# Antineoplastic 31-Norcycloartanones from *Solanum cernuum* Vell.

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Triterpenoids with 31-norcycloartanone structure were isolated for the first time from the *Solanum* genus. Cycloeucalenone and 24-oxo-31-norcycloartanone were the main constituents of the dichloromethane extract of *Solanum cernuum* Vell. leaves [7% (w/w) and 1.47% (w/w)]. Both triterpenoids were tested against human tumour cell lines, and 24-oxo-31-norcycloartanone was significantly active and selective against the lung tumour cell line NCI-H460 with total growth inhibition at 1.10 $\mu$g/mL, growth inhibition 50 at 0.19 $\mu$g/mL and lethal concentration 50 at 8.43 $\mu$g/mL, while cycloeucalenone showed poor activity. A homologous series of alkanes ($C_{25}$–$C_{34}$), $\beta$-sitosterol, and the xanthophyll lutein were also identified. The antiulcer activity was assayed for the dichloromethane extract. In the gastric ulcer model induced by 95% ethanol, administration of 500, 1000 and 2000 mg/kg/*po* dichloromethane extract gave ulcer lesion indices of, respectively, 38.2, 61.0 and 81.9%, while carbenoxolone inhibited 88.9% at 200 mg/kg. In the gastric ulcer model induced by indomethacin the dichloromethane extract showed a small percentage of lesion inhibition. The ethanol extract was also analyzed and was mainly composed of glycoalkaloids, peptides and disaccharides.

*Key words: Solanum cernuum* Vell., 31-Norcycloartanones, Antineoplastic Activity

## Introduction

Medicinal plants play an important role in the discovery of new drugs. Natural products are directly or indirectly responsible for almost 40% of the drugs used in modern therapeutics, and considering antibiotics and antitumour agents this number goes up to 70% (Newmann *et al.*, 2003). The world market of phytotherapeutic drugs moves near 14 billion US$ per year, and just in Germany this number reaches 3 billions US$ followed by France and Italy. These large values are justified attending the WHO data indicating that 80% of the world population consumes medicines based on plant materials. It must be also considered that the development of new synthetic drugs demands for high costs and that the ethnopharmacology is a precious guide for the selection of plant extracts and compounds.

The *Solanum* genus is used in traditional medicine by different cultures for distinct purposes. Some species of the genus are valuable due to their nutritional and mainly medicinal properties that can justify their use in the composition of phytotherapeutic medicines. On this basis we consider that the use of the Brazilian plant *Solanum cernuum* Vell. (Solanaceae) in folk medicine is an important unexplored subject. This species grows in the states of Rio de Janeiro and Minas Gerais; it is commonly known as panacéia and braço-de-preguiça, and described as sempre florida (always flowering) (Lorenzi and Matos, 2000). Infusions of the aerial parts are used in the treatment of gastric ulcers, liver injuries, skin affection, as antitumour, depurative, diuretic, and as antihaemorrhagic and antiblennorrhœa agents (Esteves-Souza *et al.*, 2002; Araujo *et al.*, 2002; Rodrigues and Carvalho, 2001). The roots are used as infusion or as decoc-

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tion in haemorrhages treatment (Rodrigues and Carvalho, 2001). The leaves are also used as infusion with tranquilizing action for cardiac problems (Lorenzi and Matos, 2000). Despite the common use, in the Brazilian Farmacopeia there is no reference to *S. cernuum* Vell. Considering the traditional use of the genus the study of *S. cernuum* Vell. is an unexplored field of chemical and pharmacological research. Having the above statements in mind our work includes chemical and biological activity studies, and their relation with genus data. The chemical constituents of the dichloromethane extract were determined, and its ability to prevent gastric ulceration as evaluated as antitumour activity of the pure compounds from the dichloromethane extract. The ethanol extract was screened for its chemical composition.

Chemical constituents of the *Solanum* genus have been described as steroidal and alkaloid glycosides (Ono *et al.*, 2006; Ikeda *et al.*, 2003; Coelho *et al.*, 1998), sterols (Zygadlo, 1994), flavonoids (Silva *et al.*, 2003) and also nitrogen-containing non-steroidal metabolites such as *N*-acyltriamines and pyrrole alkaloids (Evans and Somanabandhu, 1980; El-Sayed *et al.*, 1998). Several pharmacological and toxicological studies of these compounds proved their broad spectrum of activities such as the action against malignant and benign human skin tumours, cytotoxicity against human tumour cell lines, and as antidiabetogenic, antihepatotoxic, antineoplastic, antiviral and antioxidant agents (Cham *et al.*, 1991; Zhou *et al.*, 2006; Yoshikawa *et al.*, 2007; Lin *et al.*, 1988; Hu *et al.*, 1999; Arthan *et al.*, 2002). For *S. cernuum* Vell. species there are no specific studies on its nature as far as we know. The single study on *S. cernuum* Vell. found in the literature concerns the evaluation of the antiulcerogenic activity of the hydroalcoholic extract of the aerial parts of the plant where a significant antiulcerative activity is referred (Araujo *et al.*, 2002).

## Materials and Methods

### *Plant material*

*S. cernuum* Vell. leaves were collected at Bragança Paulista (SP, Brazil) in September 2004. A voucher specimen (VELL N° 653) is deposited at the herbarium Frei Velloso of the São Francisco University (Bragança Paulista, SP, Brazil).

### *Extraction and isolation*

The oven-dried (45 °C) and powdered leaves (600 g) were extracted three times with dichloromethane by maceration. The residue was washed three times with 95% ethanol. Both solvents were evaporated under reduced pressure to obtain the dichloromethane (40 g) and ethanol (55 g) extracts. The dichloromethane extract was chromatographed on a silica gel 60 (Merck 7734) (10% deactivated with water) column, eluted with *n*-hexane/ethyl acetate mixtures of increasing polarity (9:1 and 7:3). The fraction compositions were followed by TLC (silica gel $F_{254}$ 0.20 mm, Merck 5554). The fraction eluted with *n*-hexane yielded a series of alkanes ($C_{25}-C_{34}$) (1.22 g, 3.05%). The triterpenoid fraction was eluted with *n*-hexane/ethyl acetate (9:1) and purified by successive CC and preparative TLC (silica gel $F_{254}$ 0.5 mm, Merck 5744), eluted with *n*-hexane/ethyl acetate mixtures. The steroid fraction was composed of cycloeucalenone (2.80 g, 7.0%), 24-oxo-31-norcycloartanone (589 mg, 1.47%) and $\beta$-sitosterol (146 mg, 0.36%). The fraction eluted with *n*-hexane/ethyl acetate (7:3) was further subject to CC purifications with dichloromethane/methanol or chloroform/diethyl ether mixtures to yield lutein (380 mg, 0.95%). The ethanol extract was divided into two parts. A fraction was subject to CC on a LiChroprep RP-18 (Merck 13900) column with successive water and methanol elution. The water elution was controlled by TLC, and compounds were detected with $\alpha$-naphthol reagent specific for sugars detection. By the alditol acetates method the water fraction proved to be mainly composed of disaccharides (Blakeney *et al.*, 1983). The methanol fractions were checked for alkaloids using the Dragendorff reagent. By FT-IR and $^1$H NMR analyses the methanol fraction proved to be composed of glycoalkaloids. The second fraction of the ethanol extract was chromatographed by HPLC using an RP-18 Thermo ODS Hypersil ($250 \times 4.6$ mm) column and elution with a water/methanol gradient. Under these conditions the ethanol extract proved to be composed of a major component of peptide nature which was detected by TLC with ninhydrine reagent, together with FT-IR and NMR analyses.

### *Physical and spectroscopic measurements*

A Perkin-Elmer 241MC polarimeter was used to measure the optical specific rotation. FT-IR spectra were recorded on a Perkin Elmer Spectrum 1000 spectrometer, as KBr pellets, and UV-Vis spectra were recorded in methanol solution

on a Milton Roy-Spectronic 1201 instrument. $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra were recorded on a Bruker ARX400 NMR spectrometer. The NMR spectra were recorded in $CDCl_3$ or $CD_3OD$ and referenced by the residual solvent signals (chloroform: $\delta$ 7.26 ppm and 77.0 ppm; methanol: $\delta$ 3.30 ppm and 49.0 ppm); $\delta$ values are expressed in ppm. The GC-EI-mass spectra were taken on a Micromass GC-TOF (GCT) instrument with a DB1 column (0.32 mm i. d.; 0.25 $\mu$m film thickness; 30 m length). The GC operating conditions employed were as follows: carrier gas, He; split, 1:10; injection port temperature, 250 °C; temperature program: 120 °C held for 3 min, increased at 5 °C/min to 300 °C, held for 10 min. The mass spectra were performed by the analytical services laboratory of REQUI*M*TE. HPLC was performed on a system equipped with a Merck Hitachi L-7100 pump, Merck Hitachi L-7450A diode array detector and a LiChroCart® RP-18 column (250 × 10 mm).

*24-Oxo-31-norcycloartanone (**2**)*

White amorphous solid (589 mg). – $[\alpha]_D^{20}$ + 53.3° (*c* 0.46, $CHCl_3$). – $^{13}$C NMR: see Table I. – GC-EIMS: *m/z* (rel. int.) = 426 (38) $[M]^+$, 411 (23), 340 (83), 325 (7), 299 (100), 287 (2), 257 (11), 243 (8), 221 (5), 136 (53).

*Antiproliferative assay*

Cell lines from different histological origins were used in the present study. Human tumour cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-3 (ovary), PC-3 (prostate), HT-29 (colon), 786-O (renal) and NCI-ADR/RES (ovary expressing multiple drug resistance phenotype) were kindly provided by the National Cancer Institute (NCI, Frederick, Maryland, USA). Stock cultures were grown in a medium of 5 mL RPMI-1640 (Gibco®, NY, USA) supplemented with 5% of fetal bovine serum (Gibco®). Gentamicine (50 $\mu$g/mL) was added to the experimental cultures. DOX was the reference drug used in these antiproliferative assays.

Cells in 96-well plates (100 $\mu$L cells/well; inoculation density ranging from 4 to $7 \cdot 10^4$ cells/mL) were exposed to various concentrations of samples in RPMI/DMSO (0.25, 2.5, 25, and 250 $\mu$g/mL) at 37 °C, 5% $CO_2$ in air for 48 h. The final concentration of DMSO (Sigma Chemical Co, St Louis, MO, USA) did not affect the cell viability. Afterwards the plates were incubated with a 50% solution of TCA (Merck®, SP, Brazil) for 30 min at 4 °C for cell fixation. After washing and drying, the cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content using SRB dye (Sigma). The absorbance data was used in order to calculate the total growth inhibition (TGI), the growth inhibition 50 (GI 50) and the lethal concentration 50 (LC 50) by a sigmoidal parametric regression using Origin 7.1 software.

*Antiulcer activity*

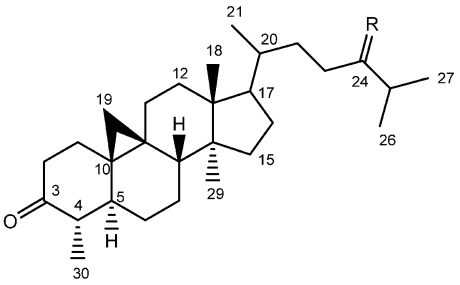
The antiulcer activity was evaluated using two different assay models for the induction of acute gastric mucosal lesions: 95% ethanol (Robert *et al.*, 1979) and indomethacin (Morimoto *et al.*, 1991). Male Wistar rats of 190–210 g weight ($n$ = 6) were used in both methods. The animals were kept, with water *ad libitum*, in proper cages, to prevent coprophagy, and fasted for 24 h. The treatments were oral administrations – *per os* (*po*). In positive control groups, animals were treated with carbenoxolone (200 mg/kg/*po*). After each experiment, the animals were sacrificed by cervical dislocation. The stomachs were removed, opened along the greater curvature and fixed between two glass plates. The lesions were measured by the Gamberini method (Gamberini *et al.*, 1991).

In the 95% ethanol-induced lesions model the animals received 95% ethanol (1 mL/kg/*po*) 1 h after the administration of the dichloromethane extract of *S. cernuum* Vell. (500, 1000, 2000 mg/kg). The animals were sacrificed 1 h after the administration of the ulcer agent for evaluation of the antiulcer activity and DE 50 determination.

In the indomethacin-induced lesions model the animals received the ulcer agent (40 mg/kg) 1 h after the administration of the dichloromethane extract of *S. cernuum* Vell. (1000 mg/kg). The animals were sacrificed 6 h after the administration of the ulcer agent for evaluation of the antiulcer activity and DE 50 determination.

## Results and Discussion

The most abundant constituents of the dichloromethane extract are triterpenes with a 31-norcycloartanone skeleton that have never been described in the *Solanum* genus. These compounds possess a C-9,19 cyclopropane unit and a methyl




R = $CH_2$ Cycloeucalenone (**1**)
R = O 24-Oxo-31-norcycloartanone (**2**)

Fig. 1. Chemical structures of compounds **1** and **2**.

group at C-4. The major compound isolated, cycloeucalenone (**1**), occurs in 7% (w/w), which represents a significant amount of the dichloromethane extract. It has a 3-oxo-$\Delta^{24(28)}$ structure and is mainly known from the fruit peel of *Musa sapientum* L. (Musaceae) (Akihisa *et al.*, 1986). It has also been obtain by oxidation of the alcohol analogue, cycloeucalenol (Cocker *et al.*, 1965). Later these authors reported the identification of its C-4 epimer (Akihisa *et al.*, 1997). Cycloeucalenone has also been described as produced from fungus source (Ondeyka *et al.*, 2005) and from *Tinospora crispa* (Menispermaceae) (Kongkathip *et al.*, 2002). In this last work **1** was tested as cardiac contractile agent. It showed slight change from the control on the right and left atrial force which means that it induces mild cardiotonic effects. So it can be considered that this metabolite could contribute to the known tranquilizing action of *S. cernuum* Vell. during cardiac problems. **1** was identified by spectroscopic studies and by comparison with published data (Dahmén and Leander, 1978). The value of specific optical rotation was important to assure the stereochemistry of cycloeucalenone (**1**), namely the orientation of the methyl group at C-4. The value that we obtained, $[\alpha]_D^{20}$ +54,9° (*c* 0.67, $CHCl_3$), is in agreement with that described in the literature, $[\alpha]_D^{20}$ +54,4° (*c* 0.89, $CHCl_3$) (Cocker *et al.*, 1965), for **1** [24$\alpha$-methyl-31-norcycloart-24(28)-en-3-one] (Fig. 1).

Compound **2** is a minor 31-norcycloartanone triterpenoid isolated from the dichloromethane extract (1.47% w/w). It proved to be 24-oxo-31-norcycloartanone by spectroscopic data in comparison with the only reference found in the literature (Akihisa *et al.*, 1998) that describes its isolation from *Musa sapientum* L. Here we report for the first time the $^{13}C$ NMR data of **2**, that was assigned by 2D NMR experiments, and also the specific optical rotation value, $[\alpha]_D^{20}$ +53.3° (*c* 0.46, $CHCl_3$). The $^{13}C$ NMR spectrum of **2** showed twenty nine resonances, of which twenty one were attributed to the triterpenoid nucleus and seven to a lateral chain. The DEPT NMR experiment permitted the differentiation of the $^{13}C$ NMR resonances into six methyl, eleven methylene, six methine, and six quaternary carbon atoms. Characteristic resonances of the triterpenoid nucleus were identical to those described for cycloeucalenone (**1**). The resonances of the remaining three secondary methyl, two methylene, two methine, and one quaternary carbon atoms were assigned by 2D NMR experiments (Table I).

Table I. $^{13}C$ NMR (100 MHz) spectral data of 24-oxo-31-norcycloartanone (**2**) ($CDCl_3$).

| C | $\delta_C$ | | HMBC |
|---|---|---|---|
| 1 | 32.8 | *t* | H-19 |
| 2 | 40.9 | *t* | – |
| 3 | 203.3 | *s* | H-30 |
| 4 | 50.0 | *d* | H-30 |
| 5 | 46.0 | *d* | H-30 |
| 6 | 25.2 | *t* | – |
| 7 | 28.0 | *t* | – |
| 8 | 47.0 | *d* | – |
| 9 | 24.9 | *s* | – |
| 10 | 24.9 | *s* | – |
| 11 | 25.9 | *t* | – |
| 12 | 35.4 | *t* | – |
| 13 | 45.4 | *s* | H-18, H-21 |
| 14 | 48.8 | *s* | H-29 |
| 15 | 32.8 | *t* | – |
| 16 | 26.9 | *t* | – |
| 17 | 52.2 | *d* | H-18, H-21 |
| 18 | 17.9 | *q* | – |
| 19 | 27.1 | *t* | – |
| 20 | 35.7 | *d* | – |
| 21 | 18.4 | *q* | H-17 |
| 22 | 30.1 | *t* | H-21 |
| 23 | 37.5 | *t* | – |
| 24 | 215.4 | *s* | H-25, H-26, H-27 |
| 25 | 40.8 | *d* | H-26, H-27 |
| 26 | 18.3[a] | *q* | H-25, H-27 |
| 27 | 18.1[a] | *q* | H-25, H-26 |
| 29 | 19.1 | *q* | – |
| 30 | 10.7 | *q* | H-4 |

$\delta$ values of compound **2** are referenced to the signal of residual $CHCl_3$ ($\delta$ 77.0 ppm).
[a] Interchangeable signals.

$\beta$-Sitosterol was also identified (Goad and Akihisa, 1997) and represents 0.36% (w/w) of the dichloromethane extract. Besides the above de-

scribed compounds a series of alkanes ($C_{25}-C_{34}$) and the potent antioxidant xanthophyll lutein (Milborrow *et al.*, 1982) were identified by chemical and spectroscopic data. They are the less and the most polar constituents of the dichloromethane extract and are present in 3.05% and 0.95% (w/w), respectively.

The search for the metabolites responsible for the antitumour action is one of the main interests of our work. As it is known different cell lines display different sensitivities towards a cytotoxic compound. The use of more than one cell line is therefore considered necessary in the detection of cytotoxic compounds. Bearing this in mind, cell lines from different histological origins were used in the present study: human tumour cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-3 (ovary), PC-3 (prostate), HT-29 (colon), 786-O (renal) and NCI-ADR/RES (ovary expressing multiple drug resistance phenotype). Cycloeucalenone (**1**) and 24-oxo-31-norcycloartanone (**2**) were tested against the above mentioned cell lines. **1** showed no interesting results but **2** exhibited significant activities in comparison with the values obtained for the reference compound DOX. **2** produced significant growth inhibition and cellular death of lung cell lines with TGI of 1.10 $\mu$g/mL, GI 50 of 0.19 $\mu$g/mL and LC 50 of 8.43 $\mu$g/mL. In Table II and Fig. 2 the total results for 24-oxo-31-norcycloartanone (**2**) against all tumour cell lines tested are presented. Here we conclude that this triterpenoid is effective and selective against the lung non-small cell line NCI-H460.

Table II. Results of the action of 24-oxo-31-norcycloartanone (**2**) against the following cell lines ($\mu$g/mL): human tumour cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-3 (ovary), PC-3 (prostate), HT-29 (colon), 786-O (renal) and NCI-ADR/RES (ovary expressing multiple drug resistance phenotype).

| Cell line | TGI | GI 50 | LC 50 |
|---|---|---|---|
| UACC-62 | >250 | 28.60 | >250 |
| MCF-7 | 158.30 | 28.70 | >250 |
| NCI-H460 | 1.10 | 0.19 | 8.43 |
| OVCAR-3 | >250 | >250 | >250 |
| PC-3 | >250 | 89.90 | >250 |
| HT-29 | >250 | >250 | >250 |
| 786-O | >250 | 47.73 | >250 |
| NCI-ADR/RES | 79.20 | 24.90 | >250 |

TGI, total growth inhibition; GI, growth inhibition 50; LC, lethal concentration 50; >250, the concentration could not be calculated because it has exceeded the highest concentration tested.

As mentioned above *S. cernuum* Vell. is traditionally used in gastric ulcer treatments and the ethanol extract proved to exhibit a significant antiulcerative activity (Araujo *et al.*, 2002). As such we investigated the dichloromethane extract for its antiulcer activity. The gastric ulcer induced by the 95% ethanol model expressed as the ulcer lesion indices (ULI) obtained from the treatment of Wistar rats with 500, 1000 and 2000 mg/kg/*po* of the dichloromethane extract were respectively 38.2,

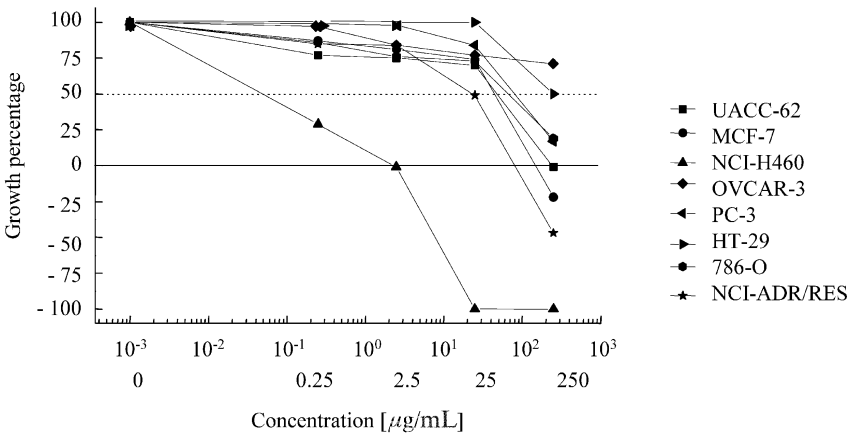



Fig. 2. Growth percentage of cell lines [human tumour cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-3 (ovary), PC-3 (prostate), HT-29 (colon), 786-O (renal) and NCI-ADR/RES (ovary expressing multiple drug resistance phenotype)] in the presence of different concentrations of 24-oxo-31-norcycloartanone (**2**).

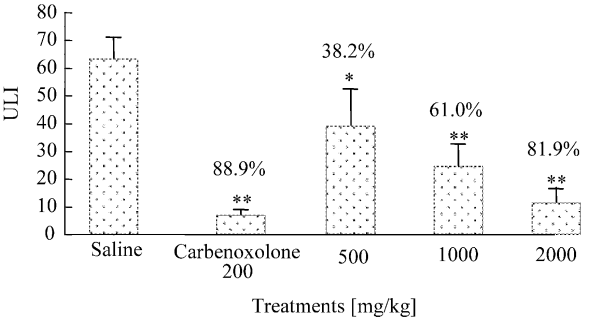



Fig. 3. Mean ± SEM of ulcerative lesions index (ULI) obtained with different doses of the dichloromethane extract of *Solanum cernuum*, saline (0.9% NaCl) and carbenoxolone (200 mg/kg) on the ethanol model. ANOVA and Dunnett's test were used for comparison (*$p < 0.05$; ** $p < 0.001$; $n = 6$). ANOVA $F_{(4.22)} = 19.95, p < 0.001$. Duncan assay, *$p < 0.01$, **$p < 0.001$.

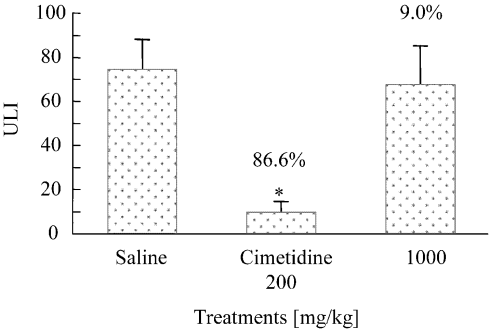



Fig. 4. Mean ± SEM of ulcerative lesions index (ULI) obtained with a dichloromethane extract of *Solanum cernumm*, saline (0.9% NaCl) and cimetidine (200 mg/kg) on the indomethacin model. ANOVA and Dunnett's test were used for comparison (*$p < 0.05$; ** $p < 0.001$; $n = 6$). ANOVA $F_{(2.13)} = 34.69$, $p < 0.001$. Duncan assay, *$p < 0.01$.

61.0 and 81.9%, while carbenoxolone inhibited by 88.9% (Fig. 3). The acute administration of absolute ethanol to rats produces gastric mucosal lesions and erosions similar to those occurring in gastric ulcer (Giordano *et al.*, 1990). The tissue damage of the gastrointestinal mucosa induced by acute ethanol toxicity may be associated with the generation of toxic reactive species which produce an unbalanced oxidant/antioxidant cellular process. When the antioxidant defence system is insufficient free radicals are accumulated causing injuries to the cell membrane, oxidative damage and cell death if the insult continues (Repetto and Llesuy, 2002). Another hypothesis proposed to explain the ethanol-induced oxidative damage to the gastric mucosa is the constrictive effect on veins and arteries of the gastric mucosa, producing congestion, inflammation and tissue injuries. Herbs are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases resulting from lipid peroxidation (Nakatami, 2000). The mild activity of the dichloromethane extract of *S. cernuum* Vell. could be regarded acting as protective factor or increasing antioxidant activity, and as such as a mechanism of local cytoprotection. The gastric ulcer induced by indomethacin (40 mg/kg) had no statistic results ($p > 0.05$) and only small percentage of inhibition lesions, *i. e.* the mechanism of action involved is not related with prostaglandin effects (Fig. 4). We can conclude from our antiulcer assays that the metabolites present in the dichloromethane extract do not contribute to the traditional use of *S. cernuum* Vell. as an antiulcerogenic. For this action more polar compounds should be responsible.

The ethanol extract was subject to chemical screening. By specific reagent analysis, NMR experiments, FT-IR and mass spectra data, the presence of glycoalkaloids was confirmed as it was expected. These compounds are neither solasodine nor solanidine which are the most common alkaloids encountered in *Solanum* species and known for their anticancer activity. Major compounds of the ethanol extract have small peptide structures and are compounds described as cytotoxic against tumour cell lines (Li *et al.*, 2003). The saccharide fraction was mainly composed of disaccharides.

Important metabolites have been identified in the dichloromethane and ethanol extracts of *S. cernuum* Vell. that can contribute to explain in part the traditional use of the species and consequently justify further studies that are now in progress.

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