Pharmacology, Biochemistry and Behavior 93 (2009) 40–46

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

# Filicene obtained from *Adiantum cuneatum* interacts with the cholinergic, dopaminergic, glutamatergic, GABAergic, and tachykinergic systems to exert antinociceptive effect in mice

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ARTICLE INFO

*Article history:*
Received 3 December 2008
Received in revised form 19 March 2009
Accepted 2 April 2009
Available online 16 April 2009

*Keywords:*
*Adiantum cuneatum*
Antinociception
Filicene
Action mechanism

ABSTRACT

In the present study, we describe the antinociceptive effect of filicene, a triterpene isolated from *Adiantum cuneatum* (Adiantaceae) leaves, in several models of pain in mice. When evaluated against acetic acid-induced abdominal constrictions, filicene (10, 30 and 60 mg/kg, i.p.) produced dose-related inhibition of the number of constrictions, being several times more potent [$ID_{50}$ = 9.17 (6.27–13.18) mg/kg] than acetaminophen [$ID_{50}$ = 18.8 (15.7–22.6) mg/kg], diclofenac [$ID_{50}$ = 12.1(9.40–15.6) mg/kg] and acetylsalicylic acid [$ID_{50}$ = 24.0(13.1–43.8) mg/kg] in the same doses as those used for the standard drugs. Filicene also produced dose-related inhibition of the pain caused by capsaicin and glutamate, with mean $ID_{50}$ values of 11.7 (8.51–16.0) mg/kg and <10 mg/kg, respectively. Its antinociceptive action was significantly reversed by atropine, haloperidol, $GABA_A$ and $GABA_B$ antagonists (bicuculline and phaclofen, respectively), but was not affected by L-arginine–nitric oxide, serotonin, adrenergic and the opioid systems. Together, these results indicate that the mechanisms involved in its action are not completely understood, but seem to involve interaction with the cholinergic, dopaminergic, glutamatergic, GABAergic and tachykinergic systems.



## 1. Introduction

Medicinal plants, considered those with therapeutic properties, have been used since the beginning of human civilization to treat different diseases, and the use of this effective strategy for the promotion of human health has significantly increased in recent years. This fact is related to several factors, including the safety, effectiveness and better quality control of the phytomedicines available on the market today. A significant portion of the drugs considered as basic and essential by the World Health Organization (WHO) are obtained from plants, and several synthetic drugs are obtained using natural products as models. However, the potential of the biodiversity as a source for new drugs is still unexplored, since of all the species plants estimated on the planet (250,000–300,000), less than 10% have been investigated in relation to their chemical, pharmacological or biological aspects. Although in recent years, notable progress has been made concerning the development of natural therapies, there is an urgent need to discover effective and potent analgesic agents (Calixto et al., 2000). Our research group has focused on the discovery of plants from the Brazilian biodiversity with therapeutic properties, especially for the treatment of inflammatory and dolorous processes, and relevant results have been obtained.

*Adiantum cuneatum* Langsd. and Fisch. (Adiantaceae) is a common plant which is widespread in South America, especially in Brazil, where it is known as "avenca". It is used as an ornamental plant, and is also frequently used in folk medicine, as a diuretic, an expectorant, an emollient, and to treat coughs, urinary disorders, alopecia, and menstrual problems (De Feo et al., 1992). In Brazil, it is often used to treat respiratory diseases and reduce pain (Michalak, 1997). We have already demonstrated that the extract, fractions and some compounds, including filicene (Fig. 1), exert pronounced antinociceptive action in mice (Bresciani et al., 2003). In this paper we have extended the evaluation of the antinociceptive activity of filicene using other models of nociception in mice, attempting to evaluate its pharmacological interaction with diverse pathways of neurotransmission and neuromodulation.

## 2. Materials and methods

### 2.1. Plant material

*A. cuneatum* was collected in March 2000 at a farm in Lageado Bonito, Caxambú do Sul, near the town of Chapecó, in the West of

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doi:10.1016/j.pbb.2009.04.004

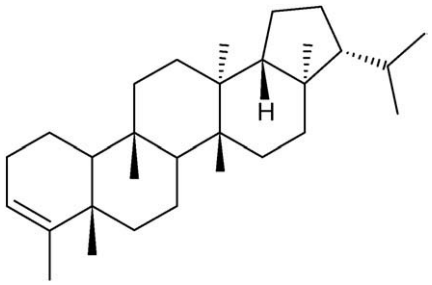


**Fig. 1.** Molecular structure of filicene isolated from *A. cuneatum* leaves.

Santa Catarina, Brazil. It was identified by Prof. Ademir Reis (Dept. Botany, UFSC) and a voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí) under number VC Filho 025.

### 2.2. Isolation of filicene

Filicene was isolated as previously described (Bresciani et al., 2003) from a hexane fraction, with a yield of 0.19% (from dried leaves).

### 2.3. Drugs and reagents

The following substances were used: acetic acid, formalin, acetaminophen, acetylsalicylic acid, diclofenac and morphine hydrochloride (Merck, Darmstadt, Germany); $N^{\omega}$-nitro-L-arginine, L-arginine hydrochloride, capsaicin, clonidine hydrochloride, L-glutamic acid hydrochloride, naloxone hydrochloride, yohimbine hydrochloride, p-chlorophenylalanine methylester (PCPA), haloperidol, ketanserin, apomorphine, phenylephrine, atropine (Sigma Chemical Co., St. Louis, USA), prazosin (Pfizer, New York, NY), baclofen, phaclofen, and muscimol (RBI, USA), methysergide (Sandoz AG, Baesel, Switzerland), and EDTA, phosphate buffer solution (PBS) (Merck Brazil). The drugs were dissolved in saline, with the exception of capsaicin which was dissolved in absolute ethanol. Filicene was dissolved in Tween 80 and diluted in 0.9% NaCl, immediately before use. The final concentration of Tween 80 and ethanol did not exceed 2% and did not have any effect *per se*. All the test doses of the drugs were chosen based on our pilot experiments, and due to the limited amounts available, it was only possible to test them orally in a model.

### 2.4. Animals

The experiments were conducted using male Swiss mice (25–35 g), housed at 22 ± 2 °C under a 12-h light/12-h dark cycle (lights on at 06:00) and with access to food and water *ad libitum*. The animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after gaining approval of the protocol by the Institutional Ethics Committee (113/2005-03 CEP-UNIVALI) and followed the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). The numbers of animals (6–8 per group) and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. Each animal was used only once (except in the hot plate test) and afterwards, the animals were sacrificed by exposure to $CO_2$.

### 2.5. Acetic acid-induced nociception

The abdominal constrictions were induced according to the procedures described previously (Collier et al., 1968). The animals were pre-treated with the compound (6–60 mg/kg i.p.) or (100–500 mg/kg, p.o.) 30 or 60 min before injection with acetic acid (0.6%, 0.45 ml/mouse), respectively. The control animals received a similar volume of vehicle. After the challenge, pairs of mice were placed in separate boxes and the number of abdominal constrictions was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of constrictions in mice pre-treated with the filicene. Acetaminophen, acetyl salicylic acid and diclofenac sodium were used as positive control to compare the potency of the antinociceptive effect of filicene in this model.

### 2.6. Glutamate-induced nociception

In an attempt to provide more direct evidence concerning the possible interaction of filicene with the glutamate systems, we also investigated whether filicene antagonized glutamate-induced licking of the mice paws. The procedure used in this test was similar to that described previously by Beirith et al. (2002). The animals were observed individually for 15 min following glutamate injection (30 µmol paw). The amount of time spent licking the injected paw was timed using a chronometer and was considered indicative of nociception. The animals were treated with the compound (10–60 mg/kg), 30 min before the glutamate injection. The control group received a similar volume of vehicle (10 ml/kg i.p.) used to dilute the compound.

### 2.7. Capsaicin-induced nociception

The method used was similar to that described previously (Sakurada et al., 1992). The animals were placed individually in transparent glass cylinders of 20 cm in diameter, which served as observation chambers. Following the adaptation period, 20 µl of capsaicin (1.6 µg/paw prepared in a phosphate-buffered solution) was injected under the skin of the dorsal surface on the right hind paw. The mice were pre-treated with filicene i.p. (10–60 mg/kg) 30 min before injection of the irritant. The control animals received a similar volume of vehicle (10 ml/kg, i.p.). After this process, pairs of mice were placed individually in different glass cylinders of 20 cm in diameter for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered indicative of nociception. The control animals received a similar volume of the vehicle (10 ml/kg, i.p.) used to dilute these drugs.

### 2.8. Hot-plate test

The hot plate test was used to measure the response latencies according to the method described previously by Eddy and Leimback (1953) with minor modifications. In these experiments the hot plate (UGO BASILE) was maintained at 56 °C ± 1 ° C. The animals were placed in glass cylinders of 20 cm in diameter, and the time between placement of the animals in the cylinder, and shaking or licking of the paws, or jumping, was recorded as the response latency index. All the animals were selected in advance on the basis of their reactivity in the model. A latency period of 30 s was defined as complete analgesia. The animals were treated with a vehicle (10 ml/kg, i.p.), filicene (30 mg/kg, i.p.) and morphine (5 mg/kg, s.c.) 30 min before the experiments.

### 2.9. Open-field test

To exclude the possibility that a possible filicene antinociceptive effect could cause depression of the central or peripheral nervous system, the animals were analyzed in the "open field" test, which enabled us to determine the effect of the drugs on motor performance, independent of their suffering at the level of the central or peripheral nervous system (Rodrigues et al., 2002). The test was performed in a wooden box measuring 40 × 60 × 50 cm, with the floor divided into 12 equal squares. The parameters observed were: ambulations (the number of squares crossed with all four paws, which was used to evaluate locomotor activity) and the number of rearings (which indicate exploratory activity).

**Table 1**
Antinociceptive effect of filicene and some analgesic drugs used in the acid acetic-induced pain model.

| Treatment | Acetic acid $ID_{50}$ (mg/kg)* | MI (%) |
|---|---|---|
| Filicene | 9.17 (6.27–13.18) | 83.66 |
| Acetaminophen | 18.8 (15.70–22.6 | 88.0 |
| Diclofenac | 12.1 (9.4–15.6) | 93.0 |
| Acetylsalicylic acid | 24.0 (13.1–43.8) | 83.0 |

*95% confidence limits are given in brackets, MI, maximal inhibition with a dose of 60 mg/kg.

### 2.10. Analysis of possible mechanism of action of filicene

To address some of the mechanisms by which filicene causes antinociception in the acetic acid-induced visceral nociception, the animals were pre-treated with different drugs. The doses of the drugs used were selected based on the literature data and also based on previous results of our laboratory. To assess the possible participation of α-adrenergic receptors in the antinociception caused by filicene, the animals were pre-treated with yohimbine ($\alpha_2$ antagonists, 0.15 mg/kg, i.p.) or prasozin ($\alpha_1$ antagonists, 0.15 mg/kg, i.p.) 15 min before administration of filicene (30 mg/kg, i.p.) phenylephrine ($\alpha_1$ agonist, 10 mg/kg, i.p.) or clonidine ($\alpha_2$ agonist, 0.15 mg/kg, i.p.). The pain response caused by the injection of acetic acid (0.6%) was analyzed 30 min after the administration of filicene or agonists. Other groups of animals received only filicene (60 mg/kg, i.p.), phenylephrine, clonidine or the vehicle (saline, 10 ml/kg) 30 min after acetic acid injection.

In separate experiments, we investigated the possible participation of the nitric oxide L-arginine pathway in the antinociceptive effect caused by filicene. To this end, the animals were pre-treated with L-arginine (precursor of nitric oxide, 600 mg/kg, i.p.) and after 15 min, they received the compound (60 mg/kg, i.p.), or $N^G$-nitro-L-arginine (a nitric oxide inhibitor, L-NOARG, 75 mg/kg, i.p.). The pain caused by the injection of acetic acid was analyzed 30 min after treatment of the animals with filicene or L-NOARG. The other groups received filicene or the vehicle (saline, 10 ml/kg) 30 min before the injection of acetic acid.

In other experiments, we investigated the possible participation of the opioid system in the analgesic effect caused by filicene. The animals were pre-treated with filicene (30 mg/kg, i.p.), morphine (5 mg/kg, s.c.) 30 min before acetic acid injection. In a separate group of mice, we analyzed the effect of naloxone (5 mg/kg, i.p.) injected 15 min beforehand, against the analgesic effect caused by both morphine and filicene. The control animals received a similar volume of 0.9% NaCl (10 ml/kg, i.p.).

The possible participation of the GABAergic system in the filicene-induced analgesic effect was investigated. For this purpose, the animals were pre-treated with bicuculline, a $GABA_A$ antagonist (1.0 mg/kg, i.p.) or phaclofen, a $GABA_B$ antagonist (2.0 mg/kg, i.p.) and after 15 min, they received filicene (30 mg/kg, i.p.), muscimol (2.0 mg/kg, i.p.) or baclofen (2.0 mg/kg i.p.), which are $GABA_A$ and $GABA_B$ receptor agonists. The pain caused by acetic acid injection was analyzed 30 min after treatment of the animals with filicene, muscimol or baclofen. The other groups received the compound or vehicle (saline, 10 ml/kg), 30 min prior to acetic acid injection.

To assess the contribution of serotonin to the analgesic profile of filicene, the animals received p-chlorophenylalanine (PCPA, an inhibitor of serotonin synthesis 100 mg/kg, i.p. once a day, for 4 consecutive days), and methysergide (non-selective serotonin antagonist, 5 mg/kg, i.p.) or ketanserin (5-$HT_2$ antagonist, 1 mg/kg, i.p.), 15 min prior to the administration of filicene (30 mg/kg, i.p.). The pain response caused by acetic acid injection was analyzed 30 min after the administration of filicene. Another group of mice was treated with the vehicle (saline, 10 ml/kg) 30 min prior to acetic acid injection.

To determine the role of the cholinergic system, the animals were pre-treated with atropine (10 mg/kg, i.p.) a non-selective cholinergic antagonist, followed 15 min later by filicene (30 mg/kg, i.p.), or acetylcholine (1.0 mg/kg i.p.). The pain caused by acetic acid injection was analyzed 30 min after treatment of the animals with felicene and acetylcholine. The other groups received filicene or the vehicle (saline, 10 ml/kg) 30 min prior to acetic acid injection.

Finally, we also investigated the participation of the dopaminergic system in the analgesic profile of filicene. The animals were pre-treated with haloperidol (0.2 mg/kg, i.p.) a non-selective dopaminergic antagonist, followed 15 min later by felicene (30 mg/kg, i.p.), or apomorphine (1.0 mg/kg i.p.). The pain caused by acetic acid injection was analyzed 30 min after treatment of the animals with filicene and apomorphine. The other groups received filicene or the vehicle (saline, 10 ml/kg) 30 min prior to acetic acid injection.

### 2.11. Statistical analysis

The results are represented as mean ± SEM, except the $ID_{50}$ (i.e., the dosage of extracts which reduced responses by 50% relative to the control values) and are presented as geometric means, accompanied by their respective 95% confidence limits. The $ID_{50}$ values were determined by linear regression GraphPad software (GraphPad Software, San Diego, CA). The statistical significance between the groups was calculated by analysis of variance followed by the Student–Newman–Keuls test. *P* values of less than 0.05 were considered indicative of significance.

## 3. Results

### 3.1. Acetic acid-induced nociception

Considering that filicene previously exhibited the antinociceptive reaction against the writhing test by the i.p. route (Table 1), we have

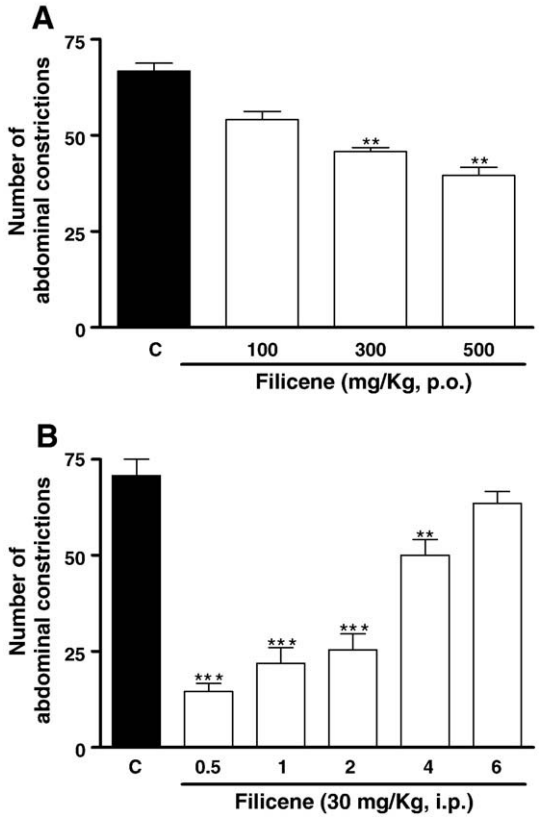


**Fig. 2.** Effect of filicene given orally (panel A) and i.p. (panel B) in the acetic acid-induced abdominal constrictions test in mice. Each column represents the mean for 6–8 animals and the vertical lines indicate the S.E.M. The asterisks denote significance levels, when compared with the control groups, $^{**}P<0.01$, $^{***}P<0.001$ (Newman Keul's multiple comparison test). Panel B also represents the time of effect (time-course) of filicene in same test.

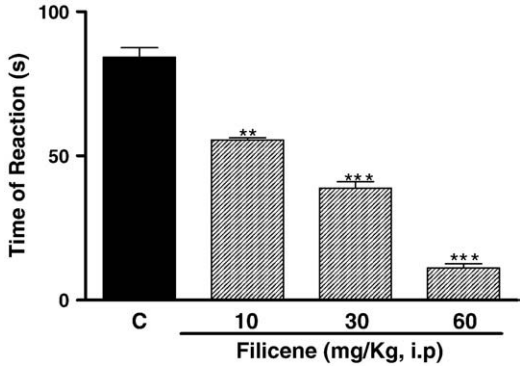


**Fig. 3.** Effect of filicene given intraperitoneally in the capsaicin test in mice. Each column represents the mean for 6–8 animals and the vertical lines indicate the S.E.M. The asterisks denote significance levels, when compared with the control groups (closed column; animals treated with the vehicle), $^{**}P<0.01$, $^{***}P<0.001$ (Newman Keul's multiple comparison test).

now evaluated its effects by another route. Filicene (p.o.) also produced significant, but non-dose-related inhibition (48.9 ± 1.34%), proving to be less effective by this route (Fig. 2A). The compound produced marked antinociception as early as 30 min after i.p. administration, an action that remained significant up to 4 h after administration (Fig. 2B). Thus, i.p. administration 30 min before the experiments was selected for all further studies.

### 3.2. Capsaicin-induced nociception

The results of this test (Fig. 3) indicate that this compound reduces capsaicin-induced pain in a dose-dependent way, presenting a calculated $ID_{50}$ value of 11.7 (8.51–16.0) mg/kg and inhibition of 87.8 ± 1.27%. The compound was effective against neurogenic pain, as observed in the first phase of the formalin test.

### 3.3. Glutamate-induced nociception

The results indicated in Fig. 4 show that different doses of filicene exhibited significant inhibition of glutamate-induced nociceptive response in mice, with an $ID_{50}$ value less than 10 mg/kg and inhibition of 76.8 ± 3.27%.

### 3.4. Hot-plate test

In this experiment we observed that filicene did not cause any significant change in response latency.

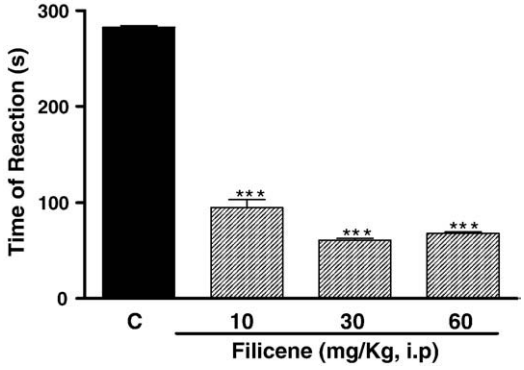


**Fig. 4.** Effect of filicene given intraperitoneally in the glutamate test in mice. Each column represents the mean for 6–8 animals and the vertical lines indicate the S.E.M. The asterisks denote significance levels, when compared with the control groups (closed column; animals treated with the vehicle), $^{***}P<0.001$, (Newman Keul's multiple comparison test).

**Table 2**
Effect of various drugs on the antinociception caused by filicene assessed in the acetic acid-induced abdominal constrictions.

| Treatment | Dose (mg/kg$^{-1}$) | Number of constrictions |
|---|---|---|
| Control | 0 | 62 ± 2.8 |
| Naloxone | 5 | 58 ± 3.2 |
| Morphine | 5 | 8.2 ± 1.9*** |
| Filicene | 30 | 12.8 ± 2.2*** |
| Morphine + naloxone | 5/5 | 52 ± 3.42$^{\dagger}$ |
| Filicene+ naloxone | 30/5 | 14.4 ± 1.84$^{NS}$ |
| Control | 0 | 46.4 ± 2.12 |
| L-arginine | 600 | 45.24 ± 3.12 |
| L-NOARG | 75 | 11.48 ± 2.6*** |
| Filicene | 30 | 12.4 ± 1.68*** |
| L-NOARG + L-arginine | 75/600 | 44.0 ± 4.2$^{\dagger}$ |
| Filicene + L-arginine | 60/600 | 13.6 ± 1.76 $^{NS}$ |
| Control | 0 | 48.5 ± 2.29 |
| Prazosin | 0.15 | 42.16 ± 4.19 |
| Phenylephrine | 10 | 4.0 ± 0.89*** |
| Phenylephrine + prazosin | 10/0.15 | 48.4 ± 2.53$^{\dagger}$ |
| Yohimbine | 0.15 | 48.6 ± 3.24 |
| Clonidine | 0.1 | 4.5 ± 1.39*** |
| Clonidine + yohimbine | 0.1/0.15 | 48.6 ± 2.6$^{\dagger}$ |
| Filicene | 30 | 15.35 ± 3.2 |
| Filicene + yohimbine | 30/0.15 | 14.5 ± 2.33$^{NS}$ |
| Filicene + prazosin | 30/0.15 | 16.54 ± 3.36$^{NS}$ |
| Control | 0 | 44.72 ± 3.8 |
| Methysergide | 5 | 42.64 ± 2.6 |
| Ketanserin | 1 | 43.82 ± 2.8 |
| Filicene | 30 | 19.4 ± 2.6** |
| Filicene + ketanserin | 30/1 | 14.8 ± 3.4$^{NS}$ |
| Filicene + methysergide | 30/5 | 12.6 ± 4.2$^{NS}$ |
| Control | 0 | 46.2 ± 2.47 |
| PCPA | 100 | 42.8 ± 3.42 |
| Morphine | 5 | 4.28 ± 0.89*** |
| Filicene | 30 | 8.49 ± 1.2*** |
| Morphine + PCPA | 5/100 | 38.2 ± 2.52 |
| Filicene + PCPA | 30/100 | 9.12 ± 1.4 $^{NS}$ |

NS, not significant. $^{**}P<0.01$, $^{***}P<0.001$ significantly different compared with the control value. $^{\dagger}P<0.05$ significantly different compared with morphine, L-arginine, phenylephrine , clonidine.

### 3.5. Analysis of the possible mechanism of action of filicene

The results of the analysis of a possible mechanism of action of filicene are summarized in Table 2 and Figs. 5–7. As shown in the Table 2, pre-treatment of the animals with naloxone given 15 min before the injection of morphine largely reversed the antinociception caused by

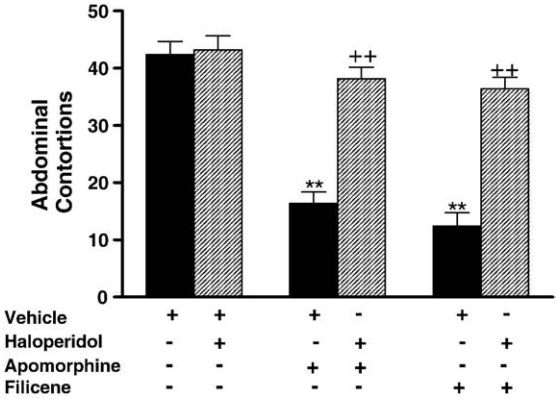


**Fig. 5.** Effect of pre-treatment of animals with haloperidol (0.2 mg/kg, i.p.), on the antinociceptive profiles of apomorphine (1.0 mg/kg, i.p.) and filicene (30 mg/kg, i.p.), against acetic acid-induced writhing in mice. Each column represents the mean of 6–10 animals and the error bars indicate the S.E.M. $^{++}P<0.01$, comparing agonist (apomorphine, filicene) plus antagonists (haloperidol) versus agonist plus vehicle (control); $^{**}P<0.01$ compared with the corresponding control values (animals injected with the vehicle alone).

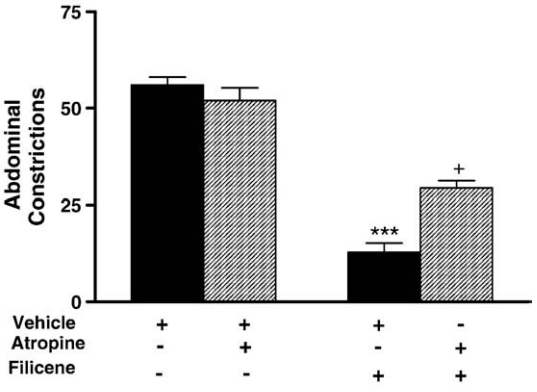


**Fig. 6.** Effect of pre-treatment of animals with atropine (0.2 mg/kg, i.p.), on the antinociceptive profiles of filicene (30 mg/kg, i.p.) against acetic acid-induced writhing in mice. Each column represents the mean of 6–10 animals and the error bars indicate the S.E.M. $^{+}P<0.05$, comparing agonist (filicene) plus antagonists (haloperidol) versus agonist plus vehicle (control); $^{***}P<0.001$ compared with the corresponding control values (animals injected with the vehicle alone).

morphine when analyzed in acetic acid model, leaving the antinociceptive effect of filicene acid unaffected. The pre-treatment of the animals with α-adrenergic antagonists, prazosin (0.2 mg/ kg, i.p.) or yohimbine (0.2 mg/kg, i.p.) 30 min beforehand, caused marked inhibition of the analgesic effect induced by phenylephrine (10 mg /kg. i.p.) and clonidine (0.2 mg /kg. i.p.), but failed in to revert the analgesic effect of filicene. The treatment of the animals with L-arginine (600 mg/kg, i.p.), a precursor of nitric oxide synthase, completely reversed the antinociceptive action caused by injection of L-NOARG (75 mg/kg, i.p., 30 min before) but not antinociception caused by filicene. Pre-treatment of the animals with PCPA (100 mg/kg), methysergide (5.0 mg/kg) or ketanserin (1.0 mg/kg) did not reduce the antinociceptive action of filicene. Pre-treatment of the animals with atropine (60.0 mg/kg) and haloperidol (0.2 mg/kg), non-selective antagonists of the muscarinic and dopaminergic receptors, reversed the analgesic response of acetylcholine, apomorphine and filicene, respectively (Figs. 5 and 6). Finally, baclofen (2.0 mg/kg), a $GABA_B$ antagonist, and bicuculline (1.0 mg/kg) a $GABA_A$ antagonist, reversed the analgesic response of muscimol (2.0 mg/kg), and phaclofen (2.0 mg/kg) respectively. These antagonists also altered the analgesic response to filicene (Fig. 7).

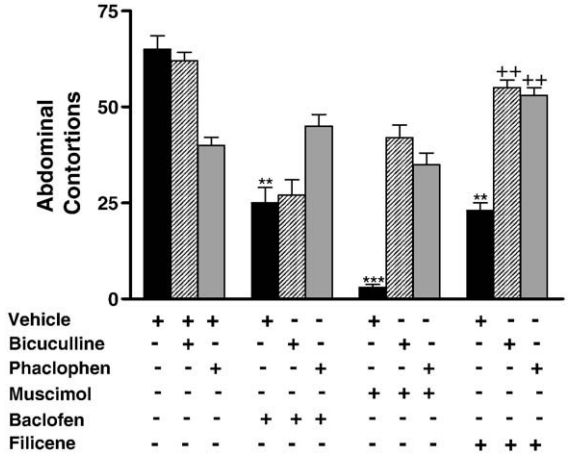


**Fig. 7.** Effect of pre-treatment of animals with bicuculline (a $GABA_A$ antagonist, 1.0 mg/kg), phaclophen (a $GABA_B$ antagonist, 2.0 mg/kg, i.p.) on the antinociceptive profiles of muscimol (a $GABA_A$ agonist, 2.0 mg/kg, i.p.), baclofen (a $GABA_B$ agonist, 2.0 mg/kg, i.p.) and filicene (30 mg/kg, i.p.) against acetic acid-induced writhing in mice. Each column represents the mean of 6–10 animals and the error bars indicate the S.E.M. $^{+}P<0.05$, comparing agonist (muscimol, baclofen and felicene) plus antagonists (bicuculline and phaclophen) versus agonists plus vehicle (control); $^{**}P<0.01$, $^{**}P<0.001$ compared with the corresponding control values (animals injected with the vehicle alone).

**Table 3**
Effect of filicene on locomotor activity (open field test) in mice.

| Treatment (mg/kg, i.p.) | Crossing number | Rearing number |
|---|---|---|
| Vehicle | 111.4 ± 4.3 | 57.21 ± 2.8 |
| Filicene 10 | 98.74 ± 3.8 | 54.56 ± 2.1 |
| Filicene 30 | 96.36 ± 5.2 | 59.16 ± 4.3 |
| Filicene 60 | 102.74 ± 3.2 | 62.38 ± 4.3 |

Data are expressed as mean ± S.E.M. of 8–10 animals.

### 3.6. Open-field test

Filicene did not alter the locomotor activity in the open field test in doses at which it completely inhibited the abdominal constrictions (Table 3).

## 4. Discussion

Considerable efforts have recently been made to discover new analgesic agents with increased efficacy and improved side effect profiles, and a high number of compounds obtained from medicinal plants have been extensively studied, with relevant results (Calixto et al., 2000, 2001). Filicene is the main triterpene obtained from *A. cuneatum*. The results of the present study confirm and also extend the preliminary studies on the antinociceptive effect of this compound (Bresciani et al., 2003). Filicene was studied using the acetic acid and capsaicin-induced abdominal constrictions tests, the hot plate test and the glutamate-induced pain test, in order to determine its interaction with different neurotransmitters. The use of different models is significant in the detection of antinociceptive properties in a substance, considering that the use of a variety of stimuli recognizes different types of pain and reveals the actual nature of the antinociceptive test drug (Bergerot et al., 2006). Filicene exhibited antinociceptive activity in three of the four animal models studied. However, both the absolute and the relative potencies of this compound varied, depending upon the experimental model used. Filicene exhibited greater antinociceptive activity of 'inflammatory' pain (acetic acid-induced abdominal constrictions) in the animal model, and was less effective in models of 'acute' pain (glutamate and capsaicin tests). Past studies have postulated that acetic acid acts indirectly by inducing the release of endogenous mediators, stimulating the nociceptive neurons which are sensitive to nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids (Collier et al., 1968). Acetic acid-induced writhing reaction in mice, described as a typical model for inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents.

Sakurada et al. (1992) proposed the capsaicin-induced pain model for the study of compounds that act on pain of a neurogenic origin (Sakurada et al., 2003). Capsaicin is a neurotoxic compound extracted from red pepper which, when applied to the skin or injected into animals, produces irritation, a painful reaction, and subsequent desensitization to chemically-induced pain (Jancsón et al., 1981). Studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids (glutamate and aspartate) nitric oxide, and pro-inflammatory mediators in the periphery, and transmits nociceptive information to the spinal cord (Sakurada et al., 2003). Our results indicate a significant reduction in neurogenic nociception caused by the intraplantar injection of capsaicin, showing that filicene caused significant effects in this model. The capsaicin-induced neurogenic paw licking response was similar to the first phase of the formalin test (Bresciani et al., 2003). Compounds with this action may be good candidates for the treatment of neuropathic conditions, in which effective treatment is difficult (Akada et al., 2006).

We also investigated the interaction of glutamatergic systems on filicene-induced analgesia. Several glutamatergic receptors, such $NMDA_R$ and metabotropic glutamate receptors (mGluRs), have been known to be involved in the modulation of glutamate-induced nociception (Yoon et al., 2006). Beirith et al. (2002) found that the nociceptive response

induced by glutamate appears to involve peripheral, spinal and supraspinal sites of mediated action which are modulated by its receptors (NMDA and non-NMDA) as well as by the liberation of nitric oxide or by some nitric oxide-related substance. We verified here that filicene was effective in reducing glutamate-induced pain.

Concerning the mechanism through which filicene exerts its antinociceptive action, a more realistic view would be to mention that we tested the pharmacological interaction between filicene and several pathways of neurotransmission and neuromodulation. At first, the antinociception elicited by the compound appears to be independent of the activation of important endogenous analgesic systems. However, subsequent results show that in part, the opposite is true. In fact, the antinociceptive action of filicene, in contrast to that reported for morphine, was not reversed by naloxone, a non-selective opioid antagonist, and its effect was not observed in the hot plate test. Furthermore, the α1- and α2-adrenoceptors seem unlikely to be involved in the antinociceptive action of filicene, evidenced by the fact that selective antagonists of these receptors failed to alter the antinociception caused by the compound in conditions where they produce significant inhibition of antinociception provoked by the selective agonists. Our results also show that the antinociceptive profile of this compound does not appear to be related to the interaction of the L-arginine–nitric oxide pathway. Another interesting finding of the present study relates to the involvement of the GABAergic pathway on the antinociceptive effect of filicene. Several works have reported the involvement of GABAergic pathway as a system which is also involved in endogenous modulation of pain (Stamford, 1995; Hayashida et al., 2008). The modulation of ionotropic gamma-aminobutyric acid (GABA) receptors (GABA-gated Cl (−) channels) by a group of natural products, including flavonoids, was studied in electrophysiological experiments (Goutman et al., 2003). Baclofen, GABA derivative, is a potent $GABA_B$ receptor agonist that is clinically used to treat spasticity, and many types of neuropathic pain, but also induces analgesia in certain animal models of pain (Aran and Hammond, 1991; Shafizadeh et al., 1997; Franek et al., 2004; Jorns and Zakrzewska, 2007). Our results confirm that baclofen markedly antagonizes acetic acid-induced nociception. In addition, $GABA_A$ or the $GABA_B$ antagonists bicuculline or phaclofen, reversed the antinociceptive effect of filicene, demonstrating that the antinociceptive effect of this compound also involves the action of the GABAergic pathways.

In our study, methysergide and ketanserin (non-selective serotonin receptor antagonists) did not inhibit filicene-induced antinociception, and treatment with PCPA (at a dose that decreases the cortical content of 5-HT and significantly reverses morphine antinociception) failed to revert the antinociceptive action of filicene. Thus, taken together, these findings strongly suggest that the serotonergic system is not involved in the antinociceptive effect of filicene.

The cholinergic system is also involved in antinociception. Antimigraine drugs, such as sumatripan, were antagonized by the muscarinic antagonist atropine and the acetylcholine-depletor hemicolinium, in several animal models (Ghelardini et al., 1996). In addition, both systemically and intrathecally administered cholinergic agonists produce antinociception, while cholinergic antagonists decrease the pain threshold (Radek et al., 2004). It has recently been demonstrated that activation of muscarinic acetylcholine receptors (mAChRs) inhibits spinal nociceptive transmission by potentiation of GABAergic tone through M(2), M(3), and M(4) subtypes (Zhanq et al., 2008). However, other mechanisms and sites of action of cholinergic substances are not known. Our results show that atropine, given systemically, reversed the antinociceptive effect caused by filicene, suggesting that antinociception detected for this compound depends, in part, on the cholinergic system.

Finally, we also investigated the interaction between the dopaminergic system and the antinociception caused by filicene. Little is known about the pathophysiological modulation of spinal dopaminergic transmission, although it is known that both inputs and sustained acute noxious stimuli accelerate dopamine turnover in the dorsal horn, suggesting an enhancement in the activity of the descending dopaminergic pathways. Dopaminergic mechanisms may play a part in the accompanying antinociception (Millan, 1999). Our results show that haloperidol, administered systemically, reverses the antinociceptive effect caused by filicene, suggesting that antinociception is dependant on the dopaminergic system.

## 5. Conclusions

In summary, the results obtained confirm and extend our previous studies, indicating that filicene presents an important antinociceptive effect in various pharmacological pain models. It was effective when administered by the i.p. and p.o. pathways, suggesting that this substance is also absorbed by the gastrointestinal system. The pleiotropic nature of the antinociceptive actions exerted by filicene was also revealed, suggesting that complex interactions with the cholinergic, dopaminergic, glutamatergic, GABAergic and tachykinergic systems might occur. We demonstrated that there are direct interactions with the dopaminergic, cholinergic, and GABAergic systems because its antinociceptive action was significantly reversed by the atropine, haloperidol, $GABA_A$ and $GABA_B$ receptor antagonists. The indirect form, the glutamatergic and tachykinergic systems, appear to play a critical role in the antinociception caused by filicene, because the nociception induced by these agents decreased by the pre-treatment with the compound, but additional experiments are necessary to confirm this hypothesis. The present work also demonstrates that the antinociception caused by filicene does not involve interaction with the opioid system, the serotonergic system, or the adrenergic system.

## Acknowledgements

The authors are grateful to Univali for its facilities and technical support, and to the CNPq and FAPESC-SC for their financial support.

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