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Artigo

Potential benefits of the 19-*nor*-clerodane *trans*-dehydrocrotonin on the central nervous system

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RESUMO: "Potenciais benefícios do 19-*nor*-clerodano *trans*-desidrocrotonina isolado de *Croton cajucara* sobre o sistema nervoso central". Neste estudo avaliou-se o efeito analgésico do diterpeno 19-*nor*-clerodano *trans*-desidrocrotonina (DCTN) isolado de *Croton cajucara* Benth (Euphorbiaceae), bem como seu efeito no sistema nervoso central utilizando-se diferentes tipos de modelos de animais roedores. A administração intraperitoneal deste diterpeno, no teste da placa quente, revelou sua atividade analgésica moderada. No entanto, no teste de contrações abdominais desencadeadas por ácido acético, a DCTN apresentou forte atividade antinociceptiva com DE_{50} de 44,88 mg/kg. Doses elevadas de DCTN (100 mg/kg) apresentaram moderada atividade depressiva do sistema nervoso central (SNC), não tendo sido evidenciado ação antidepressiva. Após algumas considerações da ação de DCTN em algesia periférica, concluiu-se que esta substância pode ser utilizada como um potente agente analgésico, sem afetar o SNC.

Unitermos: *Croton cajucara*, Euphorbiaceae, *trans*-desidrocrotonina, sistema nervoso central.

ABSTRACT: This study examined the effect of *trans*-dehydrocrotonin (DCTN), a 19-*nor*-clerodane diterpene isolated from *Croton cajucara* Benth (Euphorbiaceae), as analgesic and its effect on the central nervous system (CNS) of rodents using different animal models. The DCTN intraperitoneally exhibited mild analgesic activity on hot-plate test, but exhibited strong antinociceptive activity against acetic acid-induced abdominal writhing and the ED_{50} was calculated to be 44.88 mg/kg. At higher doses (100 mg/kg) it exhibited mild CNS depressant activities in laboratory animals. Moreover, it has negligible antidepressant activity. After taking consideration of the drug interaction, the DCTN can be used as a potent analgesic agent in case of peripheral algesia, without affecting the CNS.

Keywords: *Croton cajucara*, Euphorbiaceae, *trans*-dehydrocrotonin, central nervous system.

INTRODUCTION

Croton cajucara Benth. (Euphorbiaceae) known in the Amazon area of Brazil, as 'sacaca', is a medicinal plant used in the form of tea for the ailments such as diarrhea, diabetes, stomachache, fever, jaundice, hepatitis, malaria and inflammation of the liver (Maciel et al., 2000; Maciel et al., 2002a,b; Souza et al., 2006; Perazzo et al., 2007; Agra et al., 2008). The 19-*nor*-clerodane *trans*-dehydrocrotonin (DCTN), (Figure 1), a very important biologic clerodane reported from this *Croton*, showed striking correlation with the folk traditional therapeutic use of *Croton cajucara*, proving to possess antitumor, antiulcerogenic, hypoglycemic, hypolipidaemic, antiatherogenic, antioestrogen, antigenotoxicity, antiinflammatory and antinociceptive activities (Maciel et al., 2000; Maciel et al., 2005; Maciel et al., 2006a,b; Costa et al., 2007). Generally, clerodane diterpenes have attracted attention because of their antifeedant properties, which appears to be specific to certain insects (Rodriguez-Hahn & Esquivel, 1994). As part of our ongoing research on *Croton cajucara*, in this paper we have undertaken some pharmacological studies of its major bioactive metabolite DCTN on the central nervous system.

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MATERIAL AND METHODS

The 19-*nor*-clerodane DCTN doses and route of administration

The pure DCTN was isolated according to previously published methodology (Maciel et al., 1998; Maciel et al., 2003), dissolved in 10% DMSO and diluted with normal saline (0.9% NaCl solution in distilled water). We used 25, 50 and 100 mg/kg doses of DCTN via the intraperitoneal (i.p.) route.

Animals

Male and female Swiss-Webster strain mice (20-25 g body weight) were used for all the experiments conducted with DCTN. The animals were provided with food and tap water *ad libitum*. The animals were maintained at constant room temperature (22.0 ± 1.0 °C), humidity 55-65% and 12 hours light dark cycle. Animals were divided in groups of 6, unless otherwise stated, with each group balanced for sex and body weight. For the control animals were given equal volume of saline (0.9% NaCl solution).

DCTN evaluations of analgesic effect

Hot plate method

The analgesic study was conducted by the "Hot-Plate" (Socrel-DS37, UGO Basile, Italy) method, described by Woolfe & MacDonald (1944). The hot-plate was maintained at a constant temperature of 55 ± 0.5 °C. Each animal was placed on the hot surface and the time of response to this thermal stimulus, indicated by the licking of hind and/or fore paws or by kicking of the legs or by trying to jump-out, was recorded. For comparison, the same experiment was conducted with standard analgesic drug, Morphine sulphate [MPS] (0.5 μg/kg).

Acetic acid-induced abdominal writhing assay

Muscular contraction induced by 0.6% solution of acetic acid (AA) (0.25 ml/mice). The plant extracts or the vehicle were administered intraperitoneally (i.p.) to mice, 30 min before the acetic acid (AA) injection. After AA administration, mice were placed in boxes. The number of muscular contractions was counted after 15 minutes after injection and data represents the average number of the total number of writhes observed (Koster et al., 1959). The percent protection by experimental drug was calculated as follows:

% Protection = 100 - (treated mean/control mean) x 100

For comparison, the same experiment was conducted with standard analgesic agent, acetylsalicylic acid [ASA] (5.0 mg/kg).

Neuropharmacological study

Spontaneous motor activity test

Six mice (20-25 g) were placed on an automatic activity cage (Model-7400) with recorder (Model-7401, UGO Basile, Italy), of which the floor was made with stainless steel grids. Through these steel grids 0.6 volts and 50mA current was passed. The positive and negative terminals were mounted consecutively. When the mice were placed over these steel grids the terminals were connected and movement of the mices was detected by the detector. These movements were thus recorded and printed on thermal papers (UGO Basile, Italy). In this study, we used diazepam [DZP] (12.5 μg/kg) and amphetamine [AMP] (1.0 mg/kg), as standard CNS depressant and stimulant, respectively (Khan, 1996).

Rotarod test

The method was a modification of that previously described by Kinnard & Carr (1957). Mice were placed on a rotating rod (Rotamex 4/8, Columbus Instruments, Ohio, USA), 2.0 inch in diameter, rotating at 10 to 25 rpm. Sideward movements on the rod were limited by two circular discs set about 4 inches apart. During 4 minutes of pre-training on the rod, about 70% of the mice stayed on the rod for at least 120 consecutive seconds. These mice were administered with test drugs, confined in a small box, and 30 minutes latter given two test trials, each lasting a maximum of 120 seconds. The falling of the mice from the rod before the end of 120 sec trial period was monitored through photocells and this was recorded (Pearl, 1969).

Hole cross test

The method of Takagi et al. (1971) was employed for this experiment. A steel partition was fixed at the middle of a cage 30 x 20 x 14 cm, in size. A hole of 3 cm in diameter was made at the height of 7.5 cm in the center of the plate. The number of passages of a mouse through that hole from one side of the cage to another was recorded for a period of 2 minutes at 0, +30, +60, +120 and +240 minutes. Similar recordings were made for the control animals.

Open-field test

This experiment was carried out by the method of Gupta et al. (1971). The floor of an open field of half-square meter was divided into a series of squares, each alternatively colored black and white. The apparatus had a wall of 40 cm height. The number of squares traveled by the animals were recorded for a period of two minutes.

Pentobarbital-induced sleeping time

This test was done according to the method of Dandiya & Collumbine (1959). Thirty minutes after drug treatment, pentobarbital, at a sub-hypnotic dose of 40 mg/kg was administered intraperitoneally. The onset and the duration of pentobarbital narcosis were noted.

Forced swimming test

In this study, 12 mice were used per group. They were housed singly in wire cages with free access to food and water. Naive animals were individually forced to swim inside vertical glass cylinders (height: 40 cm, diameter: 18 cm) containing a 15cm depth of water. The glass cylinder was placed in a water bath maintained at 25 ^{o}C temperature. After 15 min in the water, the rats were removed and allowed to dry for 15 min in a heated enclosure (32 °C) before being returned to their home cages. 24 hour later, the animals were tested in the cylinders for 5 min, and the total duration of immobility was measured. The mice were judged immobile whenever they floated passively in a slightly hunched but upright position with their heads above the surface. An injection schedule (Porsolt et al., 1977, 1978), which was adopted to optimize the pharmacological effect and at the same time, to imitate more closely clinical uses was also used. Drugs were given 1 hour before the 5 min test. The first injection was given at the end of the drying period (e.g., 15 min after removal from the water in the first test).

Statistical analysis

Data obtained from the experiments are expressed as Mean and Standard Error of the Mean (Mean ± S.E.M.). Unpaired t-tests were performed by computer software SPSS (Statistical Package for Social Science) version 10.0.1, to test the level of significance. Probability (p) value of 0.05 or less was considered as significant. In this paper $p < 0.05$, $p < 0.01$, $p < 0.001$ are represented by a single (*), double (**) and triple (***) asterisk(s), respectively.

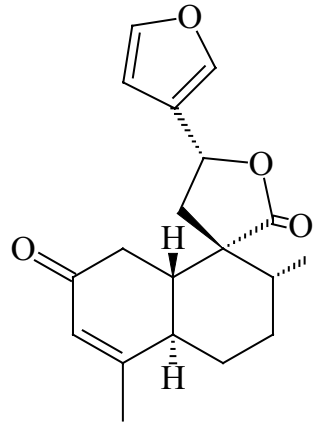
RESULTS AND DISCUSSION

Our previously pharmacological studies performed with isolated terpenoids from the stem bark of *Croton cajucara*, e.g., DCTN, CTN and acetyl aleuritolic acid (AAA) proved that the clerodane DCTN is the lead biological compound (Maciel et al., 2000; Maciel et al., 2006a,b; Costa et al., 2007). As a continuing research with this bioactive compound, in this work we optimize its therapeutic potential on the central nervous system, advancing on the several animal models. As expected (Carvalho et al., 1996) DCTN by intraperitoneal (i.p.) administration exhibited no analgesic activity at dose of 25 mg/kg on the hot-plate test. Meanwhile, in this work, we observed that at a dose of 50 mg/kg it exhibited mild analgesic activity after 120 min of i.p. drug administration. At most high dose used in these experiments (100 mg/kg) DCTN showed mild to moderate analgesic activity on the hot-plate test after 60 min of drug administration. Data recorded at different observation times such as 30, 60, 120 and 240 min, are statistically significant as shown in Table 1.

According to Carvalho et al. (1996) DCTN exhibited strong antinociceptive activity against acetic acid-induced abdominal writhing inhibition assay, in that at 25 mg/kg dose, it inhibited 44.25% abdominal writhing and data arrived from this dose is statistically significant ($p = 0.033$). In this work we observed that at 50 and 100 mg/kg doses, it inhibited abdominal writhing by 66.85% and 81.92%, respectively. The median effective dose (ED_{50}) was calculated 44.88 mg/kg (R^2 value is 0.5629) (Table 2). In order to improve the experiments with this compound, we submitted DCTN to spontaneous motor activity test, in which no effect was observed. Meanwhile, at the 100 mg/kg dose, after 120 min of i.p. drug administration, this compound exhibited mild CNS depressant activity (Table 3).

The tested clerodane DCTN exhibited almost no effects on the hole cross test with 25 and 50 mg/kg doses. Negligible depressant activities exhibited with i.p. 100 mg/kg dose of the DCTN after 60 min of drug administration. This depressant effect persisted for about 60 to 120 min, because at the 240 min observation period the depressant effect was abolished (Table 4).

In the open-field test with 25 and 50 mg/kg doses, DCTN exhibited mild central depressant activities ($p<0.05$) recorded. With a dose of 100 mg/kg it exhibited mild to moderate central depressant activity. It achieved its highest depressant activity after 120 min of i.p. drug administration. Data collected from this dose at 30 min



DCTN

Figure 1. Chemical structure of *trans*-dehydrocrotonin (DCTN).

Table 1. Tabular representation of the time taken to perceive after the administration of DCTN in comparison with standard analgesic drug, morphine sulphate (MPS).

Groups	Dose (mg/kg)	Pain Perception Time (Perception Time in Seconds), Mean±S.E.M. (p value)				
		0 min	30 min	60 min	120 min	240 min
CON (n=18)	100	16.42±1.003	15.17±1.103	17.0±1.483	15.88±1.268	13.66±1.202
DCTN (n=18)	25	24.83±3.39 (0.352)	11.17±1.52 (0.434)	9.96±0.56 (0.154)	9.0±1.39 (334)	9.97±1.31 (0.890)
	50	27.0±1.0 (0.013)**	28.5±3.82 (0.008)**	25.0±2.62 (0.001)***	30.5±1.34 (0.000)***	30.17±3.43 (0.001)***
	100	21.67±4.01 (0.881)	23.5±0.99 (0.008)**	42.17±2.55 (0.000)***	42.17±1.4 (0.000)***	35.0±5.4 (0.005)**
MPS (n=7)	0.5	24.3±4.177 (0.038)*	494.30±22.81 (0.000)***	411.17±27.84 (0.000)***	398.3±9.98 (0.000)***	385.17±9.13 (0.000)***

Note: *p<0.05, **p<0.01, ***p<0.001.

Table 2. Tabular presentation of the effect of DCTN on acetic acid (AA)-induced writhing test.

Groups	Doses (mg/kg)	Average Number of Writhings Observed, Mean±S.E.M (p value)	% Protection	ED_{50} (mg/kg)	R^2
CON (n=15)	100	44.0±2.449	-	-	-
DCTN (n=30)	25	24.67±1.96 (0.033)*	44.25		
	50	14.67±0.84 (0.004)**	66.85	44.88	0.5629
	100	8.0±0.97 (0.001)***	81.92		
ASA (n=10)	5.0	15.63±4.719 (0.001)***	64.49	-	-

Note: *p<0.05, **p<0.01, ***p<0.001.

Table 3. Tabular presentation of the effect of DCTN on spontaneous motor activity tests.

Groups	Doses (mg/kg)	Time of Study (No. of Movements), Mean ± S.E.M. (p value)				
		0 min	30 min	60 min	120 min	240 min
CON (n=14)	100	439.67±49.29	415.5±101.56	272.67±40.96	227.17±43.01	165.83±19.93
DCTN (n=21)	25	345.0±14.42 (0.196)	240.75±9.52 (0.563)	170.0±20.41 (0.331)	186.25±6.62 (0.604)	160.75±29.64 (0.524)
	50	369.25±14.82 (0.956)	254.25±8.29 (0.789)	189.25±29.0 (0.800)	174.5±39.64 (0.696)	199.0±31.04 (0.696)
	100	372.5±14.47 (0.900)	254.5±19.39 (0.808)	187.5±18.15 (0.682)	92.25±2.02 (0.000)***	119.5±18.26 (0.042)*
AMP (n=6)	1.0	423.8±27.96 (0.798)	376.4±68.18 (0.767)	405.8±46.44 (0.059)	268.83±14.62 (0.421)	327.0±54.39 (0.015)*
DZP (n=6)	12.5 µg/kg	449.0±24.16 (0.877)	269.0±34.12 (0.240)	224.6±29.75 (0.385)	153.8±10.02 (0.164)	178.8±63.18 (0.838)

Note: *p<0.05, ***p<0.001.

Table 4. Tabular presentation of the effect of DCTN on hole cross tests.

Groups	Dose (mg/kg)	Hole cross tests (number of movements), Mean±S.E.M. (p values)				
		0 min	30 min	60 min	120 min	240 min
CON (n=15)	100	3.17±0.55	3.61±0.51	2.94±0.44	3.17±0.44	3.39±0.37
DCTN (n=18)	25	3.0±0.32 (0.132)	3.8±0.58 (0.334)	3.6±0.68 (0.909)	3.8±0.37 (0.274)	5.8±0.92 (0.162)
	50	3.33±0.42 (0.396)	2.5±0.34 (0.271)	2.33±0.33 (0.075)	1.67±0.21 (0.001)***	2.33±0.49 (0.129)
	100	4.83±0.6 (0.616)	4.33±0.42 (0.047)*	1.83±0.4 (0.022)*	1.83±0.31 (0.009)**	2.5±0.5 (0.173)

Note: *p<0.05, **p<0.01, ***p<0.001.

Table 5. Tabular presentation of the effect of DCTN on open-field tests.

Groups	Dose (mg/kg)	No. of movements, Mean±S.E.M. (p value)				
		0 min	30 min	60 min	120 min	240 min
CON (n=30)	100	109.6±4.2	62.9±5.41	61.8±5.46	60.2±4.82	54.37±5.72
DCTN (n=18)	25	106.33±2.99 (0.534)	56.33±1.96 (0.261)	45.83±075 (0.007)**	46.83±1.08 (0.01)**	42.83±0.6 (0.05)*
	50	102.5±4.06 (0.242)	45.17±6.32 (0.05)*	43.67±2.12 (0.004)**	46.83±2.15 (0.016)*	42.0±1.55 (0.045)*
	100	107.83±2.56 (0.724)	50.17±0.79 (0.027)*	41.17±1.25 (0.001)***	33.67±1.84 (0.000)***	36.33±3.68 (0.01)**

Note: *p<0.05, **p<0.01, ***p<0.001.

Table 6. Tabular presentation of the effect of DCTN on rotarod test (on mice).

Groups	Doses (mg/kg)	Time of Study (no. of Movements), Mean±S.E.M. (p value)				
		0 min	30 min	60 min	120 min	240 min
CON (n=12)	100	68.3±10.45	76.5±13.31	94.1±10.5	75.8±14.04	94.6±11.53
DCTN (n=18)	25	91.25±25.53 (0.452)	77.0±22.41 (0.985)	41.75±17.2 (0.045)*	59.5±27.98 (0.627)	74.5±26.96 (0.529)
	50	99.75±19.59 (0.218)	83.0±22.68 (0.814)	86.75±23.11 (0.786)	98.25±16.49 (0.332)	93.75±23.04 (0.975)
	100	106.5±7.81 (0.01)**	65.75±31.4 (0.768)	70.5±14.51 (0.233)	62.75±25.64 (0.674)	57.25±4.05 (0.001)***

Note: *p<0.05, **p<0.01, ***p<0.001.

Table 7. Tabular presentation of the effect of DCTN on pentobarbital-induced sleeping time.

Groups	Doses (mg/kg)	Sleeping time, Mean±S.E.M. (p value)	
		Onset of sleeping (in sec)	Duration of sleeping (in sec)
CON (n=58)	100	380.21±27.64	3772.24±182.29
DCTN (n=28)	25	564.0±32.5 (0.000)***	3084.0±756.48 (0.203)
	50	582.0±36.93 (0.000)***	4104.0±286.38 (0.342)
	100	405.0±55.26 (0.696)	4095.0±375.34 (0.456)

Note: ***p<0.001.

Table 8. Tabular presentation of the effect of DCTN on forced swimming test (on mice).

Groups	Dose (mg/kg)	Swimming time (in sec), (p values)	% Deviations
CON (n=12)	100	137.55±15.98	-
DCTN (n=36)	25	131.42±7.64 (0.734)	4.46
	50	104.5±8.45 (0.087)	24.03
	100	103.8±2.17 (0.062)	24.54

(p = 0.027), 60 min (p = 0.001), 120 min (p < 0.001) and 240 min (p = 0.01) are significant (Table 5). Meanwhile, DCTN had no effect on the rotarod test at different doses (25, 50 and 100 mg/kg) (Table 6).

In the pentobarbital-induced sleeping time test, the effect of this compound is very much ambiguous. At low dose (25 mg/kg, i.p.) it exhibited mild stimulant effect. But in higher doses (50 and 100 mg/kg, i.p.) it exhibited mild depressant effect on the mice. At the dose of 100 mg/kg, the depressant effects of *t*-DCTN decreased in little extent (Table 7).

On the forced swimming test, DCTN exhibited negligible antidepressant effect on the laboratory animals (mice) (Table 8).

Acetic acid and hot-plate tests are normally used to study the peripheral and central analgesic effects of drugs, respectively (Koster, 1995; Eddy & Leimback, 1953). In the present study, we confirmed that DCTN attenuated the acetic acid, but not the hot-plate thermal stimulation. Therefore, it is probable that this diterpene

could produce its analgesic effect via peripherally (not centrally) as it was previously observed by Carvalho (1996), who proved that in the acute toxicity, there were no observed symptoms that justify the central nervous system action of DCTN, such as stereotypy, ataxia, and convulsion. Moreover, the LD_{50} of this DCTN was 555 mg/kg (p.o) for mice, which is 10 times higher than the ED_{50} (Carvalho, 1996). As part of our ongoing investigation we observed that DCTN in higher doses increased the sleep latency. Meanwhile, in lower dose, it decreased the total duration of sleeping. According to Benet et al. (1996) experiments we could suggest now that these observations may supports the possible evidence that at low dose DCTN acts as an enzyme inducer increasing the hepatic metabolizing enzyme responsible for the biotransformation of barbiturate. Therefore, at higher doses it may decreased the biotransformation of barbiturate in the liver by inhibiting the enzymes responsible for barbiturate metabolism, in this case DCTN acts as an enzyme inhibitor in the liver.

For the folkloric use of 'sacaca' in the treatment of liver disease, Kubo et al. (1991) studied its activity on the liver. They assayed the methanol extracts *in vitro* for hepatotoxic activity using carbon tetrachloride and galactosamine induced cytotoxicity in primary cultured rat hepatocytes as model systems. However, the extract did not exhibit any protective activity. Moreover, it showed unexpectedly potent hepatotoxicity. It could be due to the presence of other compound(s) in the methanol extract or may be due to DCTN. So the use of this compound should be used very carefully, until the study on the hepatic system in an *in vivo* test system has been done. On the other hand, Maciel et al. (1998; 2000; 2003; 2005) through an extensive phytochemical research did not find any cytotoxic compound in the leaves or stem bark of *Croton cajucara*. According to Maciel et al. (2000) ethnobotanical investigations, this *Croton* may not produce hepatotoxicity if used as a tea preparation to be taken at a dose of a cup twice a day during 2 to 4 weeks. It has been supported by Rodríguez & Haun (1999) who found on *in vitro* experiments that DCTN showed only hepatic damage after administered in subchronic treatment.

As part of our pharmacological research with *Croton cajucara* the assessment of the possible mutagenic potential of DCTN was evaluated in Swiss mouse bone marrow cell *in vivo*, submitted to acute intraperitoneal treatment, by micronucleus and chromosomal aberration tests showed that DCTN is not genotoxic nor cytotoxic regardless of the route of exposure (Agner et al., 1999). It was also proved that this compound is an antigenotoxic agent, in which DCTN at doses of 50 and 75% of the LD_{50}, via intraperitoneal treatment or gavage injection, was antimutagenic with regard to cyclophosphamide (Agner et al., 2001). However, the dose of 25% of the LD_{50} was only antimutagenic when administered by gavage. Additionally, we found now that DCTN to mouse bone devoid of any central nervous system side effects, and also does not affect the cardiovascular functions, such as, systolic and diastolic blood pressure and heart rate even with a very high dose (10^{-2}M) (unpublished data) (Silva et al., 2005). So considering the CNS and cardiovascular effects it is almost safe. The high yields of DCTN and its low toxicity jointed with other pharmacological evidences support that DCTN is a new potential peripheral analgesic agent.

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