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# Protective effect of *Artemisia douglasiana* Besser extracts in gastric mucosal injury

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## Abstract

The aim of this work was to evaluate markers of oxidative stress in ethanol-induced gastric ulcers and the protective antioxidant activity in-vivo of Artemisia douglasiana Besser extracts in ethanol-treated rats. Ethanol-induced oxidative damage is believed to be associated with generation of reactive oxygen molecules, which leads to oxidative stress. A. douglasiana is used in folk medicine as a cytoprotective agent against peptic ulcer. Different bioassays were performed: in-vivo stomach chemiluminescence, tert-butyl hydroperoxide initiated chemiluminescence (in-vitro chemiluminescence), total antioxidant capacity (TRAP) and catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in stomach homogenates. When ethanol ($3\,g\,kg^{-1}$) was administered, the in-vivo chemiluminescence increased by 107%, in-vitro chemiluminescence by 108%, SOD by 130% ($P<0.001$), and catalase and TRAP decreased by 43 and 59% ($P<0.05$ and 0.001, respectively). *A. douglasiana* ($400\,mg\,kg^{-1}$) pretreatment decreased in-vivo chemiluminescence by 41% ($P<0.05$), in-vitro chemiluminescence by 66% ($P<0.001$) and SOD by 56% ($P<0.001$) and increased catalase by 14% and TRAP by 168% ($P<0.001$, respectively) but GPx activity was not significantly different from the ethanol group. These results illustrate the significant antioxidant activity of A. douglasiana extract in-vivo and in-vitro.

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## Introduction

Gastric mucosal injury may be produced by ethanol intake. This compound can induce damage associated with generation of reactive oxygen molecules, which leads to oxidative stress with mitochondrial depolarization (Hirokawa et al 1998), DNA damage and a significant increase in xanthine oxidase activity and malondialdehyde, together with a decreased glutathione concentration (Drake et al 1998; Marotta et al 1999).

Another mechanism proposed for ethanol-induced gastric mucosal damage is that ethanol constricts the collecting venules and dilated arterioles, resulting in mucosal congestion, that leads to mucosal injury (Saeki et al 2000). Gastric lesions induced by ethanol are dose dependent and are prevented by endogenous prostaglandin ($PGE_2$), which inhibits gastric motility and increases mucus secretion (Araki et al 2000).

Gastric mucus plays an important role in protecting the mucosa against oxygen free radicals. A decrease in gastric mucus renders the mucosa more susceptible to injuries by oxidants (Seno et al 1995). Ethanol intake erodes the adherent mucus gel layer in the glandular part of stomach (Piezzi et al 1992).

Recent years have witnessed a renewed interest in plants as pharmaceuticals because the plants synthesize a variety of phenolic compounds with antioxidant activity that can play a role in protection against molecular damage induced by reactive oxygen species. Flavonoids are the major class of plant-derived chain-breaking antioxidants (Vaya et al 1997).

In Argentina, leaves of *Artemisia douglasiana* Besser, known by the vulgar name of matico, are used in folk medicine. The infusion, prepared by boiling leaves of matico, is used as a cytoprotective agent against peptic ulcer and also for the external treatment of sores and ulcers. The extract of *A. douglasiana* prevents gastric injury induced by absolute ethanol (Giordano et al 1992).

The main active cytoprotective principle of *A. douglasiana* is dehydroleucodine, a sesquiterpene lactone of the guiainolide type (Figure 1). Dehydroleucodine increases the synthesis of gastric mucosal glycoproteins (Guardia et al 1994), confirmed by histological studies in gastric and duodenal mucus (Piezzi et al 1995). It prevents the formation of the gastric mucosal lesions induced by absolute ethanol and by other necrotizing agents (Giordano et al 1990). The mechanism of the protective action of dehydroleucodine remains unknown but it seems to be related to PGE (María et al 1998).

The aim of this work was to evaluate markers of oxidative stress in an experimental model of gastric damage induced by the acute administration of ethanol in rats. The protective antioxidant activity in-vivo of *A. douglasiana* aqueous extracts and dehydroleucodine were evaluated in the stomach of ethanol-treated rats. Different bioassays were carried out: in-vivo stomach chemiluminescence, *tert*-butyl hydroperoxide initiated chemiluminescence (in-vitro chemiluminescence), total antioxidant capacity (TRAP) and catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity.

## Materials and Methods

### Chemicals

*tert*-Butyl hydroperoxide was from Aldrich Chemical Co (Milwaukee, WI) and 2,2′-azo-bis (2-amidinopropane) was from Polysciences (Warington, PA). Trolox and all the other chemicals were purchased from Sigma Chemical Co (St Louis, MO).

### Plant material

*Artemisia douglasiana* Besser was collected in El Durazno, San Luis, Argentina. The collection, classification and botanical identification of *A. douglasiana* Besser (Keck 1946) was made in May 1998, in San Luis, Argentina. A voucher specimen was deposited in the Herbarium of the Universidad Nacional de San Luis, numbered 55, 1443.

*Plant extract*

Extract was prepared following the recommendations of the CYTED (Anonymous 1995). Fifty grams of air-dried plant was soaked in 500 mL hot distilled water for 30 min. The extract was obtained by centrifugation and the supernatant was filtered through a Seiltz filter, after the mixture had been left standing for 20 min.

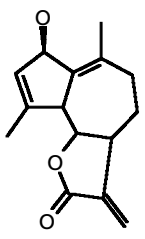

**Figure 1** Structure of dehydroleucodine (molecular weight 244).

### Extraction, purification and isolation of dehydroleucodine

Air-dried material (1.95 kg dry weight) was soaked in chloroform at room temperature (3 × 48 h). The combined extracts were coned and then dissolved in 95% ethanol and a 4% lead acetate solution added at 45 °C. After 24 h the aqueous cloudy solution was filtered through a celite pad and the filtrate coned. The mixture was then extracted three times with chloroform and the resulting solution was coned under vacuum. The gummy residue obtained (56 g) was chromatographed in a medium-pressure chromatography system. Ethyl acetate–*n*-hexane (3:7) was used as eluent. The early fractions yielded, upon rechromatography, $\alpha$-amirin, $\beta$-amirin and scopoletin, identified by comparison with authentic samples. The later fractions yielded a mixture of related sesquiterpene lactones. Rechromatography of these fractions afforded dehydroleucodine (17.32 g), ludartin (932 mg) and 1$\beta$,10$\beta$-epoxydehydroleucodine (6.5 mg). Ludartin and dehydroleucodine were identified by comparison with authentic samples.

*Dehydroleucodine*

mp 131 °C, $[\alpha]_D^{25}$: +77.0° (*c*0.89, $CHCl_3$). $^1H$ NMR ($CDCl_3$) $\delta$ 6.21 (1, *br s*, H-3), $\delta$ 3.13 (1, *d*, $J$ = 9 Hz, H-5), $\delta$ 4.01 (1, *dd*, $J$ = 9, 10 Hz, H-6), $\delta$ 2.35 (1, *ddd*, $J$ = 10, 9, 7, 1.7 Hz, H-7), $\delta$ 6.16 (1, *d*, $J$ = 2.5 Hz, H-13), $\delta$ 5.44 (1, *d*, $J$ = 2.5 Hz, H-13′), $\delta$ 2.40 (3, *s*, H-14), $\delta$ 2.27 (3, *s*, H-15).

### Experimental model for induction of gastric lesions

Fifty-five female Wistar rats, approximately 200 g, were fasted for 24 h and deprived of water for 6 h before the experiments. All rats were housed in wire-bottomed cages throughout the study to prevent coprophagy. Experiments were performed on six groups of rats. Group 1 (n = 8) received 1 mL of 0.9% (w/v) NaCl orally. Group 2 (n = 8) received 1 mL of aqueous extract of *A. douglasiana* orally. Group 3 (n = 8) received dehydroleucodine at a dose of 40 mg kg$^{-1}$. The three groups were anaesthetized 2 h after being treated. Group 4 (n = 13) was subjected to the standard gastric ulcerogenic procedure (Robert et al 1979) consisting of a single gastric administration of ethanol (3 g kg$^{-1}$). Group 5 (n = 8) received 1 mL of aqueous extract of *A. douglasiana* (400 mg kg$^{-1}$) 60 min before the oral administration of ethanol. Group 6 rats received dehydroleucodine (40 mg kg$^{-1}$) 60 min before the oral administration of ethanol. One hour later, the last three groups of rats were anaesthetized. All the experiments were performed simultaneously.

All procedures were performed in accordance with the Institutional Animal Care Committees and the European Union Guidelines for ethical treatment of animal experimentation.

### In-vivo chemiluminescence

The abdomen was opened and washed with NaCl 0.9% (w/v) to remove blood from the peritoneal cavity. The stomach was opened along an incision following the greater curvature and food remains were removed. The whole rat was covered with aluminium foil, in which a 1-$cm^2$ window was cut, allowing exposure of the stomach only. Determinations were performed with a Johnson Foundation photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA). The chemiluminescence was determined 15 min after the rats were anaesthetized with 15% (w/v) urethane (i.p., 1.5 g $kg^{-1}$). The in-vivo chemiluminescence of the stomach was expressed as the number of counts per second per square centimeter of exposed organ surface (Evelson et al 1997).

### Preparation of rat stomach homogenates

After in-vivo chemiluminescence, the rats were killed and the stomachs were removed. Rat stomachs were homogenized with 120 mM KCl and 30 mM phosphate buffer, pH 7.4. Samples were centrifuged at 600 *g* for 10 min at 4 °C. The pellet was discarded and the supernatant was used as stomach homogenate (Llesuy et al 1990).

### In-vitro chemiluminescence

The *tert*-butyl hydroperoxide in-vitro chemiluminescence of stomach homogenates was measured at 30 °C, in a Packard Tri-carb scintillation counter, 3330 model, in the out-of-coincidence mode. Rat stomach homogenates were suspended in 4 mL of 120 mM KCl and 30 mM phosphate buffer, pH 7.4. Chemiluminescence was initiated by the addition of 3 mM *tert*-butyl hydroperoxide, and maximal emission was linearly related to the protein concentration in the range 0.2–1.2 mg $mL^{-1}$. Results are expressed as counts per minute per mg of protein (Gonzalez Flecha et al 1991).

### Total relative antioxidant potential (TRAP)

Homogenate total antioxidant potential (TRAP) was measured by chemiluminescence. The addition of 10 $\mu$L of homogenate to 20 mM 2,2-azobis (2-amidinopropane) (ABAP) in phosphate buffer and 40 $\mu$M luminol in 0.1 M NaOH decreased the chemiluminescence to basal levels, for a period proportional to the amount of antioxidants present in the sample (induction time, $\delta$). The system was calibrated with Trolox (vitamin E hydrosoluble analogue). The results were expressed in $\mu$M Trolox (Lissi et al 1992).

### Activity of antioxidant enzymes

Catalase activity of the stomach homogenates was determined by measuring the decrease in absorption at 240 nm of an $H_2O_2$ solution according to the method of Chance (1954).

Superoxide dismutase activity of stomach homogenates was determined from the inhibition of the autocatalytic adrenochrome formation rate employed at 480 nm, according to the method of Misra & Fridovich (1972).

Glutathione peroxidase of stomach homogenates was assayed by measuring the oxidized glutathione-mediated oxidation of NADPH at 340 nm, according to the method of Wendel (1981).

### Protein determination

The protein concentration of stomach homogenates was measured by the method of Lowry et al (1951), employing bovine serum albumin as standard.

### Statistical analysis

Values are expressed as mean values ± standard deviations (s.d.). The statistical significance of differences between mean values was analysed by analysis of variance and Tukey test and indicated by $P$ values ($P < 0.05$).

## Results

### In-vivo chemiluminescence

Light emission from in-situ organs is related to the in-vivo steady-state concentration of reactive oxygen species. Chemiluminescence was measured 1 h after the ethanol administration, because maximal damage and lesions of the gastric mucosa were observed at that time (data not shown). Ethanol administration produced a 107% increased in light emission ($29 \pm 4$ vs $12 \pm 2$ counts $s^{-1}$ $cm^{-2}$) (Table 1).

The oral administration of *A. douglasiana* extract before ethanol administration decreased the in-vivo chemiluminescence from the stomach by 41% compared with the ethanol group ($17 \pm 1$ vs $29 \pm 4$ counts $s^{-1}$ $cm^{-2}$). Dehydroleucodine did not decrease the chemiluminescence induced by ethanol (Table 1).

### In-vitro chemiluminescence

Figure 2 shows the profile of light emission of stomach homogenates from typical examples of all the groups of rats studied. When *tert*-butyl hydroperoxide is added to rat organ homogenates there is a burst of photoemission, the kinetics depending on the type of organ (Llesuy et al 1990). In this work, stomach homogenates showed maximum chemiluminescence 22 min after the addition of 3 mM *tert*-butyl hydroperoxide for the control group and after 15 min for the ethanol-treated group. Previous treatment with dehydroleucodine before ethanol administration produced a maximal chemiluminescence at 20 min (Figure 2). When the rats were pre-treated with *A. douglasiana* extract, notably reduced *tert*-butyl hydroperoxide-initiated chemiluminescence was observed in stomach homogenates of rats 60 min after ethanol administration (Figure 2).

The maximum luminescence from stomach homogenates of ethanol-treated rats was 108% higher than control rats

**Table 1** Spontaneous organ chemiluminescence (in-vivo) and *tert*-butyl hydroperoxide-initiated chemiluminescence (in-vitro) of the different groups of rats studied.

| Group | In-vivo chemiluminescence (counts $s^{-1}$ $cm^{-2}$) | In-vitro chemiluminescence (counts $min^{-1}$ (mg protein)$^{-1}$) $\times$ $10^3$ |
|---|---|---|
| Control | 12 ± 2* | 36.5 ± 7.9* |
| *Artemisia douglasiana* | 15 ± 2* | 26.4 ± 5.1* |
| Dehydroleucodine | 14 ± 1* | 30.2 ± 7.1* |
| Ethanol | 29 ± 4 | 76.1 ± 11.2 |
| *Artemisia douglasiana* + ethanol | 17 ± 1* | 25.9 ± 5.0* |
| Dehydroleucodine + ethanol | 29 ± 2† | 67.4 ± 7.8† |

Data are means ± s.d. Ethanol was orally administered 60 min after oral treatment with *Artemisia douglasiana* extract or dehydroleucodine. *$P < 0.05$ compared with ethanol-treated group; †$P < 0.05$ compared with control group.

(($76.1 \pm 11.2$) $\times$ $10^3$ vs ($36.5 \pm 7.9$) $\times$ $10^3$ counts $min^{-1}$ (mg protein)$^{-1}$). The array decrease of the maximal intensity with *A. douglasiana* extract pre-treatment was 66% compared with the ethanol group (($25.9 \pm 5.0$) $\times$ $10^3$ vs ($76.1 \pm 11.2$) $\times$ $10^3$ counts $min^{-1}$ (mg protein)$^{-1}$). The effect of dehydroleucodine was considerably smaller than that of *A. douglasiana* extract, showing no statistical significance (Table 1).

### Total relative antioxidant potential (TRAP)

Ethanol treatment decreased TRAP levels by 59% when compared with the control group ($128 \pm 19$ vs $310 \pm 43$ $\mu$M of Trolox). Both *A. douglasiana* and dehydroleucodine treatment prevented the TRAP decrease elicited by ethanol administration, nearly reaching the level of total charge of antioxidants present in stomach homogenates of control rats. The observed protection was higher when the whole extract was employed (Table 2).

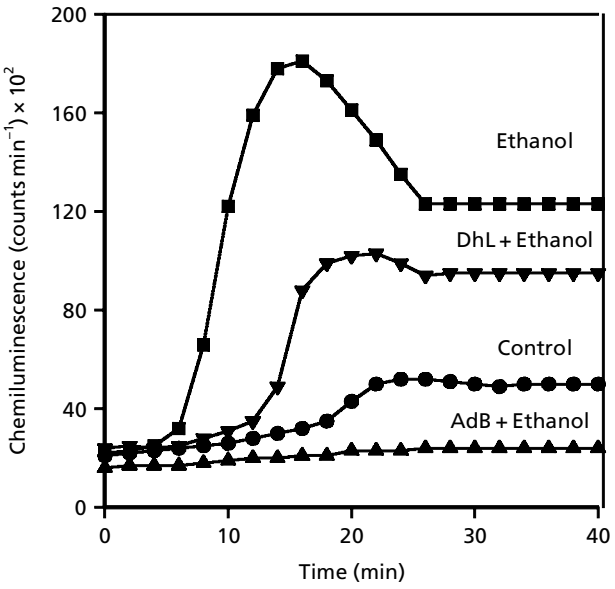


**Figure 2** The kinetic curve of *tert*-butyl hydroperoxide-initiated chemiluminescence of stomach homogenates of one rat from each group studied. Ethanol increased the light emission by 105% with respect to the control group. *Artemisia douglasiana* extract (AdB) decreased the chemiluminescence by 66% but dehydroleucodine (DhL) did not shown any change with respect to the ethanol-treated group.

### Activity of antioxidant enzymes

The activity of catalase decreased by 43% when the rats were treated with ethanol, compared with the control group ($0.40 \pm 0.10$ vs $0.70 \pm 0.01$ pmol (mg protein)$^{-1}$). *A. douglasiana* and dehydroleucodine administration did not generate any changes in catalase levels compared with the control group. When the catalase activity of *A. douglasiana*- and dehydroleucodine-pretreated groups were compared with rats that had received only ethanol, they prevented the decrease elicited by ethanol (Table 2).

The SOD levels in stomach homogenates were determined in the different groups. Ethanol administration produced a 130% increase in SOD levels compared with control rats ($1.60 \pm 0.10$ vs $0.70 \pm 0.08$ U (mg protein)$^{-1}$). This increase was totally avoided by previous administration of *A. douglasiana* extract or purified dehydroleucodine (Table 2).

Glutathione peroxidase activity did not show any significant differences between the rats treated with ethanol, *A. douglasiana* or dehydroleucodine and the control group ($15 \pm 3$ nmol $min^{-1}$ (mg protein)$^{-1}$).

## Discussion

Oxidative stress is defined as a disturbance in the pro-oxidant/antioxidant balance in favour of the pro-oxidant state (Sies et al 1985).

Acute oral ethanol administration produces a significant oxidative stress in the gastric mucosa of rats, under physiological conditions. In previous studies, Szabo et al (1984) and Mutoh et al (1990) reported that acute ethanol ingestion enhances liver lipid peroxidation. The increased rates of hydroperoxide-initiated chemiluminescence observed by Boveris et al (1985) in homogenates and mitochondria from the liver of tumour-bearing mice

**Table 2** Antioxidant enzyme activity (catalase and SOD) and total antioxidant capacity (TRAP) of the different groups of rats studied.

| Group | Catalase (pmol (mg protein)$^{-1}$) | SOD (U (mg protein)$^{-1}$) | TRAP ($\mu$M Trolox) |
|---|---|---|---|
| Control | 0.70 ± 0.01* | 0.70 ± 0.08* | 310 ± 43* |
| *Artemisia douglasiana* | 0.70 ± 0.10* | 0.70 ± 0.02* | 345 ± 21* |
| Dehydroleucodine | 0.70 ± 0.10* | 0.70 ± 0.10* | 308 ± 22* |
| Ethanol | 0.40 ± 0.10 | 1.60 ± 0.10 | 128 ± 19 |
| *Artemisia douglasiana* + ethanol | 0.70 ± 0.10* | 0.70 ± 0.02* | 343 ± 20* |
| Dehydroleucodine + ethanol | 0.60 ± 0.03* | 0.50 ± 0.03* | 259 ± 24* |

Data are means ± s.d. Ethanol was orally administered 60 min after oral treatment with *Artemisia douglasiana* extract or dehydroleucodine.
*$P < 0.05$ compared with ethanol-treated group.

could be explained by a lower level of endogenous antioxidants and free radical scavengers in the tissue.

In this study, our results show that acute administration of ethanol induces oxidative stress in the stomach and causes gastric lesions. It is the first time that light emission has been reported in a live stomach. It is an indirect assay for the in-vivo steady-state level of peroxyl radicals; the assay is non-invasive, non-destructive and specific for the organ. The stomachs treated with ethanol showed a significant increase in the in-vivo chemiluminescence. The increased photoemission reflects an increased level of oxy and peroxyl radicals that can be understood on the basis of free radical reactivity as an oxidative stress in the biological structure (Evelson et al 1997).

Aqueous extract of *A. douglasiana* and dehydroleucodine prevent the gastric injury induced by ethanol (Giordano et al 1992). Our results indicated that when the aqueous extract of *A. douglasiana* is administered together with ethanol, it inhibits in-vivo chemiluminescence. Dehydroleucodine, however, was not so effective in decreasing the light emission increased by ethanol administration.

Another result that supports the oxidative stress condition is the increase in *tert*-butyl hydroperoxide-initiated chemiluminescence in the stomachs of ethanol-treated rats. When this parameter was analysed, we observed that the *A. douglasiana* extract reduced the chemiluminescence intensity, but that administration of dehydroleucodine did not produce the same effect.

These results indicated that the oxidative stress produced by ethanol can be prevented by *A. douglasiana* extract but dehydroleucodine proved ineffective. Furthermore, it has been determined that the lipid peroxidation that occurs in the hydrophobic domains of the membranes accounts for a large part of the emission of chemiluminescence (Gonzalez Flecha et al 1991). In contrast, the major antioxidant components of *A. douglasiana* are mostly hydrophilic (Giordano et al 1990) and this might reduce their ability to reach these domains. Indeed, dehydroleucodine is a hydrophobic substance, but the antioxidant activity depends not only on its molecular structure and its redox properties, but also on its location in the membrane.

TRAP is the methodology aimed at evaluating the total antioxidant charge present in the sample and total antioxidan reactivity (TAR) reflects the efficiency as an antioxidant substance (Romay et al 1996). The procedure is based on the measurements of induction times in a system where the free radicals are produced at a constant rate, measuring the time required to deplete all active antioxidants when radicals are produced at a known rate. Addition of free radical scavengers to a solution containing ABAP-derived radical cation leads to an increase of the induction time.

Ethanol treatment reduced TRAP values by 59% when compared with the control group, showing that total antioxidant endogenous compound is consumed in stomach of ethanol-treated rats.

The data reported in a previous work (María et al 2000) indicate that the extracts of *A. douglasiana* and dehydroleucodine showed antioxidant capacity. This activity is larger in *A. douglasiana* (TRAP = 95 $\mu$M Trolox and TAR = 30 $\mu$M Trolox) than dehydroleucodine (IC50 (concentration inhibiting activity by 50%) = 15 and 117 $\mu g\,mL^{-1}$ for *A. douglasiana* and dehydroleucodine, respectively). This difference could be due to the presence of several antioxidants in the plant extract that may be responsible for the antioxidant capacity. It is known that flavonoids are present in the aqueous extract of plants (Hanasaki et al 1994; Cao et al 1997) and that the antioxidant activity depends on the *o*-dihydroxy (cathecol) structure of the B ring, the 2,3-double bond in conjugation with a 4-oxo function and the presence of both 7- and 5- and, additionally, 3-hydroxyl groups. Dehydroleucodine presents only oxy functions and double conjugate unions. As the substitution of the molecule nucleus with hydroxyl groups is essential to acquire significant antioxidant activity, it is possible that dehydroleucodine acts as an antioxidant compound, but its activity is rather inefficient (Figure 1).

When the activity of antioxidant enzymes in stomach homogenates of ethanol-treated rats was studied, we found a significant decrease in the catalase activity, indicating an oxidative stress condition. Dehydroleucodine allowed recovery of the same levels of catalase as in the control group, increasing this activity by 50% with respect to the ethanol group, but *A. douglasiana* produced an increase of 75% with respect to the ethanol group.

SOD activity showed increased values in the ethanol-treated group compared with control rats, suggesting that the oxidative stress condition could increase the levels of

$O_2^-$ or induce the activity of the enzyme (Repetto et al 1999). *A. douglasiana* extract and dehydroleucodine allowed decrease of the SOD activity, recovering the control values. SOD gene(s) are induced by diverse stress conditions and this seems to play a crucial role in environmental stress responses and tolerance (Wingsle & Karpinski 1996).

The glutathione peroxidase activity was not significantly different when ethanol-treated rats were compared with the control group. *A. douglasiana* extract and dehydroleucodine caused no change in this enzyme activity.

## Conclusions

Our data indicate that the acute administration of ethanol to rats leads to oxidative stress, a condition which is indicated by the increased chemiluminescence in-vivo, indirectly indicated by the increase in chemiluminescence in-vitro and the changes in the activity of some of the antioxidant enzymes. Ethanol may potentiate the damage associated with the increased steady-state levels of reactive oxygen species and could act by inhibiting the activity of catalase and GPx and increasing the activity of SOD.

The previous administration of *A. douglasiana* extracts produced a significant decrease in chemiluminescence, in-vivo and in-vitro, and recovered the antioxidant enzymatic and non-enzymatic protection. This effect suggests that this extract could decrease the oxidative stress condition generated by ethanol.

Dehydroleucodine proved to be less efficient as an antioxidant compound because it did not provide protection from pro-oxidant species but could recover the antioxidant activity of SOD and catalase and the total endogenous plasma antioxidant content.

The therapeutic action of the extract of *A. douglasiana* could be due, in part, to its capacity for scavenging oxygen free radicals, which may be involved in ulcer and inflammatory diseases. The different antioxidant action and protective behaviour of *A. douglasiana* and dehydroleucodine could be due to the presence of polyphenolic compounds in the plant extracts.

Dehydroleucodine increases the synthesis of gastric mucosal glycoproteins and its therapeutic function seems to be related to prostaglandin synthesis. The synergism of the cytoprotective action of dehydroleucodine and the antioxidant activity of *A. douglasiana* opens the possibility that these substances could act as active antioxidants in the in-vivo stomach, which appears important for the development of new gastric protectors.

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