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The role of nitric oxide in aloe-induced diarrhoea in the rat

Angelo A. Izzo [a], Lidia Sautebin [a], Francesca Borrelli [a], Rocco Longo [b], Francesco Capasso [a,*]

[a] *Department of Experimental Pharmacology, University of Naples 'Federico II', via D. Montesano 49, 80131 Naples, Italy*
[b] *Carlo Sessa Pharmaceutical Laboratories, viale Gramsci 212, 20099 Sesto S.G. Milan, Italy*

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Abstract

The role of nitric oxide (NO) on aloe-induced diarrhoea was studied in the rat. Nine hours after oral administration, aloe produced diarrhoea at doses of 5 g kg^{-1} (20% rats with diarrhoea) and 20 g kg^{-1} (100% of rats with diarrhoea). Lower doses of aloe (0.1 and 1 g kg^{-1}) did not produce a diarrhoeal response. Pre-treatment (i.p.) of rats with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME 2.5–25 mg kg^{-1}) reduced the diarrhoea induced by aloe (20 g kg^{-1}) 9 h after its oral administration. L-NAME (25 mg kg^{-1}) also reduced the increase in faecal water excretion produced by aloe (20 g kg^{-1}). L-arginine (1500 mg kg^{-1}, i.p.), administered to rats pre-treated with L-NAME (25 mg kg^{-1}), drastically reduced the effect of L-NAME on diarrhoea and increase in faecal water excretion induced by aloe (20 g kg^{-1}). Given alone, L-arginine did not modify aloe-induced diarrhoea. Basal Ca^{2+}-dependent NO synthase activity in the rat colon was dose-dependently inhibited by aloe (0.1–20 g kg^{-1}) and by aloin (0.1–1 g kg^{-1}), the active ingredient of aloe. These results suggest that endogenous NO modulates the diarrhoeal effect of aloe.

Keywords: Anthraquinone; Laxative; Intestine; Intestinal secretion; Colon; Nitric oxide (NO) synthase; Aloe; Aloe-emodin

1. Introduction

Aloe is the solid residue obtained by evaporating the latex which drains from the transversally cut leaves of several species of *Aloe* (Westendorf, 1993). Two distinct preparations of *Aloe* plants are most used medicinally. The leaf exudate (named aloe) is used as a laxative, and the mucilaginous gel (named aloe vera) from the leaf parenchyma is used as a remedy against a variety of skin disorders (Capasso and Gaginella, 1997). Aloe leaf exudate also possesses antidiabetic (Ghannam et al., 1986) and cardiac stimulatory activity (Yagi et al., 1982). Other preparations from *Aloe* plants are obtained from the whole leaf (total extract), used internally as a drink in a wide range of human diseases (Capasso et al., 1998), and the wood (lignaloe or aloe of the Bible), once used as an 'incense' (Tyler, 1993).

The laxative effect of aloe is due to the presence of anthranoid glycosides derivatives (mainly aloin) which are metabolized by the colonic flora to reactive aglicones anthrones (mainly aloe-emodin). Although there is no doubt that aloe exerts its action on the colonic mucosa, its mechanism of action is still unclear. It is believed that aloe (or its active ingredient aloe-emodin) acts by disturbing the equilibrium between the absorption of water from the intestinal lumen via an active sodium transport (Ishii et al., 1990) and the secretion of water into the lumen by a prostaglandin-dependent mechanism (Collier et al., 1976; Capasso et al., 1983). Platelet-activating factor (PAF) also could contribute to the laxative effect, as aloe-emodin stimulates the release of PAF in human ileal and colonic mucosa (Tavares et al., 1996).

In the past 5–6 years, it has become clear that nitric oxide (NO) is one of the mediator of the secretory effect of several laxatives, including castor oil, the diphenylmethanes bisacodyl and phenolphthalein, magnesium sulphate and anthraquinones (senna and cascara) containing laxatives (Gaginella et al., 1995; Uchida et al., 1997; Izzo et al., 1998a). Indeed NO is elevated in the colon of rats treated with laxatives and N^G-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, reduces their diarrhoeal response.

NO is a lipid soluble gas biosynthetized from the amino acid L-arginine by enzyme NO synthase (Moncada and Higgs, 1993). The isoforms of NO synthase are currently classified as constitutive and inducible. The constitutive

* Corresponding author. Tel.: +39-81-7486415; Fax: +39-81-7486403

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isoform (endothelial and neuronal NO synthase) are Ca^{2+}/calmodulin-dependent, whereas the inducible NO synthase is Ca^{2+}/calmodulin-independent and inducible by bacterial endotoxins, cytokines or following gut damage (Whittle, 1994; Izzo et al., 1998b). Among numerous and potent biological effects, NO stimulates intestinal anion secretion (MacNaughton, 1993; Wilson et al., 1993) and influences intestinal motility (Boeckxstaens et al., 1991).

In order to establish whether or not the mechanism of laxative action of aloe involves NO, we have studied the effect of L-NAME and L-arginine (the substrate for NO synthase) on aloe-induced diarrhoea in rats. In addition, we have examined NO synthase activity following aloe (or aloin) administration.

2. Materials and methods

2.1. Animals

Male Wistar (Harlan-Nossan, Corezzana, Italy) rats, weighing 180–200 g, were used after 1 week for adaptation to the housing conditions. Standard food (Mucedola, Settimo Milanese, Italy) was withheld 20 h before the experiments but there was free access to drinking water.

2.2. Diarrhoea and faecal water excretion

In preliminary experiments, the diarrhoeal dose–response to aloe (0.1–20 g kg^{-1}, p.o.) was determined. One hour after aloe treatment and each hour for 9 h, the individual rat cages were inspected (by an observer unaware of the treatment) for the presence of characteristic diarrhoeal droppings; their absence was recorded as a positive result, indicating protection from diarrhoea (Izzo et al., 1994; Izzo et al., 1996). In further experiments, the rats were treated intraperitoneally with L-NAME (2.5–25 mg kg^{-1} i.p., 15 min before and 4.5 h after aloe 20 g kg^{-1} administration) or L-arginine (1500 mg kg^{-1} i.p., 15 min before aloe 20 g kg^{-1} administration) alone or in combination with L-NAME (25 mg kg^{-1}) and the effect evaluated 9 h after aloe administration. This dose regimen was chosen on the basis of a previous work (Izzo et al., 1997). The pellets discharged during this time-period (9 h) were collected and weighed immediately and after drying for 18 h at 50° to constant weight.

2.3. Nitric oxide synthase activity

The animals were killed by asphyxiation with CO_2 9 h after oral administration of aloe (0.1–20 g kg^{-1}) or aloin (0.1–1 g kg^{-1}). Full thickness segments of the colon were homogenized at 4°C in 4 volumes of HEPES buffer 20 mM pH 7.2 containing 320 mM sucrose, 1 mM DL-dithiotreitol, 10 μg ml^{-1} soybean trypsin inhibitor, 2 μg ml^{-1} aprotinin and 10 μg ml^{-1} leupeptin. The homogenates were centrifuged at 10 000 × *g* for 30 min at 4°C. The supernatants, i.e., the cytosolic fractions containing NO synthase activity, were stored at −70°C until use. Protein concentration in the cytosolic fraction was measured spectrophotometrically using bovine serum albumin as standard (Bradford, 1976).

NO synthase activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline, according to Salter et al. (1991). Briefly, an aliquot of the cytosolic fraction (100 μg of protein) was pre-incubated for 5 min at 37°C in 50 mM potassium phosphate buffer pH 7.2 containing 60 mM L-valine, 120 μM NADPH, 1.2 mM L-citrulline, 1.2 mM $MgCl_2$ and 0.24 mM $CaCl_2$. Samples were then incubated for 10 min at 37°C with L-[U-^{14}C]arginine (150 000 dpm) and 20 μM L-arginine. The reaction was stopped by the addition of 1.0 ml of a mixture of H_2O/Dowex-50W 1:1 v/v (200–400, 8% cross-linked, H^+-form). The Na^+-form of Dowex-50W was prepared by washing four times the H^+-form of resin with 1 M NaOH and then with bi-distilled water until the pH was less than 7.5. The resin was settled by centrifugation (11 000 × g for 3 min) in a microfuge (Beckman, Microfuge 11) and an aliquot of the supernatant was taken for scintillation counting (4 ml Pico-Aqua; Packard 1500). The activity of Ca^{2+}/dependent NO synthase was determined from the difference between the [U-^{14}C]citrulline produced by control samples and samples containing 1 mM ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA); the activity of Ca^{+2}/independent enzyme was determined from the difference between the

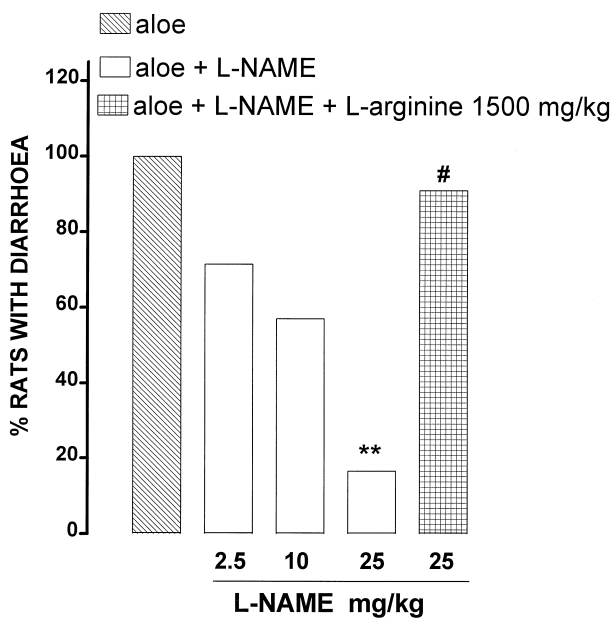

Fig. 1. Effect of graded doses of L-NAME (2.5–25 mg kg^{-1} i.p., 15 min before and 4.5 h after aloe administration) alone or in combination with L-arginine (1500 mg kg^{-1} i.p. 15 min before aloe administration) on the percentage of rats with diarrhoea 9 h after oral aloe (20 g kg^{-1}). $^{**}P < 0.01$ vs. aloe and $^{\#}P < 0.05$ vs. aloe + L-NAME 25 mg kg^{-1} ($n = 8$–12 rats).

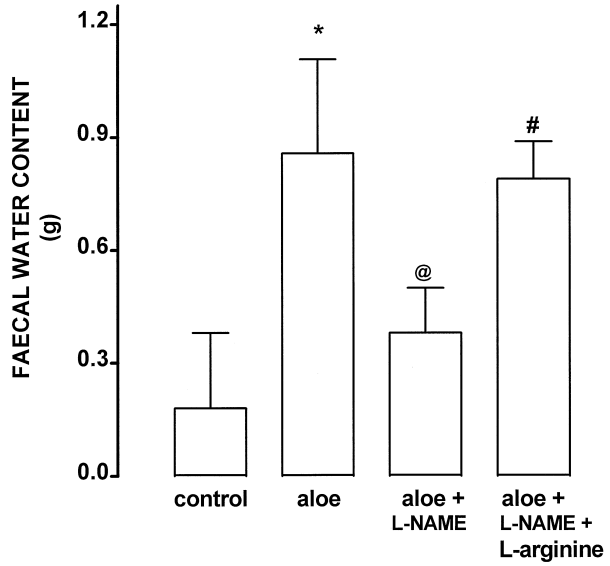

Fig. 2. Effect of L-NAME (25 mg kg^{-1} i.p., 15 min before and 4.5 h after aloe administration) alone or in combination with L-arginine (1500 mg kg^{-1} i.p. 15 min before aloe administration) on the increase in faecal water excretion induced by aloe (20 g kg^{-1}) 9 h after its oral administration. Data are mean ± S.E. mean of 10 rats. $^{*}P < 0.05$ vs. control; $^{@}P < 0.05$ vs. aloe and $^{\#}P < 0.05$ vs. aloe + L-NAME 25 mg kg^{-1}.

[U-^{14}C]citrulline produced by samples containing 1 mM EGTA and samples containing 1 mM EGTA plus 1 mM N^{G}-monomethyl-L-arginine (L-NMMA). The activity of both isoforms was expressed as nanomoles per minute per gram of tissue.

2.4. Chemicals

L-[U-^{14}C]arginine hydrochloride (specific activity 320 mCi $mmol^{-1}$) was obtained from NEN Life Science (Cinisello Balsamo, Italy). Ethylene glycol-bis (β-aminoethyl ether) *N,N,N′,N′*- tetraacetic acid (EGTA), L-arginine hydrochloride, N^{G}-nitro-L-arginine methyl ester (L-NAME) hydrochloride, N^{G}-monomethyl-L-arginine (L-NMMA) acetate and other reagents for NO synthase activity were purchased from Sigma (Milan, Italy). *Aloe ferox* (Cape aloe) water extract and aloin were obtained from Carlo Sessa Pharmaceutical Laboratories (Sesto S.G., Italy). Drugs were dissolved in distilled water. Aloe and aloin were dissolved in water.

2.5. Statistics

The Chi-square test was used to determine the significance of differences between groups with or without diarrhoea. One-way analysis of variance (ANOVA) followed by Duncan's New Multiple-Range Test and Student's *t*-test were used for NO synthase activity and faecal water excretion data, respectively. A *P* value less than 0.05 was considered significant.

3. Results

3.1. Diarrhoea and faecal water excretion

Nine hours after oral administration, aloe produced diarrhoea in rats at doses of 5 g kg^{-1} (20% rats with diarrhoea, $n = 5$) and 20 g kg^{-1} (100% rats with diarrhoea, $n = 18$). Lower doses (0.1 and 1 g kg^{-1}) did not produce a diarrhoeal response ($n = 5$ for each dose).

L-NAME (2.5–25 mg kg^{-1}) dose-dependently prevented diarrhoea induced by aloe (20 g kg^{-1}) (Fig. 1). A significant difference ($P < 0.01$) was achieved with the dose of 25 mg kg^{-1}. L-arginine (1500 mg kg^{-1}) did not modify aloe-induced diarrhoea (data not shown), but it counteracted the effect of L-NAME 25 mg kg^{-1} (Fig. 1).

Aloe (20 g kg^{-1}) also increased faecal water excretion 9 h after its administration (Fig. 2). L-NAME (25 mg kg^{-1}) strongly reduced faecal water excretion in rats treated with aloe (20 g kg^{-1}) (Fig. 2). The effect of L-NAME was counteracted by L-arginine (1500 mg kg^{-1}). L-arginine did not modify faecal water excretion in control rats (data not shown).

In control rats, L-NAME (25 mg kg^{-1} i.p., twice) did not modify significantly ($P > 0.2$) defecation [faecal wet weight (g): control 0.421 ± 0.085, L-NAME 0.389 ± 0.099, $n = 10$] and faecal water excretion [faecal water content (g): control: 0.180 ± 0.200, L-NAME 0.172 ± 0.103, $n = 10$].

3.2. Nitric oxide synthase activity

NO synthase activity, that was abolished by incubation in vitro with L-NMMA (1 mM), was detected in the

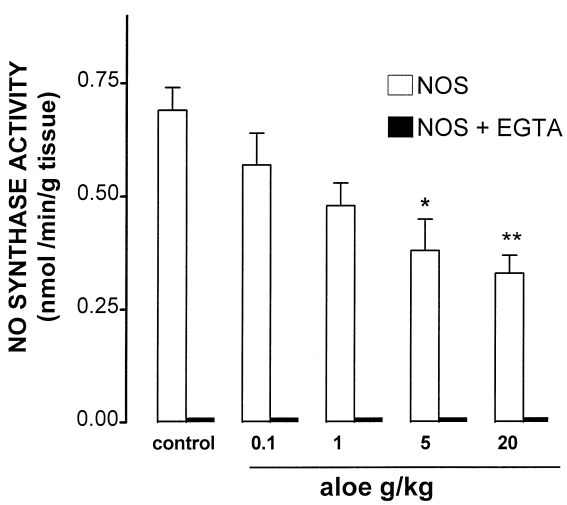

Fig. 3. Nitric oxide (NO) synthase activity in rat colonic tissue 9 h after oral administration of aloe (20 g kg^{-1}). NO synthase activity, determined as the conversion of radiolabelled L-arginine to citrulline (nmol $min\ g^{-1}$ $tissue^{-1}$), that is abolished in vitro by N^{G}-monomethyl-L-arginine (1 mM), in supernatant of colonic homogenates incubated in the absence or presence of EGTA (1 mM), is expressed as the mean values ± S.E. mean from six to eight experiments for each experimental group. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.

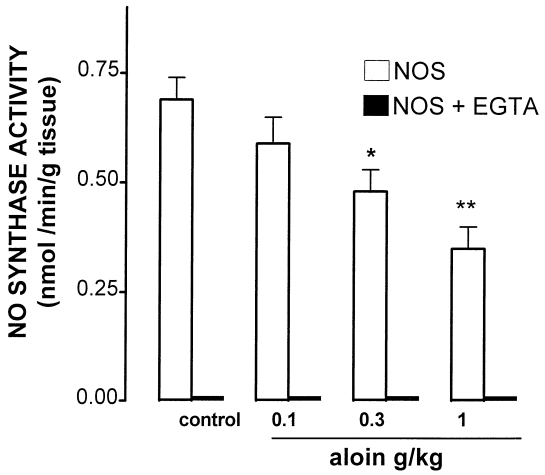

Fig. 4. Nitric oxide (NO) synthase activity in rat colonic tissue 9 h after oral administration of aloin (0.1–1 g kg^{-1}). NO synthase activity, determined as the conversion of radiolabelled L-arginine to citrulline (nmol $min\ g^{-1}$ $tissue^{-1}$), that is abolished in vitro by N^G-monomethyl-L-arginine (1 mM), in supernatant of colonic homogenates incubated in the absence or presence of EGTA (1 mM), is expressed as the mean values ± S.E. mean from six to eight experiments for each experimental group. $^{*}P < 0.01$ and $^{**}P < 0.01$ vs. control.

supernatants of homogenates of control rat colons and was 0.70 ± 0.05 nmol min^{-1} g^{-1} of tissue ($n = 15$).

At 9 h after aloe (0.1–20 g kg^{-1}) administration, a dose-dependent inhibition of NO synthase activity was observed (Fig. 3). This inhibition was significant ($P <$ 0.05–0.01) for the doses of 5 and 20 g kg^{-1}. The glycoside aloin (0.1–1 mg kg^{-1}) also dose-dependently reduced NO synthase (Fig. 4).The NO synthase activity in the supernatant from the colon of control and aloe-treated rats (as well as from aloin-treated rats, data not shown) was abolished by incubation with EGTA (1 mM) (Figs. 3 and 4).

4. Discussion

The cathartic action of aloe is well-documented. A soft stool is excreted after oral treatment with 50–200 mg aloe extract in man (Steinegger and Hansel, 1988), which is the most sensitive species. The effective dose for rats is about a hundred-fold higher (Van Os, 1976; Westendorf, 1993). In the present study aloe, administered at a dose of 20 g kg^{-1}, gave a highly reproducible response 9 h after its oral administration. We used this 9 h time period and a dose of 20 g kg^{-1} because at this time all rats exhibited evident diarrhoea.

In the present study, we have shown that the potent NO synthase inhibitor L-NAME, at doses previously reported to be effective (Izzo et al., 1994; Izzo et al., 1996), dose-dependently reduced aloe-induced diarrhoea and increase in faecal water excretion. The effect of L-NAME on both diarrhoea and faecal water excretion was prevented by L-arginine, the substrate for NO synthase, suggesting that the effect of aloe could be modulated by NO synthesis from L-arginine. It is unlikely that the action of L-NAME on aloe-induced diarrhoea results from unspecific effects of L-NAME on intestinal motility as we have shown that L-NAME did not modify defecation in control rats. Consistent with this, L-NAME did not alter gastrointestinal transit in control rats (Mascolo et al., 1993) or after skin incision or laparotomy (De Winter et al., 1997). In addition, mannitol-induced diarrhoea in the rat is not modified by L-NAME (Izzo et al., 1994). Others have shown that L-NAME increased jejunal spontaneous contractions (Calignano et al., 1992) in rats and delayed gastric emptying in dogs (Orihata and Sarna, 1994).

Several intestinal secretagogues, including endotoxin (Boughton Smith et al., 1993), bile salts (Mascolo et al., 1994) and *Clostridium difficile* (Dykhuizen et al., 1996) are able to increase NO levels in the gut. Clinical studies have shown that NO is elevated in inflammatory bowel diseases and other secretory conditions, including ulcerative colitis and Crohn's disease (Rachmilewitz et al., 1995). In addition, tackykinins (Eutamene et al., 1995), 5-hydroxytryptamine (Franks et al., 1994) and interleukin 1β (Eutamene et al., 1995), which are important mediator of secretory processes, act, at least in part, through the generation of NO. We have recently reported that the anthraquinones senna and cascara, exert their laxative effect through the liberation of nitric oxide (Izzo et al., 1996; Izzo et al., 1997). NO arises from constitutive NO synthase in the case of senna, while both constitutive NO synthase and inducible NO synthase are important in the laxative effect of cascara (Izzo et al., 1997). In the light of these results, it was expected that aloe, another anthraquinone drug, could increase NO generation in the rat colon. This assumption, that was the main purpose of the present work, was not confirmed. Indeed, our results showed that aloe produced a dose-dependent inhibition of Ca^{2+}-dependent NO synthase activity in the rat colon. Aloin, which represents about 20% of aloe crude extract, also inhibited NO synthase activity suggesting that this compound is the chemical component responsible of NO synthase inhibition observed after aloe administration. It is extremely difficult to reconcile the present results with those obtained with senna and cascara (Izzo et al., 1997). However, our data lead to suggest that the inhibition of NO synthase by aloe (or aloin) could be a mechanism to reduce the cathartic activity of aloe. In this way, aloe would limit its own ability to cause diarrhoea. It is not clear why L-arginine reverses the effect of L-NAME but not the effect of aloe. A possible explanation is that aloe (or aloin) inhibits irreversibly NO synthase in the rat colon.

The role of nitric oxide in intestinal fluid transport depends upon whether the conditions under study are physiological or pathophysiological (Gaginella et al., 1995; Izzo et al., 1998b). In physiological conditions, endogenous nitric oxide seems to be a proabsorptive molecule, based on the findings that NO synthase inhibitors produced

an increase in short-circuit current in the isolated mouse ileum (Rao et al., 1994) and reversed net fluid absorption to net secretion in the rat small intestine in vivo (Mailman, 1994; Schirgi-Degen and Beubler, 1995). However, in some pathophysiological states, NO may be produced at higher concentrations capable of evoking net secretion (Gaginella et al., 1995). Thus, NO contributes to the diarrhoeal response in trinitrobenzene sulphonic acid-induced ileitis in guinea-pigs (Miller et al., 1993) and is the mediator of the diarrhoeal response to laxatives in the rat (Gaginella et al., 1995; Uchida et al., 1997; Izzo et al., 1998a). According to this in vivo results, NO donating compounds (MacNaughton, 1993; Wilson et al., 1993) or nitric oxide itself (Tamai and Gaginella, 1993) stimulate chloride secretion in the guinea-pig and rat intestine in vitro. To our knowledge, the diarrhoea produced by aloe is the first intestinal secretory condition in which NO synthase activity is inhibited. Therefore, it is possible that NO could modulate diarrhoeal responses not only when is produced in high amounts, but also when its intestinal basal content is inhibited. Obviously our results are referred to ex vivo assays. At present, we have no evidence about the effect of aloe derivatives on NO synthase activity in vitro.

It has been reported that PAF stimulates intestinal secretion through a prostaglandin-dependent mechanism both in the rat (Buckley and Hoult, 1989; Izzo, 1996) and the human colon (Borman et al., 1998) in vitro. PAF is involved in castor oil-induced diarrhoea (Croci et al., 1997; Izzo et al., 1998a) and its intestinal content could be enhanced if NO synthase is inhibited (Caplan et al., 1994; Mascolo et al., 1996). Thus, it is possible that inhibition of NO synthase caused by aloe produces an increase in PAF synthesis which could contribute to aloe-induced secretory processes. Consistent with this hypothesis, aloe-emodin stimulates the release of PAF in the mucosa of human ileum and colon in vitro (Tavares et al., 1996).

The laxative effect of aloe also involves changes in intestinal motility, which produce an increase of intestinal transit in the colon (Capasso and Gaginella, 1997). NO is an important inhibitory neurotransmitter in the gut (Boeckxstaens et al., 1991; Whittle, 1994) and NO synthase inhibitors can affect intestinal motility in vivo (Calignano et al., 1992). Therefore, we cannot exclude the possibility that inhibition of NO synthase produced by aloe could reduce not only secretory processes, but also the increase in intestinal transit associated with aloe administration.

In summary, we suggest that inhibition of basal calcium-dependent NO synthase activity by aloe could reduce its diarrhoeal effect, based on the findings that aloe reduced basal calcium-dependent NO synthase activity and L-NAME reduced the diarrhoea and the increase in faecal water excretion induced by aloe. Aloin could be the chemical component responsible of this inhibition and it is the first secretagogue yet described to inhibit NO synthase.

Acknowledgements

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