods used by the natives of the Andes region. The grain of Quinoa contains several vitamins in low concentrations, e.g. folic acid, thiamine, ascorbic acid and riboflavin. It also contains minerals like calcium, iron, zinc, copper and manganese. The Quinoa seed, especially the embryo has a high oil content, among which are palmitic, oleic and linoleic acids. It also has endogenous antioxidants such asα- and γ-tocopherol and a high content of protein, starch and free amino acids such as lysine, histidine and tryptophan[18]. On the other hand, the tegmen of Quinoagrain have variable contents of saponins ranging from0.01 to 4.65% of its dry weight[19]. These saponins are glycosides of triterpenoid type characterized by its bitter taste, ability to form foam in aqueous solutions and its haemolytic power. This property gives a possible surfactant activity, which is consistent with studies showing that the extract of Quinoa fruit pericarp promotes haemolysis [20] and changes in intestinal permeability [21].

Recent research from our laboratory showed that hydro-alcoholic extracts of seed coat of Quinoa grain contained thiol and polyphenolic compounds, which would be responsible for the antioxidant activity observed [22].Given the importance of maintaining the homeostasis of thiol circuits and the scarce information that exist about the reducing power of herbal thiol compounds, we were interested in evaluating the redox capacity of a hydro-alcoholic extract of episperm of Quinoa on the GST activity.

Material and Methods

Chemicals

The extract from the coat of *C. quinoa* Willd seeds (30% ethanol: H_2O) was kindly donated by Laboratorios Ximena Polanco (Santiago, Chile); aliquots of a freshly prepared extract were stored at -20°C for up to 3 months. BSA (Fraction IV), Folin Ciocalteau's reagent, 5,5'-dithiobis(2-nitrobenzoicacid) (DTNB), 1-chloro-2,4-dinitrobenzene, and glutathione (GSH) were purchased at Sigma Aldrich. All reagents were of the best available quality (*p.a.*).

Animals

Adult male Sprague Dawley rats (200-250g), maintained at the vivarium of the Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago-Chile were used. Rats were allowed free access to pellet food, maintained with controlled temperature (22±1°C) and photoperiod (lights on from 7:00 to 19:00h). All procedures were performed using protocols approved

by the Institutional Ethical Committee of the Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, and according to the guidelines of the Guide for the Care and Use of Laboratory Animals (NRC, USA).

Liver cytosolic and microsomal fraction

Microsomal and cytosolic fractions were prepared from rat livers as previously reported[23]. Samples were stored at -80°C until use. Total microsomal protein was determined according to Lowry et al.[24], using BSA as standard.

Assay of GST activity

All GST activity assays were performed, as described previously[13], under conditions of linear dependence with incubation time and protein concentration. Cytosolic or microsomal protein (10 or 100µg, respectively) was used to record continuously the GST activity for 3min, after incubation with or without Quinoa extract (15min at 25°C with constant agitation). Kinetic parameters Km and Vmax for GST activity were calculated from Lineweaver-Burk analysis, using increasing concentrations of GSH (0.05-4.0mM) or 1-chloro-2,4-dinitrobenzene (0.1-1.0mM). Also, inhibitory constants for the Quinoa extract were determined by re-plotting data using Dixon and Cornish-Bowden analyses.

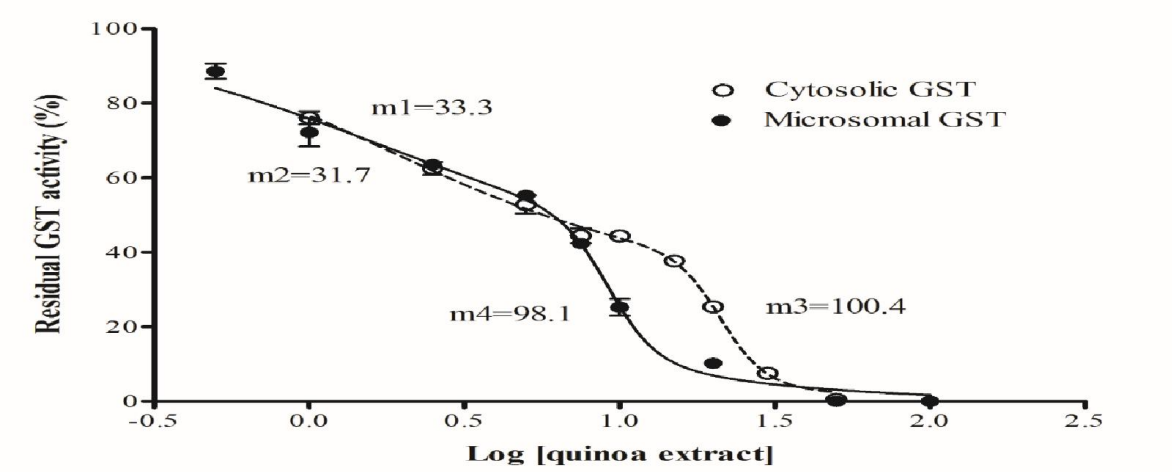
Statistical analyses

Analyses of the significance of the differences in means were performed using t-Student test. Data were considered significantly different when $p<0.05$. All statistical analyses were performed using GraphPad Prism, version 5.0.

Results

Effect of Quinoa fruit pericarp extract on microsomal and cytosolic GST activities of rat liver

Figure 1 shows the semi-log plot of Quinoa extract concentration versus the microsomal and cytosolic GST activities. Quinoa extract inhibited both enzymatic activities in a bimodal form. The slope values of the first straight lines m1 and m2 (33.3 and 31.7 for cytosolic and microsomal GST activity, respectively) obtained with low concentrations of Quinoa extract, were no significantly different, $p>0.05$ (Figure 1). By increasing the concentration of the extract on 5µL, an abrupt change in slopes was observed. This change was more abrupt for microsomal than cytosolic GST activity; the values of the slopes of the straight lines, m2 and m3however, were similar, 100.4 and 98.1 for cytosolic and microsomal GST activity, respectively (Figure 1).



For better compare the inhibitory effects of the Quinoa extract on GST activities, first and second IC50 values were obtained from two-phase non-linear regression of the data (Table 1). The inhibitor effects obtained to low concentrations of Quinoa extract on cytosolic and microsomal GST activities were similar as demonstrated by their IC50 values 1.81 and 1.73µL of extract/mg of protein ($p>0.05$).Second IC50 values obtained from Quinoa concentrations higher than 5µL however, were significantly different 21.2 and 9.2µL of Quinoa extract/mg of protein for cytosolic and microsomal activity, respectively ($p<0.05$).

Kinetic Parameters of GST In the presence of Quinoa extract

The bimodal inhibition observed in hepatic GST activities seems to be related with redox processes provoked by the reducing power of

Quinoa extract. This extract could reduce the active dimeric form of the GST and consequently, its active conformation would be altered. On the other hand, the surfactant effect on saponins present in this extract may change the microsomal membrane fluidity, thus altering its GST activity. This phenomenon also could provoke conformational changes of the GST active form, which could be associated to changes in the kinetic constants of enzymes. To separate both phenomena we determined apparent Km and Vmax of the cytosolic and microsomal GST in the presence of different concentrations of the Quinoa fruit pericarp extract. With the same aim, three points from inhibition curve of microsomal and cytosolic GST were chosen. These points were 2.5, 10 and 20μL of extract for microsomal GST assays and 2.5, 20 and 30μL of extract for cytosolic GST. Kinetic constants were calculated for each point using Lineweaver-Burk graphs (Supplementary Figure 1 and 2, Table 2).

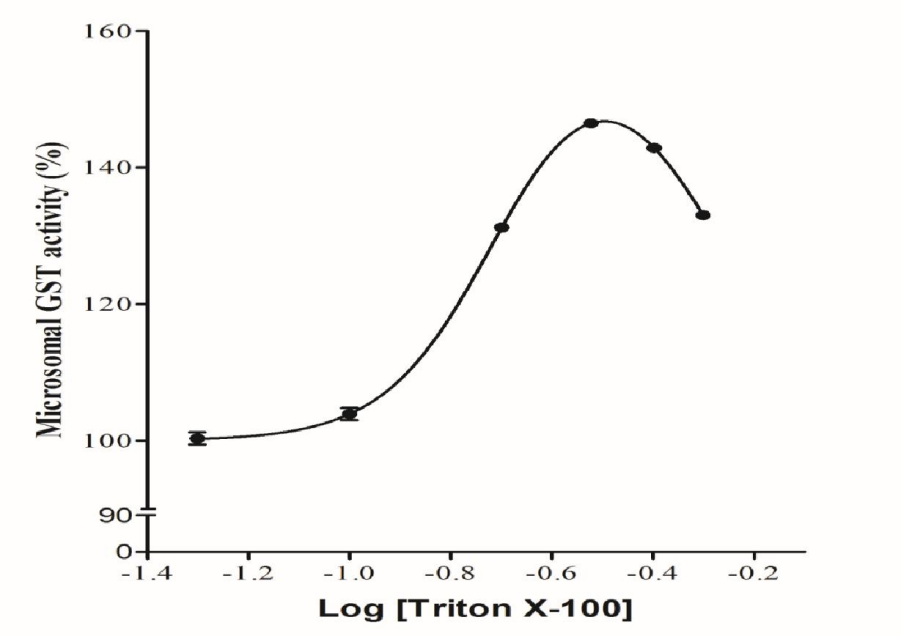


Table 1quinoa Extract Effect On Gst Activities Of Rat Liver

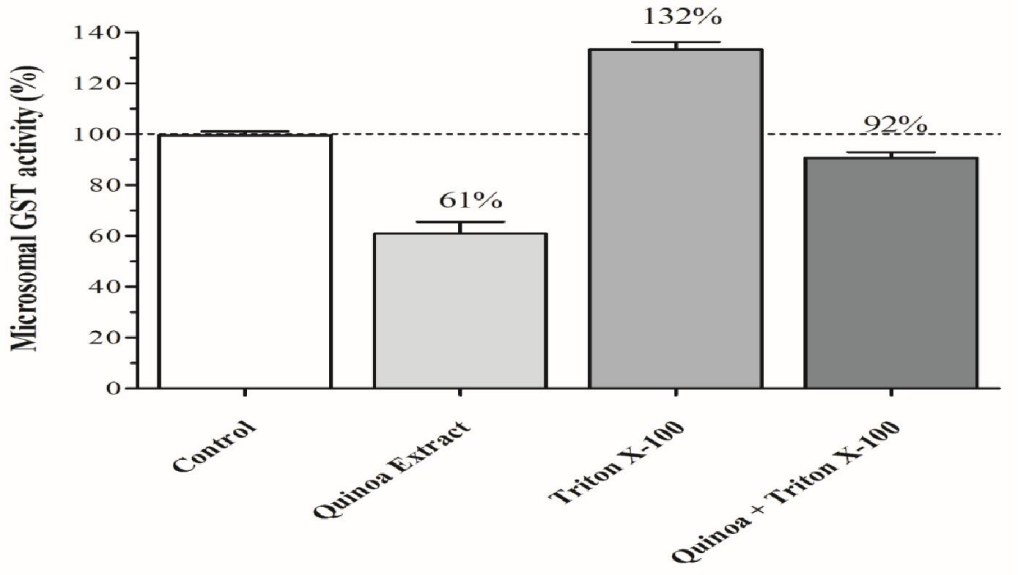
IC_{50} (μL of Quinoa extract/mg protein)	**Cytosolic GST**	**Microsomal GST**
First	1.81 ± 0.422 *	1.73 ± 0.581 *
Second	21.20 ± 0.320 **	9.20 ± 0.191 **

IC_{50} values for the inhibition of GST activity provoked by Quinoa extract were obtained from no lineal regression of data shown in Figure 1. All values correspond to the mean of at least 4 independent experiments ± SEM. **:p>0.05; **:p<0.05.*

Table 2; Effect Of Quinoa Extract On Kinetic Parameters of microsomal And Cytosolic Gst Of Liver Rat

MICROSOMAL GST				
Condition	**GSH**		**DTNB**	
	K_m (mM)	**V_{max}**	**K_m (mM)**	**V_{max}**
Control	0.46 ± 0.04	67.8 ±2.1	0.11 ± 0.01	65.4 ± 3.8
+ 2.5µL extract	0.56 ± 0.01	92.0 ± 12.5*	0.11 ± 0.03	94.4 ± 3.9*
+ 10µL extract	0.97 ± 0.04*	17.0 ± 1.4*	0.09 ± 0.01	15.0 ± 1.1*
+ 20µL extract	1.23 ± 0.03*	10.0 ± 1.1*	0.08 ± 0.01	8.0 ± 0.7*
CYTOSOLIC GST				
Condition	**GSH**		**DTNB**	
	K_m (mM)	**V_{max}**	**K_m (mM)**	**V_{max}**
Control	0.44 ± 0.01	1563 ± 82.0	0.16 ± 0.01	1468 ± 129.0
+ 2.5µL extract	0.42 ± 0.02	694 ± 8.0	0.17 ± 0.02	647 ± 86.0*
+ 20µL extract	0.46 ± 0.03	211 ± 5.0	0.20 ± 0.01	168 ± 35.0*
+ 30µL extract	0.69 ± 0.02*	100 ± 4.0*	0.16 ± 0.02	97 ± 2.0*

Saturation curves of cytosolic and microsomal GST activities were developed for GSH and DTNB in the presence of increasing volumes of Quinoa extract. Kinetic parameters were calculated using Lineweaver-Burke plots. Values of apparent V_{max} are expressed as nmol of conjugate formed/min/mg of protein. GST activity was assayed according Methods. Values represent the mean of at least 4 independent experiments ± SEM. (*) Values significantly different to respective control values, $p<0.05$.



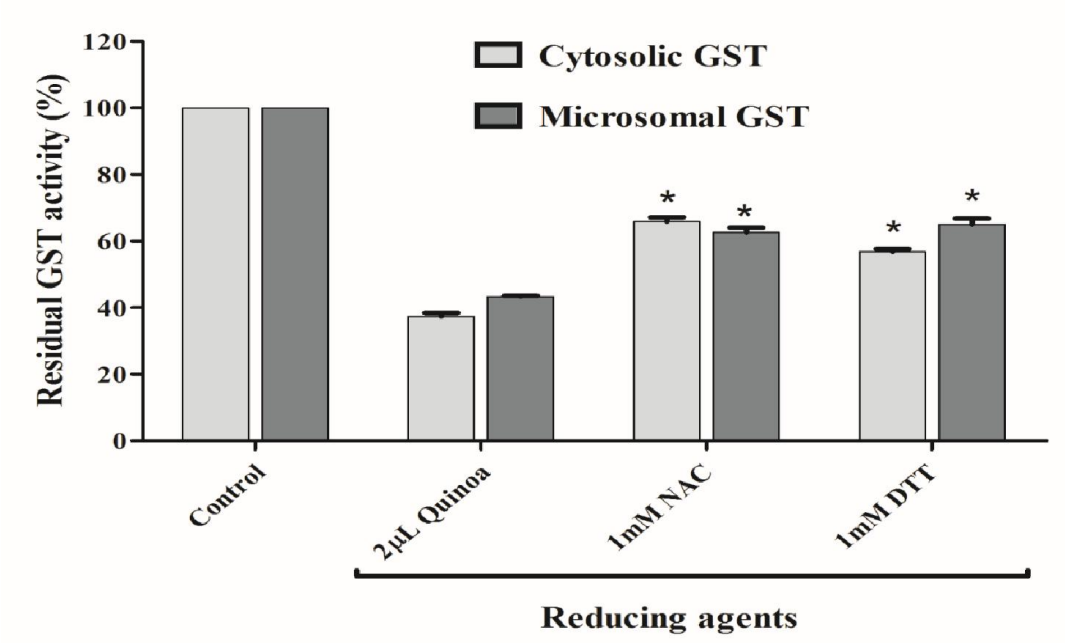
Previous data of our laboratory, showed that surfactant effect of this Quinoa extract, measured as release of red blood cell haemoglobin to the medium, was not significant until 20µL of extract/107 red blood cell [22]. Therefore, 20µL was the maximum volume of extract used to determine the kinetic parameters of microsomal GST.

Microsomal and cytosolic Vmax showed a progressive decreasing in all conditions tested probably due to reducing power of Quinoa extract. Regarding to apparent Km for DNB substrate, any of the Quinoa extract concentrations tested modified both microsomal and cytosolic apparent Km for DNB ($p>0.05$). In contrast, the control apparent GSH Km value for microsomal GST (0.46mM) gradually increased as the extract concentration was increased; the maximum value reached of this kinetic parameter was 1.23mM, which was obtained in the presence of 20µL, the maximum concentration of herbal extract tested. The control apparent GSH Km for cytosolic GST (0.44mM) however, was only increased to 0.69mM by 30µL of Quinoa extract, the highest Quinoa extract concentration tested in this case.

Triton X-100 Effect on microsomal GST activity

As a form to evaluate the surfactant effect of Quinoa extract on microsomal GST activity, the effect of Triton X-100 on this enzymatic activity was tested. Interestingly, low concentrations of this no ionic detergent provoked an increase of microsomal GST activity. The maximum increase observed (approximately 150%) was reached at Tirton-X-100 concentration of 0.3%W/V; higher concentrations of this detergent progressively decreased the microsomal GST activity, effect observed until 0.5%W/V, the highest concentration tested (Figure 2).

In this experiment the inhibitory effect of Quinoa extract, proposed as a reducing agent, was compared with N-acetyl-cysteine (NAC) and dithiothreitol (DTT). Both, NAC and DTT inhibited the activity of microsomal and cytosolic GST in similar percentage, approximately 40%. Only 2µL however, were necessary to inhibit 60% these GST activities (Figure 4).



Discussion

Herbal preparations have been proposed as a source of antioxidants in the preventive use and / or to mitigate some diseases especially those associated with oxidative stress, including aging, cancer, cardiovascular and neurodegenerative diseases[3], [16]. Polyphenolic compounds are the main herbal antioxidants studied and used both to prevent and to mitigate these pathologies. The main non-enzymatic antioxidant molecule in mammalian cells is GSH [10].Thiol compounds can protect cysteine residues in proteins through direct reduction or mediated by thio redoxins [6]. Redox homeostasis of cysteine residues is the most important for mammalian cell function [25]. Most diseases are associated with oxidative stress including neurodegenerative disorders [26], cardiovascular diseases [27], and cancer [28] are also characterized by a depletion of GSH or a decrease in GSH/GSSG ratio [29].

Data about the redox capacity of herbal thiol compounds are scarce in the literature. Previous studies from our laboratory showed that the grain seed coat extract of *C. quinoa* Willd (Quinoa), used in this study contained a polyphenol / thiol ratio equal to 4.6 (Letelier et al., 2011). Although polyphenolic compounds as thiol can act as antioxidants, these compounds differ in that the oxidation of a thiol is reversible, but oxidation of a polyphenol to quinone is very difficult in animal cell.

GSH transferase is a protein whose enzymatic activity depends on the redox state of their cysteines. That is why our interest in evaluating the ability of the grain seed coat extract of C. quinoa Willd to inhibit GSH transferase activity of rat liver. As mentioned in the introduction, the active form of the GST isoforms is its disulphide dimeric form; if this disulphide bond is reduced, the enzyme activity is lost. Our results showed that Quinoa extract was effectively able to inhibit microsomal and cytosolic GSH transferase activity of rat liver. Moreover, the semi logarithmic curve herbal extract concentration versus percentage of residual activity for both enzyme activities showed the presence of two straight lines with different slopes (Figure 1, Table 1). This could indicate that the inhibitory phenomenon observed proceed through two different mechanisms. The loss of GSH transferase activity evidenced by the first slope seems to be involved the reduction of the catalytic active dimer (Figure 1, Table 1).

This postulate is supported by analysing the values of apparent Km and apparent Vmax determined in the presence of 2.5µL/mL of the Quinoa extract, a concentration which is located approximately in the middle of the first straight (Figure 1, Table 1). In this condition the apparent Vmax of cytosolic and microsomal GST decreased approximately by half, without significant changes in the apparent Km for both GSH and the substrate 1-Cl-2,4-dinitrobenzene (Table 2). High concentrations of Quinoa extract (>10µL) caused a change in the slope of GST inhibition, thereby indicating that the mechanism involved in this case, would be different to that described above (at low concentrations of the herbal extract). This phenomenon affected in different extension to microsomal and cytosolic isoforms (Figure 1, Table 1). Since the reducing capacity of the reaction mixture increases with increasing concentration of the herbal extract, the reduction of a greater number of chemical groups of GST can be reduced, as well as other constituent proteins of the cytosol and microsomal fraction. This reductive process affects to a greater extent to the microsomal membrane. This is due to that the native conformation of this membrane is essential for expression of the catalytic activity of the microsomal GST. This statement can be supported by the observed change in the UDP-glucuronyl-transferase activity, microsomal enzyme which in the presence of detergents such as Triton X-100 is activated [30].

Interestingly, seed coat extract of Quinoa grain appears to have a high reducing power compared to N-acetyl-cysteine and DTT recognized disulphide reducing agents (Figure 6). In equal experimental conditions, cytosolic and microsomal GSH transferase activities were inhibited by 2µL of the herbal extract (18nmolequivalentsof catechin (5.2µg)and 3.88 nmol equivalents of GSH (1.19 µg)) in a significantly greater extent than that induced at concentrations of 1 mM DTT (0.163mg) and 1 mM NAC (0.154 mg). This leads us to postulate that concentrations of this Quinoa extract higher than 10µL may create a redox imbalance that affect not only the isoforms of GSH transferases, but also protein components of subcellular fractions, cytosol and microsomes. This assumption is supported by the kinetic constants determined in the presence of 10 or more microliters of Quinoa extract. Under these conditions, the apparent Vmax decreased ($p<0.05$) and the apparent Km for GSH increased ($p<0.05$) without significant changes in the apparent Km for the substrate 1-Cl-2,4-dinitrobenzene (Table 2).

The microsomal GSH Km was affected by lower concentrations of Quinoa extract than that of cytosolic GST. This difference cannot be explained by the presence of saponins in the Quinoa extract, compounds that should affect only microsomal GST activity. Detergent Triton X-100 increased the microsomal GST activity reaching 1.5 times the control GST activity at a concentration of 0.3%V/V; higher concentrations of this detergent caused a progressive decrease of this activation (Figure 2).Furthermore, the simultaneous presence of Quinoa extract and Triton X-100 in the reaction mixture, also points that the surfactant properties of Quinoa extract would not be involved in the inhibition observed; in this condition, the microsomal GST activity decreased only 8%, value corresponding to the algebraic sum of the inhibitor and activator effects provoked by Quinoa extract and Triton X-100, respectively. On the other hand, the concentration of Quinoa extract used in these experiments (20µL) do not provoke haemoglobin release of a red cell suspension, indicating the absence or very low surfactant effect of this extract[22]. The effect of triton X-100 on microsomal GST is similar to that observed for UDP-glucuronyl transferase, enzyme also localised in the liver endoplasmic reticulum [30].

Moreover, 1mM catechin (purified polyphenol) also behaved like a reducing agent but less powerful than Quinoa extract (data not shown). In this herbal extract the relationship between the concentrations of polyphenols and thiol groups was Ar-OH/R-SH=4.6. These results would indicate that herbal thiols present in Quinoa extracts could be the main responsible for the inhibition of microsomal and cytosolic GST activities observed. Because the oxidation of polyphenols to quinones is irreversible, the ability of herbal thiols to participate in redox reactions reversibly, gives them an advantage over polyphenols. Therefore, herbal extracts containing polyphenols and thiol antioxidant compounds such as Quinoa extract may be considered to develop antioxidant phyto drugs. These herbal preparations may be associated to classical therapies, especially those used in chronic diseases. Pharmacological studies however, must be previously realized to demonstrate the efficacy and security of a determined phyto drug. This is a subject of our continuing research.

Conclusions

Standardized extract of *C. quinoa* Willd fruit pericarp inhibited microsomal and cytosolic GST activities of rat liver as a concentration-response manner. This inhibitor effect was reflected in a significant decrease of the apparent Vmax and an increase of the Km for GSH. Apparent Km for the substrate 1-chloro-2,4-dinitrobenzene for both microsomal and cytosolic GSTs was not modified. The reducing effect of this extract, which causes the reduction of sulphide dimer active form of GST would explain the mechanism involved in the inhibitory effect observed. Thiol compounds but not polyphenol both present in the quinoa extract seem to be the most important reducing agent involved in the inhibitor effect on GST activity.

Abbreviations

GSH Glutathione
GST Glutathione S-transferase
ROS Reactive oxygen species

Author's contributions

MEL conceived of the study, and participated in its design and coordination and helped to draft the manuscript.
DRS participated in its design and carried out the experiments.
CGG participated in its design and helped to draft the manuscript

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