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Anti-inflammatory Extracts and Coumaroyl Ursolic Acid Derivatives from *Distictis buccinatoria*

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ABSTRACT

Three ascendant polarities extracts (n-hexane, dichloromethane and methanol) from *Distictis buccinatoria* were analyzed for their anti-inflammatory activity in the TPA mice ear edema assay. The dichloromethane extract (DbDE) shown the highest percentage of edema inhibition (90.47%; ED_{50} = 0.036 mg ear^{-1} and E_{max} = 90.9%). The chemical bio-guided study of this extract allowed to obtain an anti-inflammatory fraction DbC1F4 (95.8%; D_{50} = 0.49 mg ear^{-1} and E_{max} = 99.01%) and sub-fraction DbC2F3 (93.44% of edema inhibition). The compounds (1) 3-O-(E)-p-coumaroyl ursolic acid, (2) 3-O-(Z)-p-coumaroyl ursolic acid and (3) ursolic acid were isolated from this last fraction. These results give a scientific basis to the traditional uses of this Mexican medicinal plant, which could be standardized in the content of coumaroyl ursolic acid derivatives for future investigations of this species in treatment of inflammation related illness.

Key words: *Distictis buccinatoria*, anti-inflammatory activity, coumaroyl ursolic acid derivatives

INTRODUCTION

Distictis buccinatoria (DC) A.H Gentry previously known as *Fithecoctenium buccinatorium*, *Bignonia buccinatoria* is a perennial climbing shrub belonging to the Bignoniaceae family (Gentry, 1973, 1974). This species is locally known as Mexican blood trumpet, red trumpet, red flower, "trompetilla del Diablo", or nahualt name "tonacaxochilt" (Pool, 2007; Zepeda and White, 2008). This is a native Mexican plant found from Central to southern Mexico in rocky areas or deciduous forest 1000 m (Pool, 2007). *Distictis buccinatoria* is a climbing plant with red or orange corollas and exerted stems and stigma (Pool, 2007). Cultural importance of tonacaxochilt in Mexico has been recorded in murals painted in Catholic convents, such as; Malinalco in the state of Mexico (Zepeda and White, 2008). *Distictis buccinatoria* has been used, since, pre-Hispanic times for medical purposes (De la Cruz, 1964; Moreno, 1971). Now a days, empirical midwives and herbalists in the state of Morelos use the decoction of its leaves and flowers to treat cough, angina, inflammation, pharyngitis and coughs with blood (Rojas *et al*., 2007). Previous studies demonstrated that this plant shows anti-microbial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, bacteria and *Trichophyton mentagrophytes*, *Trichophyton rubrum* dermatophytes, as well as cytotoxicity against cancerous line cell KB (Rojas *et al*., 2007). To date, non anti-inflammatory effect or bio-guided studies regarding to

this plant has been reported. In this study, we show the anti-inflammatory activity of three ascendant polarity extracts (n-hexane, dichloromethane and methanol) from the aerial parts. In order to find the bioactive compounds, the chemical fractionation was supported with a bio-guided assay using an acute inflammation TPA test. This is the first chemical report of tonacaxochilt.

MATERIALS AND METHODS

Plant material: The Aerial parts of *Distictis buccinatoria* were collected from plants growing in Tetela del Volcan, Morelos State Mexico. This species was authenticated by Margarita Aviles and Macrina Fuentes and a sample specimen was deposited at the Herbarium of the Instituto Nacional de Antropología e Historia Morelos (INAHM) placed in the Medicinal Botanical Garden in Cuernavaca Morelos Mexico. The classified reference voucher was recorded under the code number: INAHM-2007.

General experimental procedure: NMR spectra and two-dimensional spectroscopy experiments COSY, HSQC, HMBC were taken on a Varian INOVA-400 at 400 MHz for ^{1}H NMR spectra in $CDCl_3$, CD_3OD or DMSO-d6 with tetramethylsilane (TMS) as internal standard. Chemical shifts are reported in values. High Resolution-Electro-Spray Ionization Mass Spectrometry (HR-ESI-MS) in the positive and negative ion mode was performed, using a JEOL-AX 505 HA (JEOL, Tokio, Japan) mass spectrometer. Analytical TLC was carried out on precoated Merck silica gel 60F254 or RP-18F254 plates.

Chemical reagents: 12-O-Tetradecanoylphorbol 13-acetate (TPA) and Indomethacin (Indo) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). n-hexane, dichloromethane, methanol and Silica gel 60 and chromatographic plates were obtained from Merck KGaA (Darmstadt, Germany).

Extraction and isolation: Plant Material (leaves and flowers) were dried at room temperature under dark conditions and powdered using grinder to obtain 4-6 mm size particles. This material (2.3 kg) was extracted sequentially with n-hexane, dichloromethane and methanol (1 kg/5 L) at room temperature during 24 h (three times). After filtration each solvent was eliminated by low pressure distillation at 40°C. The yield extraction (%) for each extract (w dry material/w dry extract) was determined. The resulting crude extracts were stored at 4°C until they were used.

DbDE extract fractionation: Bioguided chromatography was carry out with the most anti-inflammatory extract (DbDE, 15 g) which was fractionated by silica gel 60 (70-230 mesh, 150 g, 20×60 cm) open column chromatography eluting with n-hexane-ethyl acetate gradient system (100:0, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100, 50 mL) to give 37 samples, which were grouped according to their chemical composition in four pooled 4 fractions DbC1 (2.12 mg), DbC1F2 (4.2 mg), C1F3 (3.14 mg) and C1F4 (5.11 mg). All these fractions were tested in the pharmacological test to select the most anti-inflammatory mixture. Fraction DbC1F4 (4.5 g), which was eluted with hexane-ethyl acetate (3:7) was subjected to chromatography column previously packed with silica gel 60 (0.063-0.2 mm, 80 g) eluting with dichloromethane-acetone gradient system (1:0-0:1) to obtain 24 sub fractions that were grouped according to their similarity in chemical composition in three fractions: DbC2F1 (1.3 g), DbC2F2 (0.9 g) and DbC2F3 (2.1 g). These sub-fractions were tested in the anti-inflammatory assay test.

Chemical composition of the most active fraction: Sub-fraction DbC2F3 (0.9 g) was subjected to chromatography column (silica gel C-18, 15 g), using acetonitrile-water (8:2-1:0) to obtain 21 subfractions, which were grouped according to their chemical composition in four fractions C3F1 (46 mg) C3F2 (25 mg) C3F3 (27 mg) and C3F4 (28 mg). A mixture of 3-O-(E)-p-coumaroyl ursolic acid (1) and 3-O-(Z)-p-coumaroyl ursolic acid (2) was identified in fraction DbC3F2 and ursolic acid (3) in fraction DbC3F4. Chemical identification of these compounds was performed by spectroscopic methods (400 MHz for 1H-NMR and 100 MHz for 13C-NMR) and high resolution MS. Bidimensional experiments and comparison with published data (HMQC and HMBC) were relevant for this identification.

Animals: Female ICR mice (25-30 g) purchased by Harlan Mexico were used. Experiments were performed according to the Official Mexican Rule: NOM-062-ZOO-1999, Guidelines (Technical Specifications for the Production, Care and Use of Laboratory Animals) and international ethical guidelines to the care and use of experimental animals. Mice were maintained at temperature of 22±3°C, 70±5% of humidity with 12 h light/dark cycles and food/water *ad libitum*. Groups of seven animals were organized and assays were conducted from 8:00-13:00 h.

Acute inflammation in mice induced with TPA: Animal inflammation was induced following the previous described method (Paya *et al*., 1993; Salinas-Sanchez *et al*., 2012). Doses of 2 mg ear^{-1} f each treatment including, DbH×E, DbDE and DbME extracts, as well as fractions DbC1F1-DbC1F4, sub-fractions DbC2F1-DbC2F3 and isolated compounds DbC3F2, DbC3F4 were applied on the ear of each individual. For the most active extract (DbDE) and fraction (DbC1F4) and sub-fraction (DbC2F3) doses of 0.25, 0.5 and 1.0 mg ear^{-1} were used. Indomethacin (Ind, 1 mg ear^{-1}) was used as reference drug. All treatments were dissolved in acetone and applied topically on both ears immediately after the administration of TPA (2.5 g mL^{-1}).

Statistical analysis: Results from delta weight (Dw = wt-wnt) are expressed as the Mean±Standard error of the mean (SEM) and these were analyzed using analysis of variance (ANOVA) and Bonferroni post-test (*p<0.05).

RESULTS

Anti-inflammatory activity: The sequential extraction of *D. buccinatoria* (aerial parts) allowed obtaining a non-polar extract (DbH×E, 27.6 g, 1.2%), a medium-polar extract (DbDE, 62.1 g, 2.7%) and a polar extract (DbME, 285 g, 12.4%). The anti-inflammatory effect of these crude extracts was tested at doses of 2 mg ear^{-1} on TPA induced edema test and the percentage of inhibition of the local inflammation is summarized in Table 1. The medium-polar extract (DbDE) displayed the major activity with a percentage of the edema inhibition (90.47%) in similar way to the standard drug Indomethacin (91.91%). This extract showed a markedly dose response effect with an ED_{50} = 0.036 mg ear^{-1} and E_{max} = 90.9%.

The anti-inflammatory effect of first fractionation (DbC1F1-DbC1F4) from the most active extract evaluated at the same dose (2 mg ear^{-1}) is summarized in Table 2. In this case the fraction DbC1F4 displayed a higher percentage of inhibition of the local inflammation (95.8%) than the standard drug (Indo, 91.91%). Figure 1 shows the dose-response anti-inflammatory effect of fraction C1F4. This mixture displayed values of ED_{50} = 0.495 mg ear^{-1} and E_{max} = 99.1%.

The anti-inflammatory activity of sub-fractions (column 2, DbC2F1-DbC2F3) and sub-fractions (column 3, DbC3F2 and DbC3F4) was evaluated at the same dose of the standard drug (Indo, 1 mg ear^{-1}) and the results are summarized in Table 3. The DbC2F3 and DbC3F2 treatments displayed a higher percentage of inhibition of the local inflammation (93.49 and 92.60%, respectively) than Indomethacin (Indo, 91.91%).

Separation and identification of compounds from the anti-inflammatory treatments: Chemical structure of the bioactive compounds was determined by analysis of its ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR). These NMR analyses were recorded in DMSO-d6 solvent and all ^{13}C NMR data are reported in Table 4. Sub-fraction DbC3F2 correspond with a mixture of the known (1) triterpenoids 3-O-(E)-p-coumaroyl ursolic acid, (2) 3-O-(Z)-p-coumaroyl ursolic acid and (3) sub-fraction C3F4 displayed similar data to the ursolic acid. All of these compounds (Fig. 2), have been previously isolated from other anti-inflammatory and cytotoxic species (Liao *et al*., 2014; Liu, 1995).

Table 1: Anti-inflammatory activity of integrate extracts and Indomethacin (Indo) on TPA induced edema test in ICR mice

Treatment	Dose (mg ear^{-1})	Edema (mg) Mean±SDM	Edema inhibition (%)	E_{max}	ED_{50}
TPA	-	10.14±1.40	0.00		
Indo	1.0	0.82±0.30	91.91		
DbH×E	2.0	3.50±0.58*	68.15		
DbDE		1.06±0.25*	90.47		
DbME		8.68±1.39	22.47		
DbDE	0.125	3.24±0.76*	68.01		
	0.25	1.97±0.40*	80.60		
	0.50	1.70±0.41*	83.23	90.9 %	0.036 mg ear^{-1}
	1.00	1.50±0.42*	85.20		
	2.00	1.07±0.25*	90.47		

ANOVA, Bonferroni test, (n = 7), *p<0.05 in comparison with TPA group, DbH×E: Hexanoic extract, DbDE: Dichloromethane extract, DbME: Methanol extract

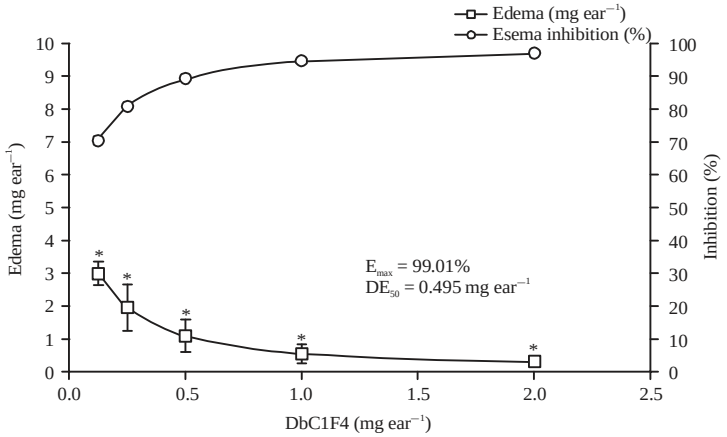

Fig. 1: Effect of different doses of the topical administration of DbC1F4 fraction on TPA induced ear edema. Indo = Indomethacin. ANOVA, *post-hoc* Bonferroni *p<0.05 (n = 7, Mean±SD, when it is compared with the negative control, TPA= 10.14 mg)

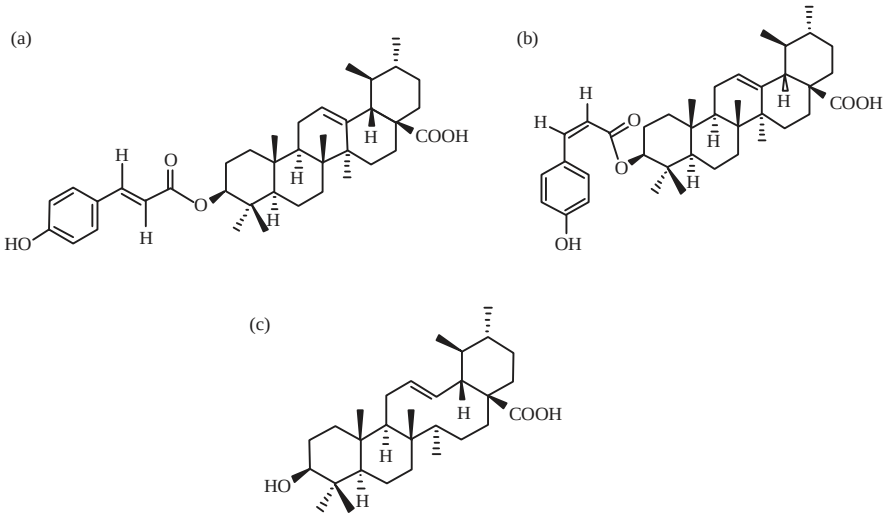

Fig. 2(a-c): Chemical constituents from the most active sub-fraction C2F3. (a) 3-O-(E)-p-coumaroyl ursolic acid, (b) 3-O-(Z)-p-coumaroyl ursolic acid and (c) Ursolic acid

Table 2: Anti-inflammatory activity of different fractions from (DbDE) and Indomethacin (Indo) on TPA induced edema test in ICR mice

Treatments	Dose (mg ear^{-1})	Edema (mg) Mean±SDM	Edema inhibition (%)
TPA	-	10.14±1.40	0.00
Indo	1.0	0.82±0.30	91.91
DbC1F1		1.23±0.43*	87.80
DbC1F2		1.27±0.64*	87.50
DbC1F3	2.0	1.60±0.45*	84.20
DbC1F4		0.43±0.09*	95.80

ANOVA, Bonferroni test, (n = 7) *p<0.05 in comparison with TPA group, DbC1F1-DbC1F4 corresponds with fractions of the first chromatographic column

Table 3: Anti-inflammatory activity of different sub-fractions from DbC1F4 on TPA induced edema test in ICR mice

Treatments	Dose (mg ear^{-1})	Edema (mg) Mean±SDM	Edema inhibition (%)
TPA	-	10.14±1.40	0.00
Indo	1.0	0.82±0.30	91.91
DbC2F1	1.0	9.20±0.43*	9.27
DbC2F2		2.47±0.64*	75.67
DbC2F3		0.66±0.45*	93.49
DbC3F2	1.0	0.75±0.33*	92.60
DbC3F4		2.54±0.66 *	74.95

DISCUSSION

Summarizing, the medium polar extract from *D. buccinatoria* (DbDE extract, 2 mg ear^{-1}) induced significant anti-inflammatory activity (90.47% of edema inhibition) in dose response way (ED_{50} = 0.036 mg ear^{-1} and E_{max} = 90.9%). The chemical bio-guided study of this extract provided fraction DbC1F4, which inhibited significantly, the local inflammation (ED_{50} = 0.49 mg ear^{-1} and E_{max} = 99.01%). In this research, the reference drug Indomethacin displayed a percentage of edema inhibition (91.1%, 1 mg ear^{-1}). The purification of fraction DbC1F4 gives the anti-inflammatory

Table 4: ^{13}C NMR spectroscopic data (400 MHz) of compounds 1-3

Position		1	2	3
1	CH_2	37.8	37.8	39.1
2	CH_2	27.8	27.8	27.0
3	CH	84.1	84.1	77.0
4	C	38.5	40.6	40.1
5	CH	52.7	54.8	54.9
6	CH_2	18.4	19.6	19.1
7	CH_2	33.1	32.8	32.8
8	C	41.7	41.2	40.3
9	CH	48.2	50.0	47.1
10	C	37.8	38.8	37.8
11	CH_2	23.5	24.3	36.6
12	CH	125.6	124.7	124.7
13	C	138.7	137.6	138.8
14	C	41.7	42.0	41.7
15	CH_2	28.3	29.4	27.6
16	CH_2	24.2	25.4	23.9
17	C	47.2	47.3	47.0
18	CH	52.7	54.8	52.5
19	CH	39.2	40.8	39.9
20	CH	39.0	40.6	39.9
21	CH_2	30.5	31.7	30.3
22	CH_2	37.0	38.2	36.6
23	CH_3	29.2	29.1	28.3
24	CH_3	17.3	17.2	16.9
25	CH_3	16.6	16.7	16.3
26	CH_3	18.2	18.1	17.1
27	CH_3	24.1	24.3	23.4
28	COOH	178.8	179.1	178.5
29	CH_3	17.3	18.5	17.1
30	CH_3	21.4	21.7	21.2
1’	C = O	167.2	166.5	
2’	CH	115.4	116.6	
3’	CH	144.4	144.7	
4’	C	126.0	126.0	
5’	CH	132.8	130.5	
6’	CH	116.2	115.1	
7’	C	159.9	158.7	
8’	CH	116.2	115.1	
9’	CH	132.8	130.5	

All spectra were acquired in DMSO-d6

sub-fraction DbC2F3 (93.44% of edema inhibition). The compounds (1) 3-O-(E)-p-coumaroyl ursolic acid, (2) 3-O-(Z)-p-coumaroyl ursolic acid and (3) ursolic acid were isolated from this last fraction (compounds 1-2 were contained in sub-fraction DbC3F2 and compound 3 in sub-fraction DbC3F4). These triterpenoids showed significant anti-inflammatory effect on acute test of TPA with 74.95 and 92.6% of inhibition of edema, respectively, when they were tested at a dose of 1 mg ear^{-1}.

Although, *D. buccinatoria* is used in traditional medicine for the treatment of inflammation related diseases, only cytotoxic, antimicrobial and antifungal activities had been described for integrate extracts from this plant (Rojas *et al*., 2007). This is the first report that confirms the traditional use of "Tonacaxochilt" as anti-inflammatory treatment. *Distictis* genus is constituted only by nine species and it practically has not been studied chemically or pharmacologically. According to the Bignoniaceae family *Tabebuia avellanedae* has been reported as anti-inflammatory and this species showed a dose-response dependant effect (v.o, 100, 200 or 400 mg kg^{-1}), when it was evaluated in the TPA test (Lee *et al*., 2012). Unlike this work, no chemical metabolites were related to the anti-inflammatory effect of *T. avellanedae*. On the other hand, here it has demonstrated that all anti-inflammatory treatments from *D. buccinatoria* contain the triterpenoids: (1) 3-O-(E)-p-coumaroyl ursolic acid, (2) 3-O-(Z)-p-coumaroyl ursolic acid and (3) ursolic acid. This last compound was related with the anti-inflammatory activity of hydroethanolic extract from *Jacaranda decurrens* (Bignoniaceae), which reduced the inflammation induced with carrageenan in an edema plantar assay (Santos *et al*., 2012). This ursolic acid has been widely described in several models of inflammatory activity and pain (Liu, 1995). Local administration of ursolic acid (0.25, 0.5, 1.0, 2.0 mg ear^{-1}) showed a DE_{50} = 0.1 mg ear^{-1} (E_{max} = 90% of edema inhibition, 2 mg ear^{-1}) in the TPA test (Banno *et al*., 2004). This effect is comparable with that obtained by the dichloromethane extract from *D. buccinatoria* (DbDE, ED_{50} = 0.036 mg ear^{-1}; 90.47% of edema inhibition) in the same pharmacological test. When, this extract was chromatographically fractionated, the treatment DbC1F4 produced a better anti-inflammatory effect than the complete extract (ED_{50} = 0.49 mg ear^{-1}; 99% of edema inhibition). The fraction DbC3F2, which corresponds with the mixture of (1) 3-O-(E)-p-coumaroyl ursolic acid and (2) 3-O-(Z)-p-coumaroyl ursolic acid was more active (92.6% of edema inhibition, 1 mg ear^{-1}) than fraction DbC3F4 (74.95% of edema inhibition, 1 mg ear^{-1}), where (3) ursolic acid was isolated. Although, these (1) 3-O-(E)-p-coumaroyl ursolic acid and (2) 3-O-(Z)-p-coumaroyl ursolic acid have been related with the cytotoxic activity of *Eleagnus oldhamii* (Liao *et al*., 2014) or antimycobacterial (Tanachatchairatana *et al*., 2008), this is the first time that these triterpenoids are described as, anti-inflammatory compounds. Considering the ethnomedical and pharmacological activities of *D. buccinatoria*, this species has significant medical potential, so it is very relevant to know the chemical identity of the bio-active compounds for the production of standardized phytochemicals.

CONCLUSION

These results give a scientific basis to the traditional uses of this species, which has been used in Mexico, since, Aztec times. Knowledge of the chemical identity of the anti-inflammatory compounds, allow us to propose new phytochemicals standardized in these compounds for future investigations of *D. buccinatoria* in treatment of inflammation related illness.

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