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Pharmaco-toxicological Study of Diterpenoids

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Abstract—*Azorella compacta*, *Azorella yareta* and *Laretia acaulis* (Apiaceae) are native species from the high Andes Mountains, northeastern Chile, and they have being traditionally used to treat asthma, colds and bronchitis, illnesses with inflammation and pain as the main symptoms. Interestingly, there are no scientific reports available on their benefits or toxicity. This study was carried out with the purpose of validating the medicinal use of these species and to discover anti-inflammatory and analgesic new molecules. As a working hypothesis, we have proposed that these medicinal species contain bioactive compounds with anti-inflammatory and analgesic effects. In this context, azorellanol, 13-hydroxy-7-oxoazorellane and 7-deacetylazorellanol, three diterpenoids previously isolated only from these plants, were subjected to farmaco-toxicological evaluation. Their topical anti-inflammatory and analgesic activities along with acute toxicities or innocuosness were also investigated. Our results indicate the absence of toxic and side effects in mice. All compounds presented dose-related inhibition of pain. 13-hydroxy-7-oxoazorellane was the most potent analgesic but it was less effective than sodium naproxen, the reference drug. Azorellanol exhibited the highest topical anti-inflammatory potency on AA (arachidonic acid) and TPA (12-deoxyphorbol 13-tetradecanoate) induced oedema, and it effect was similar to the reference drugs (nimesulide and indomethacin). Probably, its mechanism of action could be explained through the inhibition to cyclo-oxygenase activity. Our results corroborate the anti-inflammatory and analgesic effects of these species, and it justifies their use in folk medicine.

Introduction

Azorella compacta, *Azorella yareta* and *Laretia acaulis* (Apiaceae) are native shrubs that grow at the high Andes, northeastern Chile.[1] Whole plant are used in folk medicine to treat a large variety of ailments, such as bronchitis, asthma and colds.[1] However, there are no scientific reports available on their benefits or toxicity. These uses suggest us that these species could present anti-inflammatory and analgesic effects due to the presence of bioactive compounds.[2]

This study was carried out with the purpose of validate the medicinal use of these species and to discover anti-inflammatory and analgesic new molecules. As a working hypothesis, we have proposed that these medicinal species contain bioactive compounds with anti-inflammatory and analgesic effects. This paper shows the results from a pharmaco-toxicological study of three tetracyclic diterpenoids isolated only from the species in study, azorellanol (**1**), 7-deacetylazorellanol (**2**) and 13-hydroxy-7-oxoazorellane (**3**).[3,4] Previous pharmacological studies showed an antiparasitical effect of these compounds.[4,5] We have used in vivo assays to explore the topical anti-inflammatory effect, the analgesic properties and the acute toxicity or innocuousness of these selected compounds. Although they are structurally similar, they show differences at the C-7 substituents (Fig. 1).

This has allowed us to propose a structure–activity relationship and evaluate which compound is the most potent compound for oral and topical administration. The pharmacological properties evaluated here were compared with the effects of sodium naproxen (SN), nimesulide (NM) and indomethacin (IND). We postulate a mechanism of action for the anti-inflammatory and analgesic activities of these diterpenoids based upon correlations among the different in vivo assays.

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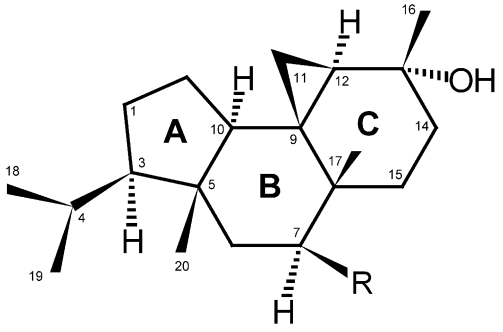

R
1 OAc
2 OH
3 =O

Figure 1.

Results and Discussion

At a 4.9 mol$\times10^{-4}$/kg dose, **1**, **2** and **3** did not exhibit acute toxicity. The mice corporal weight showed a normal variation after a 7-day observation period. Common side effects such as mild diarrhoea, loss of weight, changes in the mobility and nutritious habits were not recorded. Since there was not previous information on the toxicity of these compounds, the maximum dose to be administered was based on the SN dose that showed the maximum analgesic effect and it was 10 times increased. Table 1 show the results of the pharmacological assays for **1**, **2** and **3**, and their maximum effects (↑) and, also show the maximum analgesic activity of SN and the maximum topical anti-inflammatory effect of NM and IND on AA (arachidonic acid) and TPA (12-deoxyphorbol 13-tetradecanoate)induced oedema, respectively.[6]

All three compounds exhibited analgesic activities and the effect was dose-dependent. Even though **3** showed the higher maximum potency, all compounds have a potency value lower than SN. **1** also showed the weakest relative analgesic potency.

1 exhibited the highest topical anti-inflammatory potency on AA and TPA induced oedema, the results in both assays showed that **1** topical effect was dose-dependent. **1** potency was lower than to NM on the AA and IND on the TPA assays.

3 and **2** showed a weak potency on the AA model. **2** presented effect dose-dependent and **3** was inactive on TPA model.

The differences on the analgesic effects of **1**, **2** and **3** were smaller than those found on AA and TPA induced oedema. **3** was the most potent analgesic compound. The differences on the analgesic potency among the compounds could be based on their different physicochemical characteristics. In turn, this could translate in differences on some pharmacokinetics parameters such as gastrointestinal absorption, biotransformation and elimination. Since the acetyl group at C-7 increases lipophilicity, **1** should show a relative absortion higher than **2** and **3**. Even though **3** could have low relative absortion, its plasma concentration and half-life would be higher than **2** and **1** since the oxo group at C-7 confers resistance to hepatic biotransformation. Liver quickly transforms compounds containing hydroxy and acetyl groups into more polar molecules which are readily eliminated.[7]

Among the compounds evaluated, **1** proved to be the most potent on AA and TPA induced oedema. The acetyl group at C-7 increases lipophilicity and bioavailability, followed by stratum corneum penetration.[8] The lower potency of **2** on the AA model could be explained

Table 1. Analgesic and topical anti-inflammatory effects of **1**, **2**, **3** and reference drugs

S	Dose	%A±SEM	%TA±SEM	%TP±SEM
1	3.0×10^{-5} mol/kg	12.1*±16		
	5.7×10^{-5} mol/kg	43.6**±10		
	11.0×10^{-5} mol/kg	↑50.7**±11		
	17.4×10^{-5} mol/kg	46.6**±15		
	22.0×10^{-5} mol/kg	44.8**±14		
1	1.5×10^{-6} mol/ear		2.7±8	
	3.2×10^{-6} mol/ear		33.3**±10	
	6.3×10^{-6} mol/ear		↑38.6**±12	
1	3.0×10^{-7} mol/ear			0.0±13
	7.2×10^{-7} mol/ear			38.0**±6
	15×10^{-7} mol/ear			↑70.8**±9
	25×10^{-7} mol/ear			69.2**±10
2	1.0×10^{-5} mol/kg	6.7±5		
	3.1×10^{-5} mol/kg	38.8**±7		
	5.7×10^{-5} mol/kg	↑53.4**±16		
	10.9×10^{-5} mol/kg	32.0**±11		
2	1.0×10^{-6} mol/ear		13.2±7	
	3.4×10^{-6} mol/ear		↑21.4**±8	
	6.6×10^{-6} mol/ear		22.7**±13	
2	0.5×10^{-6} mol/ear			3.1±8
	1.5×10^{-6} mol/ear			45.2**±10
	2.6×10^{-6} mol/ear			↑79.0**±3
	4.0×10^{-6} mol/ear			66.4**±10
3	1.0×10^{-5} mol/kg	2.6±10		
	2.3×10^{-5} mol/kg	25.6**±12		
	4.9×10^{-5} mol/kg	56.8**±7		
	10.0×10^{-5} mol/kg	↑59.0**±21		
3	1.0×10^{-6} mol/ear		6.7±14	
	3.6×10^{-6} mol/ear		↑27.8**±10	
	7.0×10^{-6} mol/ear		20.3**±7	
3	1.5×10^{-6} mol/ear			5.3±9
	3.5×10^{-6} mol/ear			4.0±9
SN	4.9×10^{-5} mol/kg	↑70.0**±4	↑25.5**±4	
NM	3.2×10^{-6} mol/ear		↑48.8**±4	
IND	1.4×10^{-6} mol/ear		28.0**±9	↑81.8**±20

Without asterisks $p\geq0.01$; *$p\leq0.1$;**$p\leq0.05$; S sample; A analgesic effect; TA and TP topical anti-inflammatory effects induced for AA and TPA respectively; **1**: azorellanol; **2**: 7-deacetylazorellanol; **3**: 13-hydroxy-7-oxoazorellane; SN sodium naproxen; NM nimesulide; IND indomethacin; ↑ maximum effect. Each group represents the median±SEM of eight animals pretreated with compounds or reference drugs.

by its lower stratum corneum penetration rate. On the other hand, the high potency of **2** on the TPA model could be based on the fact that the skin or mucosa is more abraded or inflamed on TPA oedema, which in turn increases stratum corneum penetration, bioavailability and increased potency.[8]

1 is probably metabolized to **2** since the acetyl group at C-7 could be quickly biotransformed to a OH group in the skin. This transformation may contribute to a higher relative affinity with the receptor and/or to higher intrinsic activity.[7]

The **3** topical activity was very low or inactive in the AA and TPA models, respectively. This could be a consequence of a low stratum corneum penetration. Also, the **3** bioavailable fraction could show a weaker relative affinity to its receptor and/or weaker intrinsic activity for the first assays. On TPA assays, oxo group substitution at C-7 causes lack of topical anti-inflammatory effect.

Lipoxygenase inhibitors show higher potency than cyclo-oxygenase inhibitors on AA-induced oedema, while cyclo-oxygenase inhibitors are more potent on TPA-induced oedema.[9] Cyclo-oxygenase inhibitors also show analgesic activity since the drugs prevent the conversion of arachidonic acid to prostaglandins and tromboxanes. The terminal prostaglandins are extremely proinflammatory and induce pain and fever. By contrast, anti-inflammatory drugs inhibit lipoxygenase activity and leukotrienes release and do not show analgesic effects.[9]

Our results support the notion that SN and IND showed a weak potency on the AA model. However, IND showed a strong effect on the TPA model. SN and IND are stronger inhibitors of cyclo-oxygenase than NM.[10] The NM activity exhibited on AA model could be explained if we consider it as a strong free radicals scavenger.[11]

1 and **2** showed higher potency on TPA than AA induced oedema assays. This may indicate that their mechanism of action could be the inhibition of cyclo-oxygenase and could explain in part the analgesic activity showed to these compounds. The OH group at C-7 could be in part responsible of these activity. However, the analgesic activities of **3** cannot be explained as inhibition of ciclooxygenase, and this activity should have an alternative mechanism. This could also be valid for the analgesic activity exhibited for **1** and **2**.

Materials and Methods

The compounds under evaluation were isolated from petrol ether extracts of aerial parts of native medicinal species of the Apiaceae family. **1** and **2** were isolated from *A. compacta*. **1** and **3** were isolated from *A. yareta*. 1, 2 and 3 were obtained from *L. acaulis*. Their structures (Fig. 1) were assigned as reported by Loyola et al.[3,4] The purity (>95%) of the compounds was monitored by thin-layer chromatography with several solvent system of different polarity and were confirmed by spectral data (^{1}H NMR).

In vivo assays with animals: CF-1 mice of either sex-(20–25 g) were used to assess the analgesic and anti-inflammatory effects and acute toxicity. Animals under standard conditions from the Chilean Public Health Institute were fasted overnight before the experiments.

The studies of the acute toxicity were carried out to know the dose range to use in the pharmacological assays without the toxic effects for the animals. For each assay, groups of 10 mice of both sexes were allowed free access to water. **1**, **2** and **3**, suspended in 5% saline arabic gum, were orally administered via gastric catheter, at various doses. In order to detect physiological alterations, mice were weighed and observed daily during one week. The observed behaviors were: mobility and nutritious habits.[12]

The analgesic activity of each dose sample was evaluated in groups of eight mice and 16 control subjects, using an ip injection of 0.5 mL of 0.6% acetic acid.[6,13]

The analgesic effect was calculated by comparing the number of abdominal writhes between the treated and control group, which only received the vehicle. The number of abdominal writhes of each mouse was counted for 30 min, beginning 5 min after acetic acid administration. The following equation was used to calculate the mean pain percentage: $\%P = [C^{sample}/C^{control}] \times 100$, where C^{sample} is the median writhes reached in sample-treated animals and $C^{control}$ (41.6 ± 4) is the median writhes reached in control animals which received only the vehicle.[6] The analgesic effect (A), was calculated according to the following equation: $\%A = 100 - \%P$.

For the evaluation of topic anti-inflammatory activity (T), eight mice were treated with a dose sample and after 5 min they received 2 mg AA (arachidonic acid) or 2.5 μg TPA (12-deoxyphorbol 13-tetradecanoate), dissolved in 20 μL acetone. Sixteen control subjects received only AA or TPA at the same concentration. Both the sample and AA or TPA were applied to the inner (10 μL) and outer (10 μL) surfaces of the right ear. The left ear received only acetone. Mice were sacrificed by cervical dislocation and a 6 mm diameter section of the right and left ears were cut and weighed.[14] Dermal anti-inflammatory activity was evaluated according to the following equation: $\%T = [Wc - Ws/Wc] \times 100$ where Wc and Ws are the difference median values of the weights of the right and the left ear sections of the control and treated animals, respectively.[6]

For analgesic and anti-inflammatory assays, diterpenoids suspended in saline arabic gum, were orally administered 1 h before acetic acid, using an intragastric catheter. The compounds doses for each assay were equivalent to the reference drug dose showing maximum effect.[6]

Drug-induced changes were statistically estimated using the Wilcoxon test for independent data.[15] The effects were considered significant if $p \leq 0.05$. The SEM values were calculated from the mean writhes constriction and mean ears weight for treated and untreated animals.

Sodium naproxen, from Laboratorios Saval, Chile, was used as a reference drug for analgesic activity. Nimesulide and indomethacin, from Laboratorio Chile, Chile, were used as reference drugs for dermal anti-inflammatory activity.[6]

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