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Antioxidant Effect of a Combination of S-Acetyl-L-Glutathione, Vitamin E, *Silybum Marianum* Hepatic Cells under Oxidative Stress: An *In Vitro* Study

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Abstract:

Oxidative stress plays a key role in the pathogenesis of liver diseases and can be involved in the inflammatory process of liver cells. The aim of this *in vitro* study is to assess the antioxidant efficacy of three distinct components (fermented S-Acetyl-L-glutathione, *Silybum marianum* (L.) Gaern. and vitamin E all-rac-alpha-tocopheryl acetate) both individually and in combination (Glutasyl product). In addition, we also evaluated the combined antioxidant effect of the three ingredients on human hepatic cells subjected to oxidative stress induced by H₂O₂ treatment.

The results showed the dose-dependent antioxidant potential of the three components suggesting promising applications in medical contexts. The synergistic antioxidant effects observed for the Glutasyl was more than the individual components. Additionally, Glutasyl showed not-toxic activity on human hepatocytes and it could be considered useful in mitigating cytotoxicity induced by oxidative stress.

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INTRODUCTION

The liver performs several important functions, including nutrient metabolism, detoxification, and a role in regulating the endogenous antioxidant status [1,2].

Liver disorders are quite commonly reported in companion animals (i.e. dogs, cats) and they can be caused by different factors, including infection, exposure to toxic substances (i.e. some drugs), unhealthy diet, and obesity [3, 4].

In general, “oxidative stress” refers to a state in which the balance of oxidants/antioxidants (redox balance) is in favor of oxidants involved in the reactive oxygen species (ROS)[5, 6]. It is common when individuals are in an inflammatory state produced by different conditions such as cardiovascular and gastrointestinal diseases [7] and immune dysfunctions [8]. It has been proved that ROS can play a key role in the pathogenesis of liver disease[9,10]and can further damage cells involved in the inflammatory process[5,8].

In general, animal and human cells have various endogenous antioxidant systems to protect themselves from oxidative damage caused by free radicals and ROS [8]. Some of these systems consist of the presence of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) in the cells [8]. These exogenous antioxidants are the substances that can improve the endogenous antioxidant system and balance the cellular oxidative status by scavenging free radicals. The potentially protective role of natural antioxidants in different diseases has been documented in humans and pets [11,8].

In particular, S-Acetyl -L-glutathione, a yeast product produced by the fermentation process of *Saccharomyces cerevisiae* (fermented S-Acetyl -L-glutathione) as reported in the regulation EU 11/204 (2022)[12], is an important endogenous antioxidant found in different human cells [9,13]. It is involved in the elimination of toxic compounds that can accumulate inside cells and can help modulating the inflammatory response to fight infection and disease [9,14]. SAG is also a potential hepatoprotective agent preventing oxidative liver damage in dogs [2].

Another compound with hepatoprotective function combined with antioxidant effects is *Silybum marianum* [2, 4, 15]. *Silybum marianum*(L.) Gaern., commonly

known as Milk Thistle, contains silymarin, which is composed of the multiple flavanolignans including the most active constituent, Silybin [2,4,16]. The potential benefits of this ingredient on both human and veterinary studies has been extensively documented. This includes a wide range of positive effects, such as immunomodulation, anti-inflammatory properties, regenerative capabilities, antifibrotic actions, antioxidant activity, choleric effects, and hepatoprotective qualities [2, 4, 16-18].

Finally, Vitamin E exhibits a robust antioxidant effect, shielding cells from oxidative damage induced by free radicals [5]. By doing so, it plays a crucial role in preserving the integrity of cell membranes and interacts with the immune system, regulating its response. It is imperative to acknowledge that vitamin E supplementation holds significance in managing oxidative stress. Numerous studies, such as those conducted by Schlieck [19], indicate that vitamin E supplementation may contribute to the reduction of oxidative stress in dogs.

Two recent *in vivo* studies tested a new supplement containing S-acetyl-glutathione (SAG), silybin, and other antioxidant ingredients such as vitamin E, in healthy dogs and in dogs affected by liver disease [20,2]. The first study, showed the synergic effect of all the ingredients present in the supplement with an increase in glutathione peroxidase (Gpx) levels in healthy dogs [20]. While in the second study, it was showed an hepatoprotective effect and a significant increase in erythrocyte GSH levels in dogs with liver disease [2].

The primary objective of this study is to assess the antioxidant efficacy on hepatic cells of three distinct components—namely, fermented S-Acetyl -L-glutathione, *Silybum marianum* (L.) Gaern. and Vitamin E (all-rac-alpha-tocopheryl acetate)—both individually and in combination (Glutasyll product). In parallel, the secondary objective aims to evaluate the combined antioxidant impact of these three ingredients on human hepatic cells subjected to oxidative stress induced by H₂O₂ treatment.

MATERIAL AND METHODS

The active part of the Glutamax advance (CandioliSrl) product, defined as Glutasyll, is composed of three antioxidant ingredients and Maltodextrin at the following percentages: fermented S-Acetyl-L-glutathione 1.05%, Vitamin E 0.05%, *Silybum*

marianum 0.5%, Maltodextrin 98.4%. These are the minimal percentages which may vary depending on the dosage and weight of the single animal target species, but it is guaranteed as a minimum dosage of 28.2 mg of S-Acetyl-L-glutathione and 4 mg of Silybin every 15 kg of animal body weight. Glutasyll is used in the treatment of peptic ulcer, gastro esophageal reflux disease and symptomatic treatment of chronic and acute heartburn. In this study, each of the ingredient, first individually and then the whole Glutasyll product were tested for their antioxidant proprieties using a standard[2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) Assay (ABTS). Then, the cytoprotective effect of Glutasyll was tested on human hepatic cells under oxidative stress. Here we describe the two experiments conducted in this study.

Antioxidant Capacity of Glutasyll and its Components

The stock solution of the product fermented S-Acetyl -L-glutathione was prepared at a concentration of 100 mg/mL in distilled water; the stock solutions of both the products *Silybum marianum* (L.) Gaern. and vitamin E (all-rac-alpha-tocopheryl acetate) were prepared in Dimethyl Sulfoxide (DMSO) at the highest possible concentration: 5 and 1 mg/mL, respectively.

For each of the three ingredients, colorimetric reactions were carried out in a final volume of 200 μ L which included sodium acetate buffer (50 mM) at pH 4.5, ABTS(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) at 1.5 mM; HRP (Horseradish Peroxidase) at 2.5 μ M and H₂O₂ at 10 μ M. The products were tested at the following final concentrations (mg/mL): fermented S-Acetyl -L-glutathione = 10 - 5 - 2.5 - 1.25 - 0.625; *Silybum marianum* (L.) Gaern. = 0.5 - 0.25 - 0.125 - 0.063 - 0.031; Vitamin E(all-rac-alpha-tocopheryl acetate) = 0.1 - 0.05 - 0.025 - 0.013 - 0.0063.

Samples of five different concentrations were added first to a mixture containing the sodium acetate buffer, ABTS and HRP, in the wells of a 96 multi well plate, and the initial absorbance was measured at 415 nm. Then, at the end of the first reading, the solution containing H₂O₂ was added to each well, and the plate was incubated at room temperature, in the absence of light, for 5 minutes. At the end of the incubation period, the absorbance at 415 nm was read for each sample tested. The difference between the final and the initial absorbance is considered as inversely proportional to the antioxidant power of the compound used.

The antioxidant capacity of the products under test was calculated on the basis of a calibration line made with increasing concentrations of the standard antioxidant compound, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). This was done to assess the Trolox equivalent antioxidant capacity (TEAC), and data are reported as nanomole equivalents of Trolox.

Antioxidant Effect on Hepatic Cells under Oxidative Stress Treated with Glutasyll

Cell Lines and Culture Conditions

This experiment was conducted on a line of human liver cells, specifically hepatocellular carcinoma cells (Hep G2). The cells were cultured at 37 °C, 5% CO₂ in Minimum Essential Medium (MEM) supplemented with 1% L-Glutamine and 10% fetal bovine serum (FBS).

Preparation of the Tested Product

Glutasyll, was dissolved in DMSO at a concentration of 2.4 mg/ml, and subsequently diluted in culture medium for cell treatments. For the first test (cytotoxicity test), 10 scalar concentrations of the same product were used (μ g/ml): 24 - 12 - 6 - 3 - 1.5 - 0.75 - 0.375 - 0.19 - 0.095 - 0.05. Dilutions were selected based on a theoretical prediction of blood concentrations after oral administration at the therapeutic dose.

Cytotoxicity Test

In order to identify the maximum non-toxic dose suitable for conducting the bioactivity test, the following experiment was conducted.

The first day, Hep G2 cells were seeded in 96-well multiwell plates at a density of 10⁴ cells/well in growth medium supplemented with 10% FBS. The following day, the medium was removed and replaced with 100 μ L of complete medium supplemented with 1% FBS, and treatment with Glutasyll was performed on the cells. Specifically, cells were treated with 10 scalar concentrations of the tested product, obtained through 1:2 serial dilutions to determine the non-toxic doses. Sodium dodecyl sulfate (SDS) at a concentration of 1 mg/ml was used as a positive control (TOX).

Cells were incubated for 24 hours at 37°C and 5% CO₂. The following day, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well at a final concentration of 0.5 mg/ml, and the plate was incubated for an additional 2 hours at 37°C and 5% CO₂. After this incubation period, reduced MTT crystals were solubilized by

removing the medium and adding 100 μL of DMSO to each well. The absorbance at 595 nm for each sample was then measured using the Infinite $\text{\textcircled{R}}$ M NANO+ plate reader (Tecan). Cell activity was expressed as a percentage of the absorbance at 595 nm of untreated cells (NT).

Cytoprotection from Oxidative Stress Test

To determine the cytoprotective effects of Glutasyll on hepatic cells under stress, the following experiment was conducted.

Hep G2 cells were seeded in 96-well multi well plates at a density of 10^4 cells/well in growth medium supplemented with 10% FBS for 24 hours. Then, prior to the oxidative-stress induction, the cells were pre-incubated with three non-toxic concentrations of Glutasyll, in order to assess its protective effect. For this purpose, the medium was removed and replaced with 100 μL of complete medium supplemented with 1% FBS, and the cells were treated with Glutasyll at the concentrations selected from the previous cytotoxicity test (concentrations achieving at least 80% cell viability). Specifically, three 1:2 serial dilutions (0,024 – 0,012 – 0,006 mg/ml) of Glutasyll were selected, the cells were treated and such treatment was kept for 20 hours at 37°C and 5% CO_2 . To induce an *in vitro* model for oxidative stress, following the Glutasyll pre-incubation HepG2 cells were treated with H_2O_2 at a concentration of 15 mM for 4 hours. At the end of the 4-

hour period, the MTT reagent was added to each well at a final concentration of 0.5 mg/ml, and the plate was incubated for an additional 2 hours at 37°C and 5% CO_2 . After this incubation period, reduced MTT crystals were solubilized by removing the medium and adding 100 μL of DMSO to each well.

The absorbance at 595 nm of each sample was then measured using an Infinite $\text{\textcircled{R}}$ M NANO+ plate reader (Tecan). Cell activity was expressed as a percentage of the absorbance at 595 nm of untreated cells (NT). Data were statistically analyzed with the software Graphpad Prism 10, through one-way ANOVA followed by Dunnet's post-test (vs the oxidative stress control group, H_2O_2).

RESULTS AND DISCUSSION

Antioxidant Capacity of Glutasyll and its Components

Given the different buffer solubility of the three tested ingredients (fermented S-Acetyl -L-glutathione, *Silybum marianum* (L.) Gaern and Vitamin E (all-rac-alpha-tocopheryl acetate)), we decided to use five different concentrations, starting with the first available concentration according to the degree of solubilization of the single ingredient and performing 4 successive 1:2 scalar dilutions. According to the data shown in Figure 1 all the ingredients tested individually have a concentration-dependent antioxidant power. In fact,

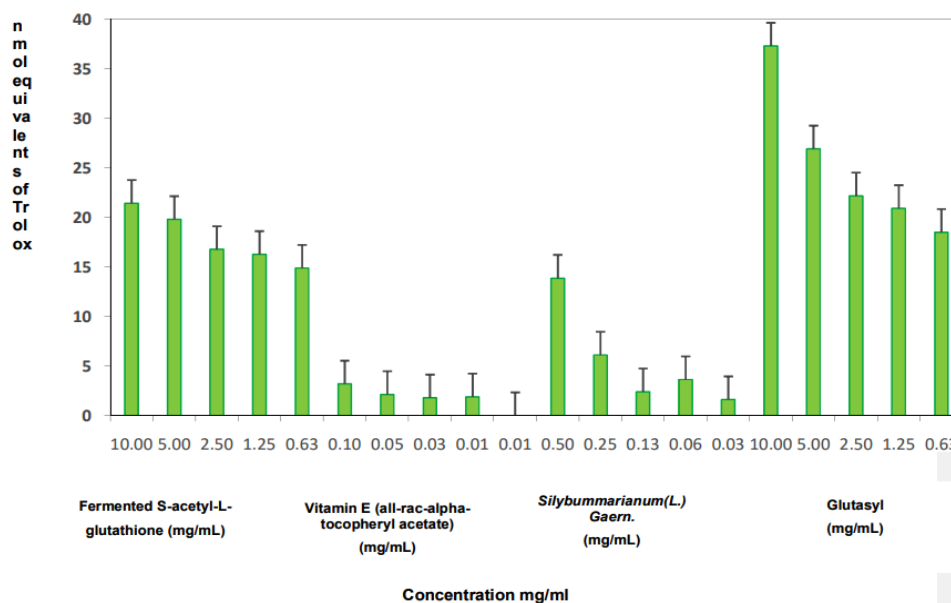


Figure 1: Trolox equivalent antioxidant capacity (TEAC) for each ingredient (fermented S-Acetyl -L-glutathione, *Silybum marianum* (L.) Gaern. and vitamin E (all-rac-alpha-tocopheryl acetate) at different concentrations tested individually and together.

decreasing the concentration of the tested product also decreases the antioxidant power.

Specifically, fermented S-Acetyl -L-glutathione being the most soluble component was tested at much higher concentrations than the other two components and showed higher antioxidant power [7,9]. Despite that, at the lowest concentration (0.625 mg/ml) its activity is comparable to the first concentration of the tested *Silybum marianum* (L.) Gaern. (0.5 mg/ml) [14].

Vitamin E(all-rac-alpha-tocopheryl acetate) appears to be the component with the lowest antioxidant activity when compared to the other two constituents tested in this experiment. However, the first available concentration of this vitamin is very low (0.1 mg/ml). Moreover, the antioxidant activity at 0.1 mg/ml of the Vitamin E(all-rac-alpha-tocopheryl acetate) is similar to the antioxidant activity at 0.13 mg/ml of *Silybum marianum*(L.) Gaern.

Finally, the graph shows a strong synergic antioxidant effect of the three ingredients when combined together at any of the five tested concentrations. This result is promising as the Glutasyll complex can be a valid support for counteracting oxidative stress when used alone or as part of a feed supplement.

The *in vitro* test data reveals that all three components exhibit antioxidant potency at the tested concentrations, with the exception of concentrations below 0.01 mg/ml, and this effect is dose-dependent. Fermented S-Acetyl -L-glutathione demonstrates superior antioxidant potency, albeit being tested at higher concentrations compared to the other two components. Conversely, Vitamin E(all-rac-alpha-tocopheryl acetate) appears to have lower antioxidant potency due to its limited solubility concentration. *Silybum marianum*(L.) Gaern. displays intermediate antioxidant potency at the tested concentrations. At 0.1 mg/ml, Vitamin E(all-rac-

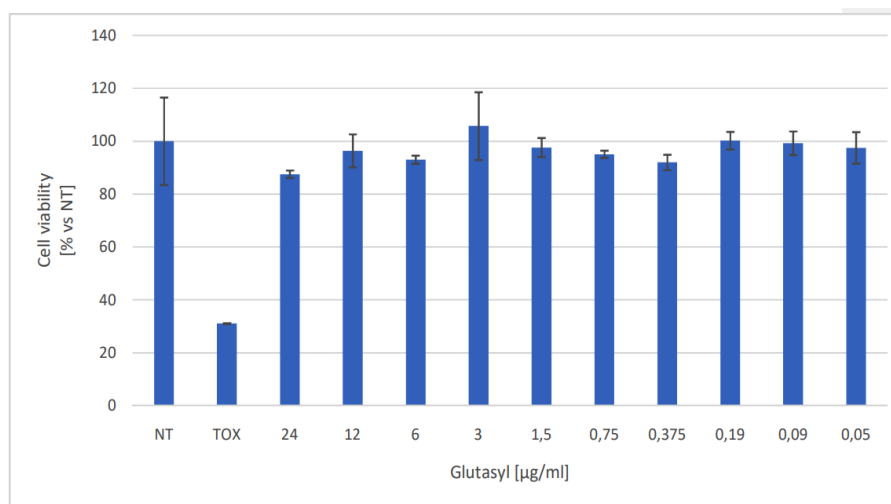


Figure 2: Cell activity test results. NT-not treated cells, TOX-1mg/mLSDS solution.

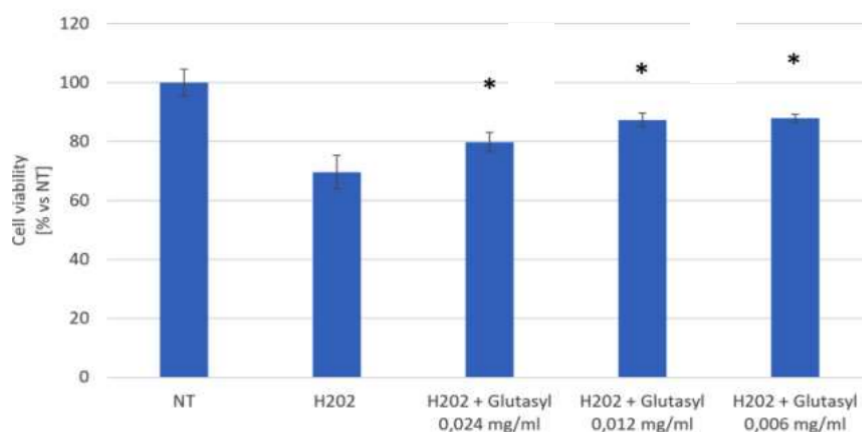


Figure 3: Cytoprotection from oxidative stress test.

Table 1: Raw data for Cytotoxicity Test

	NT	TOX	24	12	6	3	1.5	0.75	0.375	0.19	0.09	0.05
O.D.	0.602	0.211	0.589	0.624	0.814	0.632	0.643	0.602	0.602	0.668	0.641	0.620
	0.621	0.210	0.584	0.630	0.641	0.674	0.667	0.651	0.621	0.703	0.670	0.658
	0.804	0.209	0.602	0.700	0.621	0.655	0.679	0.632	0.642	0.662	0.701	0.700

Table 2: Raw data for Cytoprotection from Oxidative Stress Test

	NT	H ₂ O ₂	H ₂ O ₂ +Glutasyll 24 µg/ml	H ₂ O ₂ +Glutasyll 12 µg/ml	H ₂ O ₂ +Glutasyll 6 µg/ml
O.D.	0.679	0.538	0.532	0.626	0.617
	0.724	0.510	0.575	0.638	0.623
	0.749	0.443	0.581	0.599	0.638
	0.689	0.487	0.579	0.616	0.619

alpha-tocopheryl acetate) and *Silybum marianum*(L.) Gaern. exhibit similar antioxidant action, net of variability. Similarly, fermented S-Acetyl -L-glutathione and *Silybum marianum*(L.) Gaern. at the concentration of 0.5 mg/ml show comparable antioxidant effects. Considering the dose-dependent trends, it can be inferred that at the concentration of 0.1 mg/ml, the three tested compounds may have similar antioxidant actions. When the three ingredients were evaluated collectively in the form of Glutasyll, their antioxidant capability reached its peak at concentrations of 10 mg/ml and 5 mg/ml, surpassing the individual components. Although the antioxidant effectiveness diminished at other concentrations, the combined formulation retained the highest overall value compared to the individual components in terms of Trolox equivalent antioxidant capacity (TEAC) (Figure 1).

Antioxidant Effect of Glutasyll on Hepatic Cells under Oxidative Stress

Cell Activity Test

The cell viability test was conducted to identify non-toxic *in vitro* concentrations for use in the subsequent bioactivity test (Table 1). Hep G2 cells were treated with 10 scalar concentrations of Glutasyll, and cell activity was analyzed 24 hours post-treatment. The experiment included a negative control of untreated cells and a toxicity control (SDS 1%). The results of the test are shown in Figure 1.

Cytoprotection from Oxidative Stress Test Results

The cytoprotection test was conducted to analyze the product's ability to reverse/reduce cell death induced

by oxidative stress (Table 2). The results of the test are shown in Figure 2. As it can be observed from the graph, as expected, the treatment with H₂O₂ induced a significant reduction in cell viability, this is indicative of the induction of oxidative stress leading to suffering/death of liver cells compared to untreated cells (NT). In addition, when the cells are treated with Glutasyll, cell viability is partially rescued, and this effect is significant for every concentration tested (0,024 – 0,012 and 0,006 mg/ml; p ≤ 0,005). Thus, Glutasyll is able to alleviate the cytotoxicity induced by oxidative stress in human liver cells.

CONCLUSION

In summary, our *in vitro* study underscores the dose-dependent antioxidant potential of fermented S-Acetyl -L-glutathione, *Silybum marianum* (L.) Gaern. and Vitamin E (all-rac-alpha-tocopheryl acetate), suggesting promising applications in medical contexts. The synergistic antioxidant effects observed in Glutasyll surpass individual components, presenting a potential therapeutic avenue. Additionally, the non-toxic profile of Glutasyll on human hepatocytes presents a promising outlook for medical interventions, extending to veterinary application. This is especially noteworthy in mitigating cytotoxicity induced by oxidative stress.

CONFLICT OF INTEREST

One of the authors is employee of the Candioli Pharma S.r.l. Two of the authors are scientific consultants for the Candioli Pharma S.r.l. Candioli Pharma S.r.l is a company that may be affected by the research reported.

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