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IL FARMACO 59 (2004) 55–61
IL FARMACO
www.elsevier.com/locate/farmac

Analgesic and anti-inflammatory activities evaluation of (-)-*O*-acetyl, (-)-*O*-methyl, (-)-*O*-dimethylethylamine cubebin and their preparation from (-)-cubebin

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Received 27 July 2003; accepted 29 July 2003

Abstract

The anti-inflammatory and antinociceptive effects of the acetylated (**2**), methylated (**3**) and aminated (**4**) derivatives of cubebin (**1**), obtained by its reaction with acetic anhydride, methyl iodide and dimethylethylamine chloride, respectively, were investigated, using different animal models. The compound (**2**) was the most effective anti-inflammatory one in the carrageenin-induced paw edema in rats and was the only one which showed dose–response correlation for this assay with $r = 0.993$ and $Y = 64.58x + 0.22$. Besides, compounds (**2**) and (**4**) were more effective than cubebin in inhibiting acetic acid-induced writhing in mice, producing dose–response correlation with doses of 10, 20 and 30 mg/kg, respectively. Regarding the hot plate and the cell migration tests in rats, none of the four tested compounds showed activity. Overall, the results showed that the acetylation and amination of cubebin were efficient in enhancing its analgesic activity, as well as its anti-inflammatory activity.

Keywords: (-)-Cubebin; (-)-***O***-acetyl cubebin; (-)-***O***-methyl cubebin; (-)-***O***-dimethylethylamine cubebin; Anti-inflammatory activity; Analgesic activity; *Piper cubeba*

1. Introduction

Prostaglandins (PGs) are well known to be mediators of inflammation, pain and swelling. Cyclooxygenase (COX) enzymes are responsible for its production, starting from arachidonic acid. The compounds produced by COX pathway are well accepted as mediators of the inflammatory response. In this sense, the non-steroidal anti-inflammatory drugs (NSAIDs) are well known to act on COX enzymes. It has been recently found that there are two isoforms of COX, which differ in their basal expression, tissue localization and induction during inflammation. Hence, differences in the pharmacological profiles of various NSAIDs might be accounted for the different degrees of selectivity for COX-1 and COX-2. Besides, the potency and selectivity of NSAIDs seems to be direct involved in their gastric, renal and hepatotoxicity [1].

Cubebin was initially isolated from the crude hexane extract of the leaves of *Zanthoxylum naranjillo* Griseb by Bastos et al. [2] and was evaluated for analgesic and anti-inflammatory activities [3]. This compound belongs to the dibenzylbutyrolactone lignan group [2], which is widely distributed in the plant kingdom [4] and has been investigated by researchers from different fields of expertise, leading to the isolation of several compounds holding interesting biological activities.

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doi:10.1016/j.farmac.2003.07.012

The aim of this work was to obtain compounds (**2**), (**3**) and (**4**) by respective acetylation, methylation and amination of the hydroxyl group of (**1**) and to evaluate their possible analgesic and anti-inflammatory activities in an attempt to improve the biological activities previously described for cubebin, i.e. its analgesic, anti-inflammatory [5] and trypanocidal [3] properties. Also, Borsato et al. [6], reported the isolation of (-)-***O***-methyl cubebin from *Lychnophora ericoides* roots, as well as its activity on writhes in mice induced by acetic acid.

2. Experimental

2.1. Chemistry

2.1.1. Drugs and reagents

Acetic anhydride, dry tetrahydrofuran (THF), chloroform, sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), powdered metallic magnesium, dimethylethylamine chloride, metallic sodium bars and magnesium sulfate ($MgSO_4$) were supplied by Merck Co., Darmstadt, Germany. The absolute ethanol, dry pyridine, dry dichloromethane and sodium hydride (NaH) were acquired from Acros Co., New Jersey, USA. Toluene, hexane and ethyl acetate were supplied by Mallinckrodt Co., Xalostoc, Mexico. Silica gel aluminum sheet plates 0.25 mm F-254 were bought from Merck Co. (Darmstadt, Germany). Solvents used in the reaction were generally distilled and dried before use.

Indomethacin (Prodome Co.) and kappa carrageenin type III (Iota-Fluka-Biochemika Co.) were also acquired from the market. Morphine sulfate (Innovatec Co.) was kindly provided by the pharmacy of the University Hospital of Ribeirão Preto and CH_3I (Acros Co.) was provided by the Medicinal Chemistry Laboratory of the School of Pharmacy of Ribeirão Preto.

2.1.2. General experimental procedures

Optical rotations were measured at λ 589 nm on a Shimidt-Haenseh polartronic HH8 polarimeter using 1.0 cell. IR spectra were recorded on a Nicolet FT-IR Protegé 520 instrument. NMR spectra were recorded on Brucker ARX 400 spectrometer. Samples were dissolved in $CDCl_3$, and the spectra were calibrated at the solvent signals at δ 7.26 (^1H) or δ 77.0 (^{13}C). Splitting patterns are as follows: *s*, singlet; *d*, doublet; *t*, triplet; *q*, quadruplet; *m*, multiplet. The reaction was monitored by thin layer chromatography (TLC). The developed TLC chromatograms were observed under ultraviolet light (254–265 nm). High performance liquid chromatography (HPLC) Shimadzu, bearing LC-10ADVP pump, SPD-M10AVP arranges of diode detector and DGU-14A degasificator was used for purity determination. The analysis on a C-18 CLC-ODS Shimadzu column, using increasing gradient of MeOH: MeOH/H_2O (50%) and MeOH (100%) at 30 min was performed. An Ugo Basile Plethysmometer (no. 7140) and Ugo Basile Hot Plate (no. 7280) were used for the biological assays.

*2.1.3. (-)-Cubebin (**1**) isolation*

Powdered seeds of *Piper cubeba* L., bought from the market, were exhaustively extracted by maceration with 96% ethanol. The concentrated crude extract was partitioned between the phases of hexane and methanol/water (9:1), furnishing 430 g of the dried methanol/water fraction, which were submitted to repeated column chromatography over 1.0 kg of silica gel (12 × 120 cm). Elution with increasing proportions of hexane, dichloromethane and ethyl acetate yielded six fractions. Then, cubebin-rich fractions (1:1 hexane/dichloromethane and 100% dichloromethane) were submitted to repeated crystallization in hexane/acetone to furnish crystalline cubebin (37 g). Its chemical structure was confirmed by its ^1H NMR and IR data in comparison with the one published in the literature [7]. Its purity was estimated at 99% by both HPLC and spectral data analysis.

*2.1.4. Preparation of (-)-O-acetyl cubebin (**2**)*

(-)-***O***-acetyl cubebin was prepared by reacting 50 mg of cubebin (0.14 mmol) and 3 ml of acetic anhydride in 0.8 ml of dry pyridine. After the end of the reaction time, the mixture was poured into a flask containing toluene and evaporated under reduced pressure to eliminate pyridine. Afterwards, dichloromethane was added and evaporated under reduced pressure to eliminate toluene residue. This process furnished yellow oil containing 85% (34.25 mg/0.086 mmol) stoichiometric yielding of the product, which was purified over silica gel column by using hexane/ethyl acetate (3:2). Its purity was estimated at 98% by both HPLC and spectral data analysis.

*2.1.5. Preparation of (-)-O-methyl cubebin (**3**)*

A solution of cubebin (2.8 mmol) in 5 ml of THF was added to the suspension of NaH (1 g washed with hexane, free of paraffin grease) in dried THF (50 ml) [8] and the mixture was stirred for 30 min at room temperature. Then, methyl iodide (1 ml) was added and the reaction medium was stirred overnight under N_2 atmosphere. Excess of NaH was decomposed by adding aqueous methanol (1:1). Diluted HCl was added and the medium was partitioned three times with ethyl acetate (3 × 30 ml). The organic phase was neutralized with 5% $NaHCO_3$ aqueous solution (2 × 20 ml), 10% NaCl aqueous solution (3 × 20 ml) and 5% $NaHCO_3$ aqueous solution (2 × 20 ml), dried with $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure, yielding a brown residue, which was purified by silica gel column using hexane/ethyl acetate (4:1) as eluent, furnishing colorless oil with 91.4% (95.00 mg/2.56 mmol) stoichiometric yielding. Its purity was estimated at 98% by both HPLC and spectral data analysis.

*2.1.6. Preparation of (-)-O-dimethylethylamine cubebin (**4**)*

Cubebin 0.06 g (0.1403 mmol) in 1 ml of ethanol was added into a solution of sodium ethoxide (5 ml of ethanol, 2 EqM of Na^0) under reflux for 2 h. After that, 0.024 g (0.204 mmol) of dimethylethylamine chloride was added, the

reaction was monitored by TLC and the reflux was carried out for additional 6 h. At the end of the reaction, 5 ml of water was added, the phases were separated and the organic phase was extracted with ethyl acetate (3 × 10 ml). The organic phase was washed with 10% NaCl aqueous solution (3 × 10 ml), dried with $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by chromatography on a silica gel column eluted with methylene chloride. The product was obtained as dark yellow oil and its purity was estimated at 99% by both HPLC and spectral data analysis.

2.2. Pharmacology

2.2.1. Analgesic and anti-inflammatory activities evaluation

2.2.1.1. Animals. Male Swiss albino mice (20–25 g) were used for the writhing test. Male Wistar rats (160–170 g) were used for the hot plate test, for the paw edema assay as well as for the cell migration test. The animals were housed in groups of five in standard cages at room temperature (25 ± 3 °C) with both food and water ad libitum. Twenty-four hours before the experiments they were transferred to the laboratory and were maintained only with water ad libitum.

2.2.1.2. Administration of the compounds. All compounds were administered at 10, 20 and 30 mg/kg, except indomethacin, which was administered at 5 mg/kg. All of them were previously suspended in a 5% Tween–saline solution (negative control) and administered orally 30 min before all experiments. Morphine sulfate (4 mg/kg), used for the hot plate test (positive control), was administered subcutaneously, 5 min before the test.

2.2.1.3. Analgesic activity. *Writhing test.* This test was carried out by using the method described by Koster et al. [9]. The writhes were induced by intraperitoneal injection of 0.6% acetic acid (v/v) (80 mg/kg) into a group of five mice. The number of muscular contractions was counted for 20 min after acetic acid injection. The data represent the average of the total number of writhes observed at 5 min intervals and are expressed as writhes inhibition.

Hot plate test. The hot plate test was performed following the method of Woolfe and MacDonald [10], adapted for rats. The animals were placed on the equipment kept at 55 ± 1 °C. The reaction time was noted by observing either the licking of the hind paws or the rotation movements at 10, 20, 30, 40, 50 and 60 min after drug administration. The data represent the mean reaction time for the animals and the results are expressed as analgesic index [11].

2.2.1.4. Anti-inflammatory activity. *Rat paw edema.* The used method was described by Winter et al. [12] in 1962. A dose of 0.1 ml (100 μg) of carrageenin was injected into the right paw and 0.1 ml of saline solution was injected into the left paw. Foot volume was measured by plethysmography (Ugo Basile Plethysmometer no. 7140) at 1 h intervals after the inflammatory stimulus, for 5 h [13], but the activity was acknowledge only for the 3rd hour, in which the maximum edema occur. The results were obtained by measuring the volume difference between the right and the left paws in comparison to both the negative control group, treated with saline solution and the positive control group, treated with indomethacin.

Acute carrageenin-induced inflammatory reaction in the peritoneal cavity of rats. Four different groups of five animals were treated orally with (**2**), (**3**) and (**4**) (30 mg/kg) and with the standard drug dexamethasone (0.5 mg/kg, positive control), respectively. The negative control group received orally only a 5% Tween–saline solution. One hour later, a volume of 3 ml of carrageenin 100 μg/ml in saline sterile solution was injected into the rat's peritoneal cavity. Cell migration was quantified 4 h after the injection of carrageenin, according to the method described by Souza and Ferreira [14].

2.3. Statistical analysis

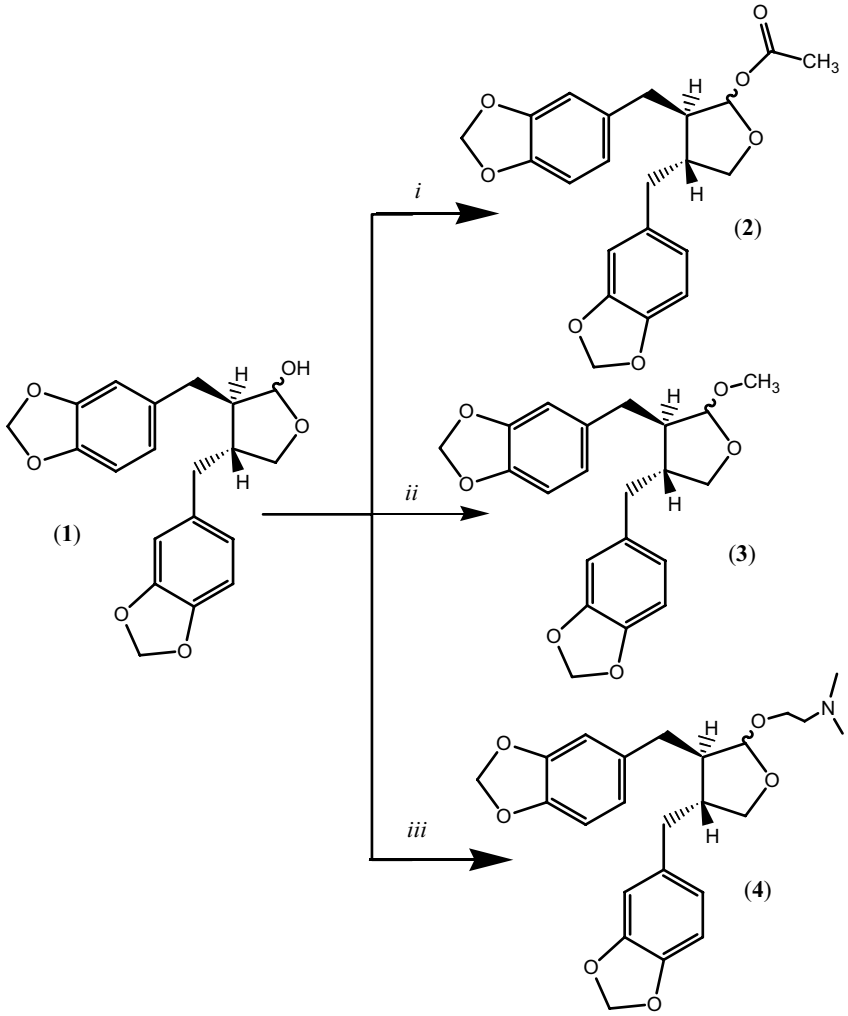
Data were analyzed statistically by one-way ANOVA and Dunnett's multiple comparison test, with the level of significance set at $P < 0.05$ and $P < 0.01$.

3. Results and discussion

The synthesis of (-)-cubebin (**1**) derivatives, (-)-***O***-acetyl cubebin (**2**), (-)-***O***-methyl cubebin (**3**) and (-)-***O***-dimethylethylamine cubebin (**4**) are outlined in Scheme 1.

Changing the chemical structure of active compounds has been the goal of medicinal chemists over the last few years aiming to improve the biological activities, to reduce the side effects as well as to obtain compounds holding new valuable biological activities [15]. Likewise, (**1**), a dibenzylbutyrolactone lignan, was modified in its original structure to yield (**2**), (**3**) and (**4**) derivatives by respectively acetylation, methylation and amination of its lactol group in an attempt to improve its analgesic and anti-inflammatory activities, by introducing different groups at the oxygen at carbon 9 (Table 1).

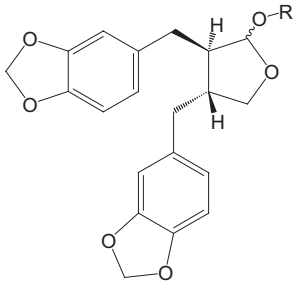
The acetylating reaction was carried out using dry pyridine to withdraw the hydrogen of the lactol group and to generate the acetyl group from acetic anhydride to be inserted in the place of hydrogen. Considering that lignan is highly sensitive in acid medium, which opens its lactol ring, an alkaline medium was used to eliminate this effect and to produce maximum yields. The reaction was carried out at room temperature for 24 h and the addition of toluene allowed totally elimination of pyridine by its evaporation under reduced pressure to produce yellow oil (Table 2). As done for the acetylating reaction, NaH was used as reagent to generate the oxygen anion to attack the carbon of methyl iodide to form the methylated product [16]. Using NaH, the reaction



Scheme 1. Synthesis of cubebin derivatives. *i*: Ac_2O, py, rt 24 h (**2**); *ii*: MeI, NaH, THF, rt, N_2, over night (**3**); *iii*: EtOH, Na^0, $ClCH_2CH_2N(CH_3)_2$, ▲ 6 h (**4**).

was performed without rupture of the lactol ring but, on the other hand, the use of BF_3 etherate/methanol opened the lactol ring because liberation of HF occurs.

Table 1
Chemical structure of (**1**) and its derivatives



1 (-)-Cubebin	-H
2 (-)- O-acetyl cubebin	-(C=O)-H3
3 (-)- O-methyl cubebin	-CH3
4 (-)- O-dimethylethylamine cubebin	-(CH2)2 – N(CH3)2

Therefore, the synthesis of (**4**), carried out by the Williamson synthesis, an important process of ether preparation, was achieved without cleaving lactone cubebin ring [17] (Scheme 1).

Crystallization solvent for cubebin, yields (%) and $[\alpha]_D^{t°C}$ are given in Table 2. The structures of the compounds were elucidated by IR and ^1H NMR data analysis (Table 3).

Regarding the administration of carrageenin, it produced a significant edema in the rat paws, which was more intense in the animals treated only with a 5% Tween in 0.9 % saline solution at the 3rd hour after carrageenin injection. Hence, (**2**), (**3**) and (**4**) (30 mg/kg), administered orally 30 min before the injection of carrageenin, inhibited the formation of edema by 68%, 65% and 70%, respectively, 3 h after the injection of the inflammatory stimulus in comparison with negative control group, while cubebin inhibited 57% (Fig. 1, $P < 0.05$). From the tested compounds, only (**2**) produced a dose–response correlation for the paw edema test, with $r = 0.993$ and $Y = 64.58x + 0.22$ (Fig. 2). This might be partially explained by the high specificity of the acetylated anti-inflammatory compounds to the serine group of the

Table 2
Crystallization solvents, melting points, % yields and formula of the compounds

Compounds	Cryst. Solvent	Melting point (°C)	Yield (%)	$[\alpha]_D^{t°C}$
1	Hexane/acetone	130-131	9*	–8.12° (25 °C) (*c* 0.46, $CHCl_3$)
2	–	oil	85	–123.33° (26 °C) (*c* 0.0057, $CHCl_3$)
3	–	oil	91	–26.85° (26 °C) (*c* 0.02, $CHCl_3$)
4	–	oil	71	–4.38° (25 °C) (*c* 0.067, $CHCl_3$)

* Yield of the pure crystalline cubebin.

Table 3
IR and ^{1}H NMR spectral data of the compounds

Compounds	IR (KBr and film[a]) ν (cm^{-1})	^{1}H NMR (methanol-d_4) δ ppm (*J* in Hz)
1	3500, 3300 (–OH), 2940, 2900 (O–C–O), 2000, 1600 (aromatic ring), 1489, 1442 (bond C=C of ring), 1038 (O–C–O), 810, 772 (C–H), 663 (C= bond), 640 (–OH)	6.80–6.40 (m; 6H; Ar-H), 6.80–6.40 (m; 4H; H-7), 6.80–6.40 (m; 4H; H-7′), 2.30–1.90 (m; 1H; H-8), 2.90–2.50 (m; 1H; H-8′), 5.20 (s; 1H; H-9), 4.05 (dd; 1H; H-9′) and 3.72 (dd; 1H; H-9′), 6.1 (s; 4H; –O–CH_2–O–)
2	3068, 3018, 2903 (O–C–O), 2043, 1608 (aromatic ring), 1750 (C=O), 1494, 1443 (bond C=C of ring), 1190, [C–(C=O)–O], 812, 759 (C–H), 667 (C= bond)	Ar-H (6.65–6.40; m, 6H); C-8 (2.30–1.95; m, 1H); C-9′ 4.04 (dd, 1H) and 4.00 (dd, 1H); –O–CH_2–O– (5.90; s, 4H); –CH_3 (1.99; s, 3H)
3	3118, 2999 (O–C–O), 2921, 1619 (aromatic ring), 1424, 1383 (bond C=C of ring), 1150–1085 (O–C–O), 1.075–1.020 (C–O), 816, 757 (C–H), 667 (C= bond)	Ar-H (6.65–6.40; m, 6H); C-8 (2.10–1.99; m, 1H); C-9 (4.65; d, 1H); C-9′ [3.8 (t; 8.3 Hz) and 3.7 (8.5 and 6.8 Hz; 1H)]; –O–CH_2–O– (5.90; s, 4H); –CH_3 [2.00; 3H and 1.99; s, 3H]
4	3118, 2999 (O–C–O), 2921, 1619 (aromatic ring), 3.650–3.450 (N–R_3) 3100–2850 (–CH_3 and –CH_2), 1580 (–N–H), 1424, 1383 (bond C=C of ring), 1258, 1028 (O–C–O), 1250 (C–N) 816, 757 (C–H), 667 (C= bond)	Ar-H (6.70–6.40; m, 6H); H-8 (2.54; m, 4H); C-8′ (2.2; d, 1H); H-9 (5.09; d, 1H); H-9′ (3.71; d, 2H); –O–CH_2–O– (5.90; s, 4H); –O–CH_2– (3.47; d, 2H); –CH_3–N– (2.27; d, 6H); –CH_2–N– (2.2; d, 1H)

s, singlet; dd, doublet of doublet; m, multiplet.

COX, causing its inhibition, as observed by Carvalho et al. [18]. Moreover, it can be observed for the obtained compounds that the introduction of the aliphatic groups to the lactol ring lead to higher anti-inflammatory activity in comparison with (**1**). Also, it is known that the third phase of the edema induced by carrageenin, in which the edema reaches its highest volume, is characterized by the presence of PGs and other compounds of slow reaction [19]. Ueno et al. [20] found that the injection of carrageenin into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of PGI_2 and other autacoids, which are responsible for the formation of the inflammatory exudate. Multiple mechanisms regulate the induction of COX-2, especially in drastic animal models. Therefore, it is suggested that the mechanism of action of (**1**) derivatives may be related to PG synthesis inhibition, as described for the anti-inflammatory mechanism of indomethacin in the inhibition of the inflammatory process induced by carrageenin [21]. Moreover, the cell migration into the damaged tissue is an important step in the inflammatory process. Therefore, using carrageenin as a stimulus, it was possible to produce an acute inflammatory response after 4 h into the peritoneal cavity of rats, with a large number of polymorphonuclear cells in the exudate. However, the administration of (**1**) derivatives did not reduce the cell migration, once the results were similar to the one obtained for the negative control group (5% Tween–saline solution). On the other hand, dexametasone provided 80% of inhibition. According to Larsen and Henson [22], the direct participation of the PGs in the chemotactic response is unlikely to occur. However, leukotriene B_4 is a potent chemotactic compound for the polymorphonuclear leukocytes, and lipoxygenase, an enzyme for the leukotriene synthesis, is very sensitive to the anti-inflammatory steroidal drugs, such as dexametasone [23].

In addition, the classification of antinociceptive drugs is usually based on their mechanism of action either on the central nervous system or on the peripheral nervous system [24]. With respect to the writhing test, the research group of Deraedt et al. [25] described the quantification of PGs by radioimmunoassay in the peritoneal exudates of rats, obtained after intraperitoneal injection of acetic acid. They found high levels of PGs, $PGE_{2\alpha}$ and $PGF_{2\alpha}$, during the first 30 min after acetic acid injection. Nevertheless, it was found that the intraperitoneal administration of acetic acid induces the liberation not only of PGs, but also of the sympathetic nervous system mediators [26]. Thus, from the results obtained for the writhing test using acetic acid, it can be observed that all tested compounds presented a dose–response correlation at the doses of 10, 20 and 30 mg/kg. Moreover, compounds (**2**) and (**4**) were more active in regard to inhibition of writhings in mice by 62.0% and 54.0%, respectively, in comparison to (**1**), which inhibit the writhings by 23.0% (Fig. 3). Its is important to point out that the results observed in this assay for compound (**3**) are similar to the findings of Borsato et al., 2000 [6], in which cubebin was slightly more active than methyl cubebin. Our results showed that methyl cubebin was slightly more active than cubebin at the dose of 30 mg/kg. However, despite these results, the differences are not statistically significant by the applied statistic test.

It is known that NSAIDs usually do not increase the pain threshold in normal tissues, like local anesthetics and narcotics do [27]. Nevertheless, the hot plate test was undertaken to verify if the (**1**) derivative compounds could show any central analgesic effect. As expected, the results obtained for cube-

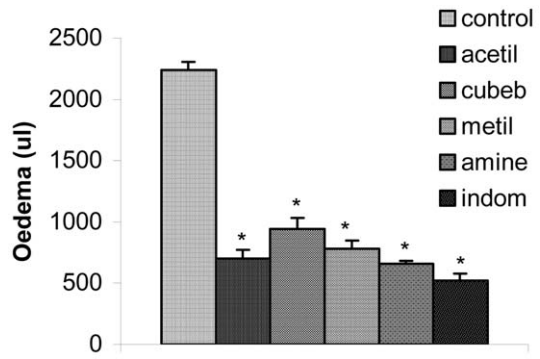


Fig. 1. Effect of the administration of cubebin, (-)-***O***-methyl cubebin, (-)-***O***-acetyl cubebin, (-)-dimethylethylamine cubebin and indomethacin, all at 10 mg/kg on the rat paw edema induced by carrageenin injection (100 μg/paw). The paw edema volume was taken at the 3rd hour after carrageenin injection. The bars chart means the average ± S.D. of $n = 5$ per group. $^*P < 0.05$.

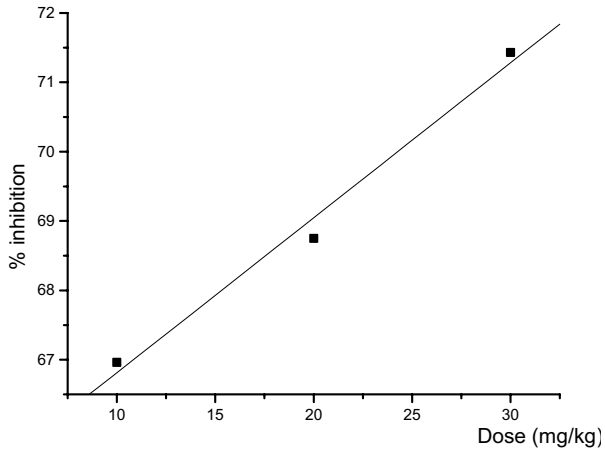


Fig. 2. Effect of the administration of (-)-***O***-acetyl cubebin at 10, 20 and 30 mg/kg on the rat paw edema induced by carrageenin injection (100 μg/paw). The paw edema volume was taken at the 3rd hour after carrageenin injection. The strait line represents the equation $Y = 64.58x + 0.22$ of the administered doses with $r = 0.993$.

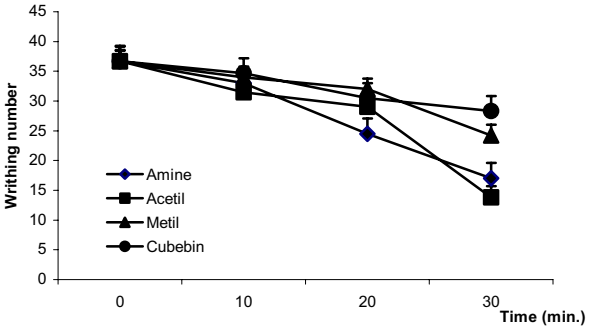


Fig. 3. Effect of the administration of cubebin, (-)-***O***-methyl cubebin, (-)-***O***-acetyl cubebin, (-)-***O***-dimethylethylamine cubebin at the doses of 10, 20 and 30 mg/kg on the writhes induced by acetic acid in mice. The marks means the average ± S.D. of $n = 5$ per group (cubebin $r = 0.99$, (-)-***O***-methyl cubebin $r = 0.95$, (-)-***O***-acetyl cubebin $r = 0.93$, (-)-***O***-diethylmethylamine cubebin $r = 0.988$).

bin derivatives did not show any significant activity. On the other hand, the results obtained for the group treated with morphine were highly significant. Thus, these compounds were considered to have no analgesic effect on the central nervous system that could contribute to its peripheral analgesic effect.

On the basis of these investigations, it may be concluded that the non-steroidal cubebin derivative compounds [(**2**), (**3**) and (**4**)], showed anti-inflammatory and analgesic activities similar to that observed for the non-steroidal drugs. It may also be suggested that the mechanism of action of the tested compounds might be associated with the inhibition of PG synthesis, as observed for most non-steroidal drugs.

Acknowledgements

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP—Proc. no. 98/14956-7, 02/02855-9, 02/07221-8, 01/012489-7) for financial support.

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