Phytochemical profile and analgesic evaluation of *Vitex cymosa* leaf extracts

Suzana Guimarães Leitão,*,1 Tereza Cristina dos Santos, #,2 Franco Delle Monache, 4 Maria Eline Matheus, 3 Patrícia Dias Fernandes, 3 Bruno Guimarães Marinho

1Faculdade de Farmácia, Departamento de Produtos Naturais e Alimentos, Universidade Federal do Rio de Janeiro, Brazil, 2Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, 3Departamento de Farmacologia, ICB, Universidade Federal do Rio de Janeiro, Brazil, 4Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università La Sapienza. Italy.

**Abstract:** *Vitex cymosa* Bertero ex Spreng., Lamiaceae, is found in Central and Amazon regions of Brazil, where it is popularly used as antirheumatic. Extracts from the leaves of *V. cymosa* were tested in analgesia models such as abdominal contortions induced by acetic acid and formalin to test peripheral analgesia; as well as the tail flick and hot plate models, to test spinal and supraspinal analgesia. A significant reduction was observed in the number of contortions with all extracts and in all doses. In the formalin model, a reduction in the second phase (inflammatory) was observed with all extracts, whereas only the *n*-butanol extract was able to act in the first, neurogenic, phase. In the tail flick model, all extracts increased latency time. Naloxone treatment reverted analgesic effect of all extracts with the exception of the dichloromethane one. All extracts developed peripheral and central analgesic activity. In the hot plate model no antinociceptive effect was observed for all tested extracts. All these results taken together suggest that *V. cymosa* leaf extracts were able to promote peripheral and central antinociceptive activity mediated by the opioid system. Twenty three substances were isolated and identified in the extracts and include flavonoids (*C*-glucosyl flavones, flavones and flavonols), triterpene acids from ursane and oleanane types, iridoids (free and glucosides), as well as simple phenols.

**Keywords:** *Vitex cymosa* Verbenaceae sensu lato Lamiaceae sensu lato antinociceptive activity medicinal plants flavonoids triterpene acids iridoids

### Introduction

*Vitex* genus, Lamiaceae, (the genus has been formerly included in the Verbenaceae s.l. and recently transferred to the Lamiaceae s.l.) is constituted by small trees or shrubs occurring in tropical and subtropical regions. Many species have been studied in relation their chemistry, which is characterized by the presence of *C*-glucosyl flavones, ecdysteroids, diterpenes and iridoids (Brito, 1992), as well as in relation to their biological activities. Antiinflammatory (Sidhartha et al., 1990; Chawla, et al., 1991), wound healing (Sidhartha et al., 1990) and antiandrogenic (Bhargava, 1989) activities, as well as inhibition of lymphocyte proliferation (You et al., 1998) and smooth muscle relaxant effect (Dayrit et al., 1987; Dayrit & Lagurin, 1994) are among those described in literature. *V. agnus-castus* is actually used in the treatment of pre-menstrual syndrome and menopausal complaints (Mills & Bone, 2000). Despite the fact that the genus is well represented in Brazil with c.a. 35 species (Leitão et al., 2008), as far as we know, only three species have been chemically or pharmacologically investigated so far (Leitão et al., 2008, Gallo et al., 2008; 2006, Sá Barreto et al., 2005, Gonçalves et al., 2001). *Vitex cymosa* Bertero is a small tree widely distributed in the Central and Amazon regions of Brazil, where it is popularly known as “tarumã-do-igapó” and “tarumã-do-alagado” (Correa, 1926). The species are used by the native populations of the State of Maranhão to treat rheumatic pains. In a previous work done by our group (Miranda et al., 2010) extracts from this plant’s barks, collected at Maranhão State, Brazil, were tested in antinociceptive models, such as the tail flick test, to evaluate the central analgesic effect. In this work a significant antinociceptive activity for the dichloromethane extract was observed. The (+)-trans-4-hydroxy-6-propyl-1-oxocyclohexan-2-one
was isolated and determined to be the active principle. In this work we investigated the analgesic properties of the dichloromethane leaf extracts of this plant.

Materials and Methods

Plant material

Leaves of *Vitex cymosa* Bertero ex Spreng., Lamiaceae, were collected in Corumbá, Mato Grosso do Sul State, Brazil in December (in fruit). The plant was identified by Dr. Vali Pott, from Embrapa, Corumbá. A voucher specimen was deposited at Universidade Federal de Juiz de Fora herbarium under number CESJ 11,711.

Plant extracts

Dried and pulverized leaves (2.2 kg) were exhaustively extracted with ethanol. After filtration and concentration under reduced pressure the aqueous residue was sequentially extracted with organic solvents of increasing polarities, to afford the new extracts: dichloromethane (VD), ethyl acetate (VA) and *n*-butanol (VB) extracts, in this order, as well as (LW), which is the water-soluble remaining extract.

Isolation and identification of substances from the extracts

Dichloromethane extract (VD)

Part of VD was first chromatographed on silica gel eluted with CH$_2$Cl$_2$ with increasing amounts of EtOAc. Fractions eluted with CH$_2$Cl$_2$-EtOAc (9:1) were re-chromatographed on Sephadex LH-20 (MeOH) yielding 21. Fractions eluted with CH$_2$Cl$_2$-EtOAc (8:2) were purified by prep. TLC (CH$_2$Cl$_2$-EtOAc, 3:1) to afford 1, and 2. Fractions eluted with CH$_2$Cl$_2$-EtOAc (7:3) were re-chromatographed on Sephadex LH-20 (MeOH), yielding 3. A second fractionation of VD was performed by CC on silica gel eluted with CHCl$_3$, with increasing amounts of EtOAc, and with EtOAc with increasing amounts of MeOH. Fractions eluted with CHCl$_3$ were re-chromatographed on silica gel to afford 4, 13, and a mixture of 15 and 17 (CHCl$_3$-EtOAc 1:1). Fractions eluted with CHCl$_3$-EtOAc 9:1 were re-chromatographed on Sephadex LH-20 (MeOH) affording a mixture of 16 and 18, as well as 5. Fractions eluted with EtOAc-MeOH 8:2 were re-chromatographed in silica gel with the organic phase of a mixture of EtOAc-acetone –H$_2$O (25:8:2), further purified on Sephadex LH-20 and, finally, by prep. TLC to afford 23 and 20. A third fractionation of VD was directly subjected to Sephadex LH-20 (MeOH) and led to the isolation of 19.

Ethyl acetate extract (VA)

VA was chromatographed on silica gel eluted with the organic phase of a mixture of EtOAc-acetone-H$_2$O (25:8:2 to 25:10:5) affording sixteen fractions. From the first two fractions, 12 and 14, were obtained by crystallization, respectively. From fraction 6, 10 and 11 were obtained by prep. HPLC (see conditions below). From fractions 12-13, a mixture of the flavonoids 6, and 7, was obtained by crystallization and, from fraction 15, the flavonoids 8, and 9, were further purified by prep. TLC.

Butanol extract (VB)

VB was fractionated by silica gel CC eluted with CHCl$_3$ with increasing amounts of MeOH affording 21, 22 and 23, as described previously (Santos et al., 2001). From fractions 10-16 (CHCl$_3$-MeOH 9:1) 6, was obtained, and fractions 17-18 afforded 7.

Animals

All experiments were performed with male Swiss mice (20-25 g) obtained from our own animal facility. Animals were maintained in a room with controlled temperature 22±2 °C for 12 h light/dark cycle with free access to food and water. Twelve hours before each experiment animals received only water, in order to avoid food interference with substances absorption. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Biomedical Science Institute/UFRJ - Ethical Committee for Animal Research.

Drugs and extracts administration

All extracts used were administered by oral gavage at concentrations of 10, 30 and 100 mg/kg in a final volume of 0.1 mL. Morphine and dipyrone were used as reference drugs and were administered by oral gavage. The control group was composed by PBS with the same amount of DMSO used in the dose of 100 mg/kg of extracts (0.005%) and had no effect per se. Naloxone was administered i.p. 15 min before oral administration of morphine or extracts.

Acute toxicity

Acute toxicity was determined following the experimental model described by Lorke (1983). A single oral dose of extracts (500 mg/kg) was administered to a group of ten mice (five males and five females). Behavior parameters including convulsion, hyperactivity, sedation,
grooming, loss of righting reflex, increased or decreased respiration, and food and water intake were observed over a period of five days. After this period animals were killed by cervical dislocation, stomachs were removed, an incision along the greater curvature was made, and the number of ulcers (single or multiple erosion, ulcer or perforation) and hyperemia was counted.

Acetic acid-induced abdominal writhing

Mice were used according to Matheus et al. (2006). Briefly, the total number of writhings following intraperitoneal (i.p.) administration of 2% (v/v) acetic acid (AA) was recorded over a period of 20 min, starting 5 min after AA injection. The animals were pretreated orally with extracts or vehicle, 60 min before administration of AA.

Formalin test

The procedure was similar to the method described by Gomes et al. (2007). Animals received the injection of 20 μL of formalin (2.5% v/v) into dorsal surface of the left hind paw. Immediately, the time that the animal spent licking the injected paw was recorded. The nociceptive response develops two phases: 0 to 5 min after formalin injection (first phase, response to neurogenic pain), and 15 to 30 min after formalin injection (second phase, response to inflammatory pain). For comparison purposes, the IC50 of morphine was calculated as 1.7 mg/kg. The animals were pre-treated with oral dose of extracts (10 to 100 mg/kg), vehicle or morphine (1.7 mg/kg), 60 min before administration of formalin.

Tail-flick test

The procedure used was similar to Matheus et al. (2006). Briefly, mice tails were immersed on a water bath set at temperature of 50±1 °C. The time necessary for the mice to withdraw the tail, in seconds (named reaction time) was registered at 20, 40, 60, 80, 100 and 120 min after administration of V. cymosa extracts (10, 30 and 100 mg/kg) or morphine (2.3 mg/kg). For comparison purposes, the IC50 of morphine was calculated as 2.3 mg/kg. Baseline was considered as the mean of reaction time obtained at 60 and 30 min before administration of V. cymosa extracts or morphine, and was defined as normal reaction of animal to the temperature. Increase in baseline (%) in tail flick test. Statistical significance between groups was performed by the application of analysis of variance ANOVA followed by Bonferroni’s test. p values less than 0.05 (p<0.05) were used as the significant level.

Results

Phytochemical identification of known compounds

Phytochemical investigation of Vitex cymosa leaf extracts led to the isolation and identification of a number of known iridoids, triterpenes and flavonoids, which are secondary metabolites commonly found in the genus. Their chemical structures were identified by means of one and two dimensional 1H and 13C NMR techniques, as well as by mass spectral data, which were in accordance with previously reported data. From the dichloromethane extract, VD, the iridoids tarumal, 21 (Santos et al., 2001) and agnuside, 23 (Boros & Stermitz, 1990); the flavonoids kampferol, 1, 3'-O-methyl-luteolin, 2, luteolin, 3, pachypodol, 4, and apigenin, 5 (Agrawal, 1989); and the triterpenes 2α,3α-hydroxy-oleanolic acid, 15, 2α,3β-hydroxy-oleanolic acid, 16, 2α,3α-hydroxy-ursolic acid, 17, 2α,3β-hydroxy-ursolic acid, 18, 2β,3β,19α-hydroxy-ursolic acid, 19, (Mahato & Kundu, 1994), and 28-O-glucosyl-2α,3α,19α-hydroxy-ursolic acid ester, 20 (Seto et al, 1984), were isolated. From the ethyl acetate extract, VA, orientin, 6, iso-orientin, 7, vitexin, 8, iso-vitexin,
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9, 2”-O-caffeoyl-orientin (Leitão & Delle Monache, 1998), 11, 2”-O-p-hydroxybenzoyl-orientin, 10, (Leitão & Delle Monache, 1998) procatechuic acid, 12, vanillic acid, 13, and p-hydroxybenzoic acid, 14, were isolated. From the n-butanol extract, VB, orientin, 6, iso-orientin, 7, tarumal, 21, viteoid II, 22 and agnuside, 23, (Santos et al., 2001) were isolated.

**Analgesic activity**

To test the antinociceptive effects of *Vitex cymosa* extracts were used models of peripheral, spinal and supra-spinal analgesia.

**Acute toxicity**

In order to evaluate the possible toxic effects of *V. cymosa* extracts, mice were treated orally with 500 mg/kg of each of them. Treated mice did not present any behavioral alterations. Also, stomachs were removed from the animals and examined under dissecting microscope. No lesions or bleedings were observed (data not shown).

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of writhings. At the doses of 30 and 100 mg/kg there was significant effect to all three fractions, VD, VA and VB (Figure 1).

**Formalin test**

Intraplantar injection of formalin evoked a characteristic biphasic licking response. For the control group, the duration of licking in the 1st phase was 48.8±11.7 s, and for the 2nd phase was 141.6±33.0 s. As shown in Figure 2, pre-treatment with different doses of VD, VA or VB had no significant effect against the 1st phase. However, 2nd phase was markedly reduced by VD, with maximal effect observed with 100 mg/kg. VA and VB developed significant effect on the 2nd phase only at doses of 30 and 100 mg/kg. However, VA effects were lesser pronounced than those of VB or VD. Results obtained demonstrated that VD was the most efficient in reducing the time that the animal spent licking the formalin-injected paw.

**Tail-flick and hot plate tests**

In view of the fact that *V. cymosa* extracts demonstrated significant antinociceptive activity on acetic acid-induced writhing and formalin tests, we decided to test its action on spinal and supraspinal models of pain - tail flick and hot plate tests. As shown in the Figure 3, all three extracts induced an increase in the time to withdrawal the tail from the bath (demonstrated as increase in baseline). Morphine (2.3 mg/kg) produced its maximum antinociceptive effect between 60 and 100 min after oral administration, with maximal values reaching 50% increase in baseline at 100 min. The antinociceptive action of morphine lasted about 120 min. Dichloromethane extract (VD) had similar curves to all doses tested, and reached maximal response 80 minutes after administration. Although VD induced an increase in the baseline, this was not a significant effect. VB extract developed a dose-response curve, with very little differences between each tested dose (10, 30, and 100 mg/kg). Twenty minutes after VB administration a significant increase in the baseline could already be observed with all doses. At 120 min, the antinociceptive effect of morphine began to decline, whereas the effect of VB continued at high levels within 120 min, and lasted 2 h after a single oral administration (data not shown). The response to VA was quite different. Significant antinociceptive activity was observed at the dose of 100 mg/kg. This effect appeared after 60 minutes of administration and was maintained until the end of the experiment.
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Figure 3. Effects of *Vitex cymosa* leaf extracts in the tail flick response. Animals were pre-treated with different doses of extracts or vehicle by oral administration. Groups are: vehicle (▲), morphine (2.3 mg/kg, ■), extracts at 10 (○), 30 (△), or 100 mg/kg (Δ). The results are presented as mean±SD. (n=10) of increase in baseline (%) calculated as described on methods section. Statistical significance was calculated by ANOVA followed by Bonferroni’s test.*p*<0.05 when compared with vehicle-treated mice. Where no error bars are shown is because they are smaller than the symbol.

**Mechanism of action**

To test the hypothesis that *V. cymosa* could have supraspinal analgesic effect, the hot plate model was used. Upon oral administration of *V. cymosa* extracts (at 100 mg/kg), no antinociceptive effect was observed (data not shown).

**Discussion**

The purpose of this paper was to establish a scientific basis for one of the traditional uses of *Vitex cymosa*, which is against rheumatic pains. All tested extracts significantly inhibited the abdominal contortions induced by acetic acid in mice. Acetic acid causes an increase in peritoneal fluid levels of prostaglandins, involving in part, peritoneal receptors (Deraedt et al., 1980) and inflammatory pain by inducing capillary permeability (Amico-Roxas et al., 1984). Although the writhing test has poor specificity (Le Bars et al, 2001), it is a very sensitive method of screening antinociceptive effects of compounds. The acetic acid-induced writhing method is able to determine antinociceptive effects of compounds and dose levels that might appear inactive in other methods (Gene et al., 1998). This results indicate that *Vitex cymosa* presents antinociceptive activity. Despite the assayed extracts possess very different chemical compositions, they all consist of a varied class...
of compounds for which some kind of antiinflammatory and/or analgesic activity has been described in literature. Flavonoids, for example, were detected in all the assayed *Vitex cymosa* extracts (VD, VA and VB). Several studies have reported the antinociceptive effects of flavonoids (Thirugnanasambantham et al., 1990; Pathak et al., 1991; Ramesh et al., 1998; Toker et al., 2004), and, of particular interest is that one reporting the antinociceptive activity of luteolin (Block et al., 1998), which was isolated from VD extract. The antinociceptive activity of this flavonoid has been evaluated by Block et al. (1998) in the acetic acid-induced abdominal constriction model in mice. Pharmacological analysis revealed that luteolin caused gradual and significant inhibition of abdominal writhings. When compared to acetylsalicylic acid, acetaminophen, dipyrone and indomethacin, by using the same pharmacological procedure, luteolin was about 8-16-fold more active than these standard drugs (Block et al., 1998). Luteolin was also detected, among other flavonoids, in extract of *Vitex rotundifolia* fruits (Emi et al., 1998). Thirugnanasambantham et al. (1990) showed that various synthetic flavone derivatives, structurally related to luteolin, displayed significant analgesic activity and suggested that some of them could be acting through an opiate-like mechanism. However, whether or not the antinociceptive action of luteolin involves such mechanism requires further investigations. Luteolin, together with apigenin, orientin and vitexin - which are present in *Vitex cymosa* extracts, were also isolated in a bioassay-guided fractionation of an antinociceptive extract from *Zea mays* stigmas (corn silk), which activity was evaluated by the p-benzoquinone-induced writhings and the formalin-induced edema models (Abdel-Wahab et al., 2002).

Concerning the isolated triterpenes, they are all hydroxyl-derivatives of ursolic and oleanolic acids, for which antinociceptive activity has been demonstrated previously by our group (Costa et al., 2003). The presence of iridoids in the active extracts is also noteworthy. Although there are some reports on the anti-inflammatory activity for this class of compounds (Ghisalberti, 1998; Recio et al., 1994; Singh, et al., 1993) including a report from *Vitex* genus (Suksamrarn et al., 2002) pharmacological data on analgesic activity for this class of compounds is scarce. There is one report on the analgesic properties of agnuside (Okuyama et al., 1998) but no reports for the iridoids tarumal or vitexoid II. It is interesting to note that *Vitex cymosa* is used in the Brazilian traditional medicine to treat rheumatoid arthritis. Also noteworthy, is the fact that one of the most prescribed phytomedicines to treat degenerative diseases of the musculoskeletal system nowadays is the South African plant *Harpagophytum procumbens* D.C., known as Devil’s Claw, which active principles are claimed to be iridoid glucosides (Stewart & Cole, 2005; Baghdikian et al., 1997).

The neurogenic and inflammatory pain was evaluated using formalin test. Drugs that act primarily as central analgesics inhibit both phases while peripherally acting drugs inhibit only the phase 2 (Rosland et al., 1990). We observed that none of *V. cymosa* extracts was able to decrease the time that the animal spent licking the injected paw on the first phase, while the second phase was inhibited by all tested extracts, specially VD. The presence of dihydroxy- and trihydroxy derivatives of ursolic and ursolic acids in this extract is noteworthy, since, in a previous work from our group, we have demonstrated the analgesic potential of a mixture of ursolic, oleanolic and micrometric acids, in the same models reported here (Costa et al., 2003). Inhibition of the second phase alone, (Rosland et al, 1990), suggests a peripheral antinociceptive activity of *Vitex cymosa*. These results corroborate with the inhibitory effect of *V. cymosa* extracts on the acetic acid-induced writhing response.

Several studies demonstrated that opioids can produce analgesia through peripheral mechanisms after inflammation of tissues (Stein et al., 1989, 1996). *V. cymosa* extracts produced antinociceptive effect in the formalin test in mice after oral administration. These findings are in agreement with others studies where antinociceptive effects of peripherally acting µ opioid agonist, loperamide, were detected in the formalin test after subcutaneous administration (Shannon & Lutz, 2002). The peripheral antinociceptive activity produced by opioid system is based on the opening of potassium channels, causing hyperpolarization in nociceptive afferents. (MILAN, 1999).

In order to verify if *V. cymosa* extracts could develop central antinociceptive effect, the models of tail flick (spinal analgesia) and hot plate (supra-spinal analgesia) were used. Extracts from the leaves of *V. cymosa* were able to increase latency time in the tail flick. In this model, after the thermal stimulus applied to the tail, superficial tissue layers are broadly stimulated and a tail withdrawal reflex is evoked, which characterizes spinal mechanism and involves sensory and motor components (Green & Young, 1951). The rapid onset with an early maximum effect, which waned very shortly after drug administration, is characteristic of the time course of action of opioid agonists (e.g. morphine, fentanyl) that mediate analgesia via central mechanisms and were described under both normal and inflammatory conditions (ACETO et al., 1997, MILLAN et al., 1987). Administration of VD, VB, and VA produced different time course of action. VD did not evoke a significant effect and, the antinociceptive effect of VA was observed only with a higher dose. VB, however, showed a rapid onset and longer-lasting action than
the centrally acting opioids, with higher effects. In this way, these results presented in the tail flick test show spinal antinoceptive activity by the *V. cymosa* extracts. The spinal antinoceptive activity is associated with a reduction in the release of excitatory neurotransmitters (glutamate, substance P etc.) at the spinal cord, and hyperpolarization of ascending neurons (Besson & Chaouch, 1987). In the hot plate test, the antinoceptive activity was not observed probably due to the difficulty in crossing the blood-brain barrier by the active molecules of *V. cymosa* extracts, revealing a probable supraspinal antinoceptive effect if the active molecules were administered by intracerebroventricular route. (Grisel & Mogil, 2000). The supraspinal antinoceptive activity is related to activation of the endogenous inhibitory control of pain. (Saadé & Jabbur; 2008).

The reduction of the antinoceptive effect of the extracts produced by the prior administration of naloxone, a non-selective opioid antagonist, shows the participation of the opioid system in the mechanism of action of extracts of *Vitex cymosa*, proving the involvement of this system in the peripheral, spinal and supraspinal antinoceptive actions. Together, these data are consistent with the interpretation that systemically administered morphine exerts its analgesic effects by interacting with both central and peripheral opioid receptors (Perrot et al., 2001; Shannon & Lutz, 2002).

**Conclusions**

The lactone (+)-trans-4-hydroxy-6-propyl-1-oxocyclohexan-2-one which was determined to be the active principle of *V. cymosa* dichloromethane bark extracts could not be isolated from the dichloromethane extracts from the leaves. The exact mechanism of action and the active principles responsible for the analgesic activities described here remain to be confirmed, but taking all these in vivo results together, it can be suggested that *Vitex cymosa* leaf extracts were able to promote peripheral and central antinoceptive activity mediated by the opioid system.

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**References**


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*Correspondence*

Suzana Guimarães Leitão
Departamento de Produtos Naturais e Alimentos, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro
CCS, Bloco A, 2o andar salas 4 e 10, Ilha do Fundão, 21941-590, Rio de Janeiro-RJ, Brazil
sgleitao@pharma.ufrj.br
Tel/Fax: +55 21 2562 6413/6425