Effect of *Aloe barbadensis* Miller juice on oxidative stress biomarkers in aerobic cells using *Artemia franciscana* as a model

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Abstract

This study reports on the induction of oxidative stress in aerobic cell systems by *Aloe barbadensis* Miller (Aloe vera) juice using the salt water crustacean *Artemia franciscana* as a model. A consistent pattern was observed where *Artemia franciscana* nauplii responded to Aloe vera juice exposure with a decrease in the overall activity of redox related enzymes. Exposure of *Artemia franciscana* to sub-lethal levels of Aloe vera juice resulted in a decreased activity of thioredoxin reductase, glutathione reductase and glutathione peroxidase by 34% (66 % enzymatic activity), 79% (21 % enzymatic activity) and 90% (10 % enzymatic activity) respectively. Similarly apparent was the trend whereby the co-exposure of the nauplii to vitamin E counteracted this effect. For each of the biomarker enzymes tested, vitamin E co-exposure resulted in enzyme activities closer to the control value (78%, 56% and 32% of control enzymatic activities for thioredoxin reductase, glutathione reductase and glutathione peroxidase activity respectively). These results indicate that exposure to sub-lethal doses of Aloe vera juice induces alterations in the cellular redox status of *Artemia franciscana* and that vitamin E addition helps the *Artemia franciscana* nauplii to overcome/block the juice induced oxidative stress.

Key words: *Aloe barbadensis* Miller, Aloe vera, antioxidant, oxidative stress, vitamin E

Introduction

As a result of oxidative metabolism, all aerobic cells generate reactive oxygen species (ROS) including superoxide radical ($O_2•^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•) (McCord and Fridovich, 1978). These toxic products place the cell under oxidative stress and can negatively affect its chances of survival. For example, OH• is known to initiate lipid peroxidation which may result in the loss of membrane integrity and the generation of toxic aldehydes (Halliwell and Gutteridge, 1984). Cells have developed a variety of mechanisms to minimise the effects of ROS. These defences consist of both enzymatic and non-enzymatic mechanisms. The important antioxidant enzymes include superoxide dismutase (SOD), catalase, thioredoxin, thioredoxin reductase, glutathione peroxidase (GPx), and glutathione reductase (GR). The non-enzymatic antioxidant defences include glutathione as well as vitamins A, C and E. When the pro-oxidative forces overwhelm the antioxidant defences, oxidative stress is established.

Oxidative stress is associated with many human diseases including lung cancer, chronic inflammation, atherosclerosis and Alzheimer's disease (Halliwell and Gutteridge, 1984). Individuals with elevated dietary intakes of non-enzymatic antioxidants such as vitamins A, C and E are less likely to suffer heart and vascular disease, diabetes and some forms of cancer (Gey, 1995). However, studies into the medicinal effects of antioxidants have proved confusing, with some studies showing therapeutic effects (Tafazoli *et al*., 2005; Coşkun *et al*., 2005), whilst other studies indicate that these antioxidants may themselves be toxic (Sirdaarta and Cock, 2008; Driver and Georgeou, 2003). It has been shown in a variety of human and animal models that the effects of vitamin E and vitamin C are dose dependent with low doses

behaving as antioxidants, while high doses themselves induce toxicity through oxidative stress (Sirdaarta and Cock, 2008; Miller *et al*., 2005).

There is growing interest in the use of natural antioxidants to protect against a variety of diseases. Consumption of beverages such as tea and fruit juices, which are rich in flavanoids and flavonoids, has been associated with decreasing serum cholesterol and systolic blood pressure, thus decreasing coronary vascular disease (Pearson *et al*., 1999). Eating the seeds of *Garcinia kola*, which contains the potent antioxidant kolaviron, has been reported to have anticarcinogenic and hepatoprotective effects (Farombi *et al*., 2005). Cloves contain high levels of antioxidant phytochemicals and have been linked with prevention of lung cancer (Banerjee *et al*., 2006). Even drinking wine is linked with protection against oxidative stress through its antioxidant phytochemicals (Pearson *et al*., 1999).

Aloe barbadensis Miller has a long history as a multi-purpose remedy being used in the treatment of fever, burns and wounds, gastrointestinal disorders, diabetes, immuno-regulation, bacterial and viral diseases and in cancer treatment (Reynolds and Dweck, 1999). The use Aloe vera juice is steadily increasing around the world, not only to treat illnesses and injuries, but also by healthy individuals as an antioxidant to counteract aging. Despite the widespread use of Aloe vera juice as an over the counter drug, its toxicology remains to be systemically studied. The juice itself is known to contain toxic compounds (Avila *et al*., 1997; Winters *et al*., 1981). Indeed, recent studies have demonstrated hepatic toxicity in otherwise healthy individuals self medicating with Aloe vera juice (Kanat *et al*., 2006; Rabe *et al*., 2005). Similarly, Aloe vera administration has been associated with the induction of

Henoch-Schonlein purpura (Evangelos *et al*., 2005). Little is known about the risk of interaction/effects of Aloe vera juice ingestion with other drugs.

Many studies have reported on the antioxidant potential of extracts (Hu *et al*., 2003; Hu *et al*., 2005). These studies have demonstrated the free radical scavenging activity of Aloe vera components. Aloe emodin in particular has high inhibitory free radical scavenging activity (Yen *et al*., 2000) and can inhibit linoleic acid peroxidation (Malterud *et al*., 1993). However, other studies have also reported on the toxic effects of Aloe vera components (Avila *et al*., 1997; Winters *et al*., 1981). Tian and Hua (2005) have demonstrated that the Aloe vera components aloe emodin and aloin may act as either a pro-oxidant or an antioxidant, their action being dependent on their concentration.

Artemia franciscana was chosen as a model to examine the induction of acute oxidative stress by Aloe vera juice in aerobic cells in this study due to the high sensitivity of *Artemia franciscana* to toxins compared with vertebrate cell systems, the commercial availability of their eggs, their simple laboratory rearing and short life cycle, their lack of requirement for specialised equipment or training and the low cost of using this test model (McLaughlin *et al*., 1998; Meyer *et al*., 1982). *Artemia franciscana* are also adaptable to different conditions (eg salinity, temperature), making this model easily reproducible between laboratories. The high salinity of the *Artemia franciscana* model also lessens the possibility of microbial interference in mechanistic studies (Sung *et al*., 2007). A related organism (*Artemia parthenogenetica*) has previously been used in a similar study to determine mechanistic detail of the induction of oxidative stress by a variety of pharmaceuticals

and detergents (Nunes *et al*., 2006). Similarly, previous studies have used *Artemia salina* for toxicity (Matthews, 1995) and antioxidant (Rudneva, 1999) mechanistic studies.

In a recent study from this laboratory (Sirdaarta and Cock, 2008), we reported on the ability of *Aloe barbadensis* Miller juice to induce oxidative stress in the salt water crustacean *Artemia franciscana* and the ability of vitamin E and its analogue Trolox to counteract this oxidative stress. The aim of this study is to further examine Aloe vera juice toxicity by examining its ability to induce oxidative stress and to assess its effect on various biomarker enzymes (thioredoxin reductase, glutathione reductase and glutathione peroxidase).

Materials and Methods

Chemical Reagents

Aloe vera juice was obtained from Aloe Wellness Pty Ltd, Australia and was stored at 4 ^{o}C until use. Vitamin E (Sigma, purity > 96%) was dissolved in 60% methanol to give a 10 mg/ml stock and diluted further to 2.5 mg/ml in deionised water for testing on *Artemia franciscana*.

Stock *Artemia franciscana* cultures

Artemia franciscana Kellogg cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater

was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/l distilled water were prepared fresh prior to use. 2 g of *A. franciscana* cysts were incubated in 1 litre synthetic seawater under artificial light at 25 ^{o}C, 2000 Lux with continuous aeration. Hatching commenced within 16-18 h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 h of hatching.

Artemia franciscana exposure to oxidative stress

A franciscana nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. For each oxidative stress test, 50 ml of saline containing approximately 10,000 *A. franciscana* were removed from the stock cultures. Each was treated with the test solutions (1.6 ml Aloe vera juice resulting in a 3% concentration in assay; juice/antioxidant cotreatment consisting of 1.6 ml Aloe vera juice (3% concentration in the assay) and 2.15ml of Vitamin E (100 μg/ml concentration in the assay) respectively). The concentrations of test treatments used were all previously determined to be sub-lethal (Sirdaarta and Cock, 2008). The control (no treatment) required no additives. All tests had artificial seawater added to give a final volume of 54 ml. All *A. franciscana* tests were incubated for 24 h at 25 ^{o}C.

Protein Homogenate Preparation

Following the oxidative stress exposure period the *A. franciscana* nauplii were sacrificed, dried by rotary evaporation and resuspended in 5 ml of ice cold 0.1 M

phosphate buffer pH 7.2 containing 0.1% Triton X-100. The resulting mixture was homogenised by sonication and centrifuged at 15,000 rpm for 5 mins. The supernatant was passed through a 10 cm x 1 cm G25 Sephadex (Sigma) column. The protein containing filtrate was dried by rotary evaporation and resuspended in 5 ml of ice cold 0.1 M phosphate buffer pH 7.2. Aliquots were stored at -10^{o}C for protein estimation and enzymatic activity determinations.

Protein estimation

Protein concentrations were estimated by the Bradford (1976) protein method adapted to microplate. Bovine serum albumin (Sigma, > 96%) was diluted in deionised water and was used as a standard.

Enzyme Biomarker Activity Determination

Glutathione reductase activity

Glutathione reductase (GR) activity was determined by an adaptation of the protocol of Carlberg and Mannervik (1985). Briefly, enzyme activity was quantified spectrophotometrically by monitoring the $\Delta A_{340.}$ Enzymatic activity was expressed as Units of enzyme activity/mg protein where Units of activity are refined as µmoles of NADPH oxidised/min.

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined by an adaption of the method of Smith and Levander (2002). Enzyme activity was quantified spectrophotometrically by monitoring the ΔA_{340} as oxidised glutathione (GSSG) is reduced back to the reduced form (GSH) by glutathione reductase. Enzymatic activity was expressed as Units of enzyme activity/mg protein where enzyme activity is expressed as nanomoles of NADPH/min/mg protein.

Thioredoxin reductase activity

Thioredoxin reductase (TrxR) enzyme activity was determined by measuring the NADPH-dependant reduction of DTNB to TNB. Enzymatic activity was determined by monitoring the NADPH dependant reduction of DTNB to TNB. This reduction to TNB was then measured by monitoring the change in absorbance at 412 nm for 10 mins. Enzymatic activity was expressed as Units of enzyme activity/mg protein where Units of activity are refined as μmoles of DTNB reduced /min.

Statistical Analysis

Data are expressed as the mean ± SD of at least three independent experiments. The Paired *T*-Test was used to calculate statistical significance between control and treated groups with a P value < 0.05 considered to statistically significant.

Results

Thioredoxin Reductase Activity

Exposure of *A. franciscana* nauplii to 3% Aloe vera juice for 24 h resulted in a significant decrease in thioredoxin reductase activity as shown in figure 1. Thioredoxin reductase activity decreased from 3.8 ± 0.5 Units/mg total protein in the untreated control protein extracts to 2.5 ± 0.2 Units/mg total protein, in the *A. franciscana* nauplii exposed to Aloe vera juice. This represents an approximate 34 % decrease in enzymatic activity. This inhibitory effect of the juice could be partially counteracted by the co-exposure of juice in the presence of the antioxidant vitamin E. As shown in figure 1, the co-exposure of *Artemia* nauplii to vitamin E along with the Aloe vera juice resulted in increase in thioredoxin reductase activity, closer to the control values. Vitamin E treatment did not completely overcome the effect of Aloe vera juice. Indeed, even with co-exposure with vitamin E, the thioredoxin reductase activity is still only approximately 78 % of the control value.

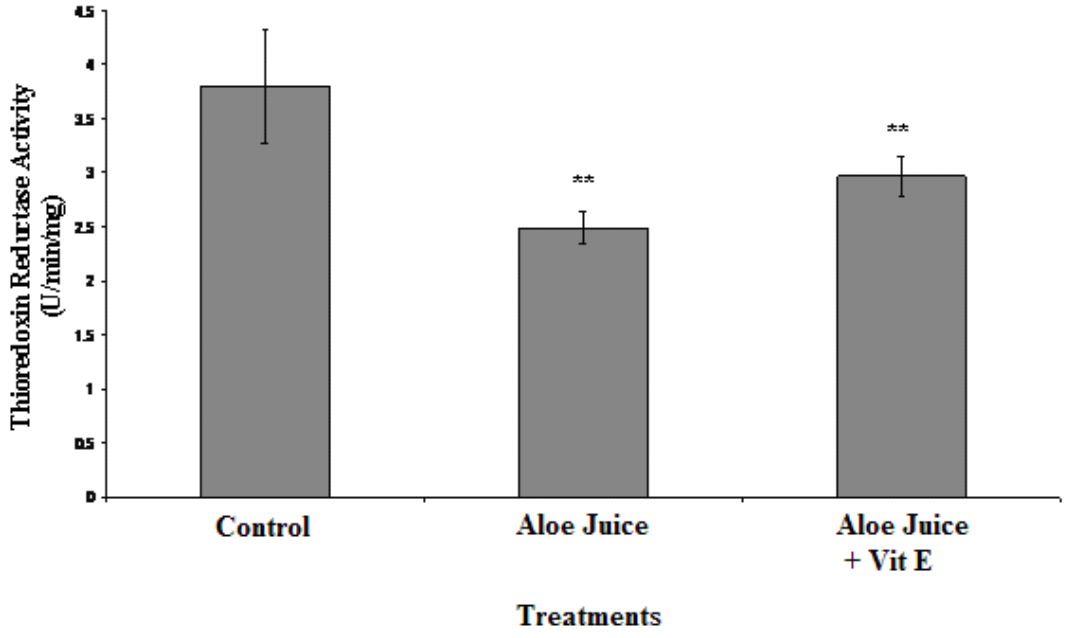

Figure 1: The effect of 24 h exposure of 3 % Aloe vera juice alone and co-exposure of 3% juice and vitamin E (100 µg/ml) for 24 h on thioredoxin reductase activity in

protein extracts from *Artemia franciscana* nauplii. Results represent the mean (± standard deviations) of triplicate assays. ** indicates a statistically significant difference to the control assay.

Glutathione Reductase Activity

Glutathione reductase activity was similarly affected by exposure to 3 % Aloe vera juice for 24 h (figure 2). Indeed, Aloe vera juice treatment resulted in a more dramatic decrease in glutathione reductase enzymatic activity than seen for thioredoxin reductase. Glutathione reductase activity decreased from 4.3 ± 0.5 Units/mg total protein in the untreated control protein extracts to 0.9 ± 0.2 Units/mg total protein. This represents an approximate 79 % decrease in enzymatic activity on exposure of the *A. franciscana* nauplii to Aloe vera juice. As with thioredoxin reductase activity, this inhibitory effect could be partially counteracted by the co-exposure of juice in the presence of the antioxidant vitamin E. The co-exposure of *A. franciscana* nauplii to vitamin E along with the Aloe vera juice resulted in an increase in glutathione reductase activity, closer to the control values (figure 2). Vitamin E treatment did not completely overcome the effect of Aloe vera juice. In extracts from *A. franciscana* co-exposed to vitamin E and Aloe vera juice, the glutathione reductase activity increases to approximately 44 % of the control value.

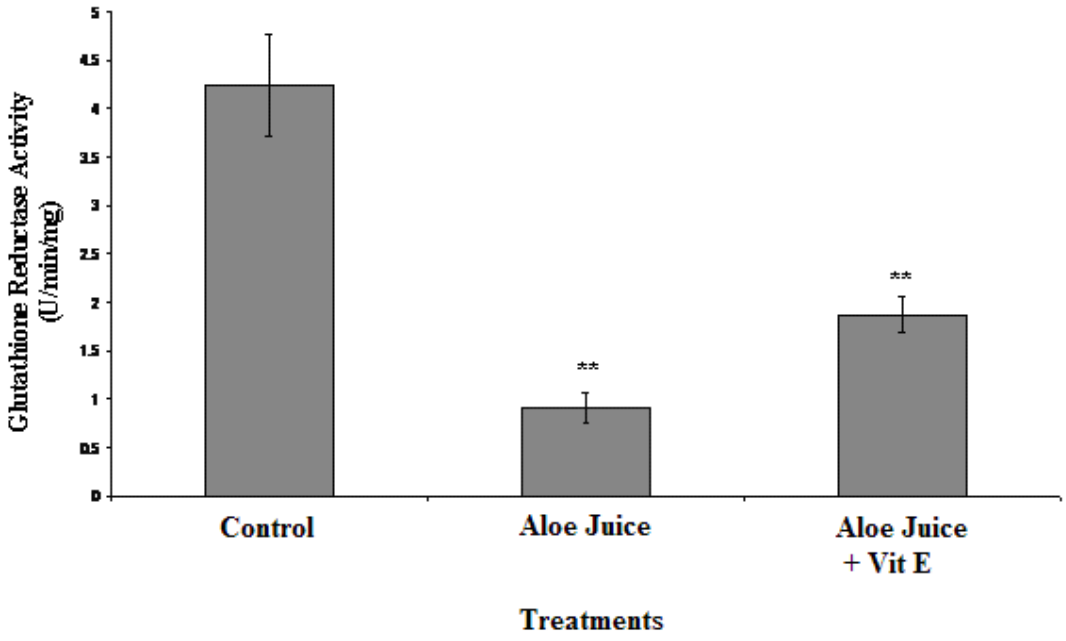

Figure 2: The effect of 24 h exposure of 3 % Aloe vera juice alone and co-exposure of 3% juice and vitamin E (100 µg/ml) for 24 h on glutathione reductase activity in protein extracts from *Artemia franciscana* nauplii. Results represent the mean (± standard deviations) of triplicate assays. ** indicates a statistically significant difference to the control assay.

Glutathione Peroxidase Activity

Exposure of *A. franciscana* nauplii to 3% Aloe vera juice for 24 h resulted in a significant decrease in glutathione peroxidase enzymatic activity as shown in figure 3. Glutathione peroxidase activity decreased from 23.4 ± 2.5 Units/mg total protein in the untreated control protein extracts to 2.3 ± 0.4 Units/mg total protein when the *A. franciscana* were exposed to 3 % Aloe vera juice. This represents an approximate 90 % decrease in enzymatic activity. This inhibitory effect of the juice could be partially counteracted by the co-exposure of juice in the presence of the antioxidant vitamin E. Co-exposure of *Artemia franciscana* nauplii to vitamin E along with the Aloe vera

juice resulted in increase in glutathione peroxidase activity, closer to the control values (figure 3). Vitamin E treatment only partially overcame the effect of Aloe vera juice. Indeed, even with co-exposure to vitamin E, the thioredoxin reductase activity is still only approximately 32% of the control value.

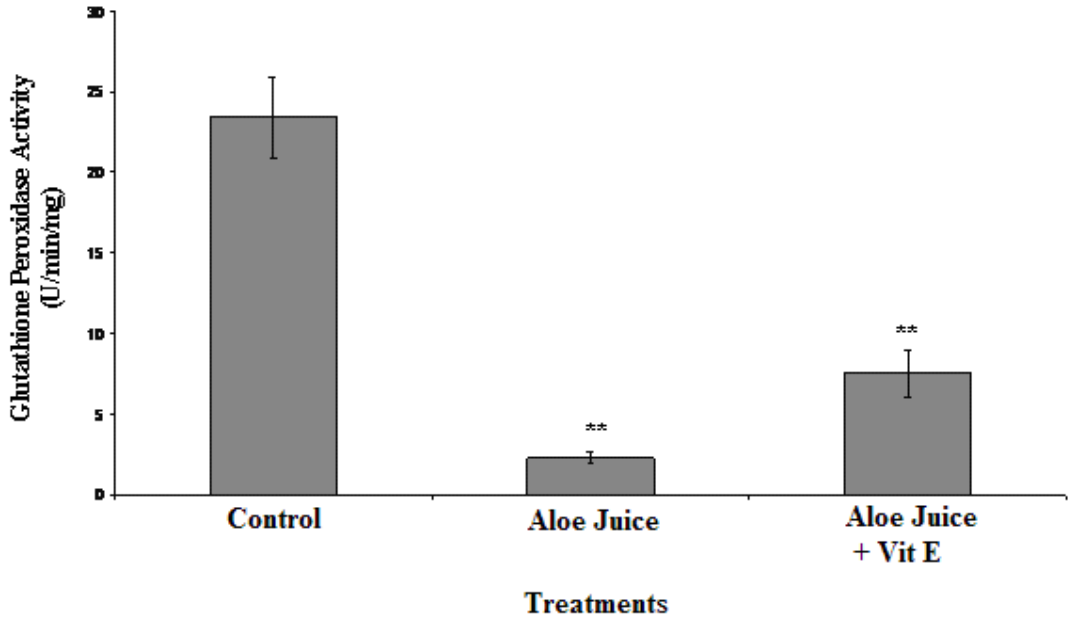

Figure 3: The effect of 24 h exposure of 3 % Aloe vera juice alone and co-exposure of 3% juice and vitamin E (100 µg/ml) for 24 h on glutathione peroxidase activity in protein extracts from *Artemia franciscana* nauplii. Results represent the mean (± standard deviations) of triplicate assays. ** indicates a statistically significant difference to the control assay.

Discussion

Organisms normally possess an array of enzymatic mechanisms devoted to assisting them to cope with oxidative stress. However, these enzymes may not be sufficient to avoid the establishment of oxidative stress when cells are exposed to toxins. Following induction of oxidative stress, damage to various cellular macromolecules

would be expected (Halliwell and Gutteridge, 1999). From a mechanistic viewpoint, examination of the levels/activities of the antioxidant enzymes can be a valuable source of information into the way a toxin works. The induction of antioxidant enzymes is dependent on a number of factors including hyperproduction of ROS and other endogenous and exogenous factors (Correia *et al*., 2003).

Previous studies in this laboratory (Sirdaarta and Cock, 2008) indicate that induction of oxidative stress may be responsible for Aloe vera juice toxicity. However, these studies did not determine the mechanistic detail. Oxidative stress induction may therefore involve the mitochondrial or non mitochondrial pathways. The work presented in this study was undertaken to further examine the mechanism of oxidative stress induction.

The current studies use the marine crustacean *Artemia franciscana* to examine the effects on antioxidant enzyme activities of Aloe vera juice exposure. Little literature exists on the antioxidant defence mechanisms in *Artemia* species. The only study we found that examines antioxidant enzymes uses a different *Artemia* species (*Artemia parthogenica*) (Nunes *et al*., 2006). The Nunes study highlights the difficulties encountered in studying *Artemia* antioxidant enzyme activities. Specifically, these authors state that antioxidant enzymatic activities in *Artemia* can be very different to activities of similar enzymes in vertebrate cells. These authors state the activities determined in their studies for thioredoxin reductase, glutathione reductase and glutathione peroxidase to be substantially lower than the corresponding activities in vertebrate cells. Indeed, one of the difficulties encountered in our studies was the relatively low enzyme activities, in the cases of thioredoxin reductase and glutathione

reductase being just above baseline levels. Conversely, Nunes *et al* report superoxide dismutase levels to be higher than those seen for vertebrate cells. Whilst these authors use a different test organism (*Artemia parthogenica* compared to *Artemia franciscana* used in our studies) it is possible a similar situation exists in *A. franciscana.* Similarly low antioxidant enzyme activities have been reported for other marine crustaceans such as *Aristeus antennatus* (Mourente and Diaz-Salvago, 1999) and freshwater crustaceans such as *Daphnia magna* (Barata *et al*., 2005).

The levels of enzyme inhibition seen when *Artemia* nauplii were exposed to Aloe vera juice (34%, 79% and 90% for thioredoxin reductase, glutathione reductase and glutathione peroxidase respectively), are indicative of a mechanism of oxidative stress induction after acute exposure of the nauplii to the toxin. These findings therefore provide further support for previous studies which indicate that Aloe vera juice acts as a toxin by inducing oxidative stress (Sirdaarta and Cock, 2008). A consistent pattern was observed where the *Artemia franciscana* responded to oxidative stress by decreasing the overall activity of redox related enzymes. Similarly apparent was the trend whereby the co-exposure of the nauplii to vitamin E counteracted this effect. For each of the biomarker enzymes tested, vitamin E co-exposure resulted in enzyme activities closer to the control value. Vitamin E addition helps *Artemia franciscana* nauplii to overcome/block the juice induced oxidative stress.

The current study used passive diffusion through the gills as the predominant entrance route for the test compounds, which would be the natural route for uptake in marine and freshwater crustaceans. However, the gills may act as selective barriers to toxin or vitamin E uptake and therefore may affect the response to their exposure. It is likely

that the uptake of the relatively insoluble vitamin E may be more dramatically affected than that of the more soluble phenolic components of the Aloe vera juice, accounting for the only partial restoration of enzymatic activity seen with vitamin E co-exposure.

In conclusion, the current studies indicate that Aloe vera juice affects antioxidant enzyme function in the *Artemia franciscana* nauplii, blocking the activity of several of the enzymes responsible for maintaining the redox state of the cell. These studies further support the mechanism of Aloe vera juice toxicity involving the establishment of a state of oxidative stress. The current study reports on the mechanism of oxidative stress induction in *Artemia franciscana*. The mechanism of oxidative stress induction in human cells may be different to the mechanism in *Artemia franciscana*. Studies using human cell lines are needed to determine if the same mechanism occurs in human cells.

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