POSTER: CHARACTERIZATION OF THE BIOLOGICAL ACTIVITIES OF THE ANTARCTIC LIQUEN *Usnea aurantiaco-atra*

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INTRODUCTION

Lichens have a large variety of uses and for some of them ethnopharmacological properties are reported. Indeed these organisms are a source of original compounds (e.g. depsides and depsidones) that have been poorly investigated for their biological activities (Muller, 2001). Nevertheless, several studies have been carried out. These studies reported a variety of very interesting properties e.g. antibiotic (Lauterwein et al. 1995), antiproliferative (Ogmundsdottir et al. 1998), antioxidant (Hidalgo et al. 1994), anti-HIV (Zhao, H et al. 1997).
As the lichen flora from the Antarctic region, remarkable for its variety and development, represents a potential source of new bioactive compounds, we have undertaken a biological screening to detect cytotoxic activity and antioxidant activity of the lichen extracts of the *Usnea Aurantiaco-Atra*.

Figure 1. Specimen of Usnea aurantiaco-atra. (Jacq) Bory

2. MATERIALS AND METHODS

2.1. Plant materials

The probes of lichen *Usnea aurantiaco-atra* were collected and identified during two Antarctic campaigns (corresponding to austral summers of 2011 and 2013) in the vicinity of the Spanish base "Juan Carlos I" in Antarctica. It is located at the Hurd Peninsula of Livingston Island (Southern Shetland Archipelago).

2.2. Extraction procedure

A Soxhlet process successively and exhaustively extracted lichen powders during 4 hours with n-hexane, dichloromethane and methanol with 600 ml of each solvent for 30 g of dried lichen powder. After solvent removal under reduced pressure (Buchi, Labortechnik, Suiza), dry residues were dissolved in dimethylsulfoxide (DMSO), obtaining 3 extracts: hexane extract, dichloromethane extract and methanolic extract.

LIchen (30 g)	Extraction yield (g)
Hexane Extract	1,04± 0,26
Dichloromethane Extrac	0,73± 0,15
Methanol Extract	4,58 ± 1,02

2.3 The extract identification

The preliminary study using CCF , CG-MS y RMN revealed that the hexanique, diclorometan and metanolic contained usnic acid, triglicerido and sugars.

For each extract, ***CC flash de silicagel 60*** was performed, with the corresponding dissolvent. Afterwards, each of the obtained fractions of every extract was brought to GS-MS where the compounds were identified by comparing them with the machine database. The significant results of metabolites are the ones with A/Total Area greater or equal 5%.

Hexane extract: it has a high percentage of usnic acid in F0. Fraction F1 presented an **Ergosta derivative -4,6,8 (14), 22-tetraen-3-one**, which has been shown to have antitumor, cytotoxic, immunosuppressive activity, and is effective in the treatment of chronic kidney disease (Zhao et al., 2010). F4 presented ***Crinosterol*** (encountered in seaweed), which has been used in the treatment of Alzheimer's due to its anti-aging properties and neuroprotective functions (Sun et al., 2014). The latter fraction is the most interesting.

In the dichloromethane extract, the F3 stood out, which presented a lower percentage of usnic acid than in the hexane extract, **Ergosta -4,6,8 (14), 22-tetraen-3-one** and a low percentage of unidentified **terpene** and also ***acids fatty***.

Methanolic extract: several fractions with usnic acid were obtained although the percentages are the lowest. It also contained very low percentage of unidentified **terpenes** and an important amount of **phenolic compounds**, aromatic hydrocarbons and sugars.

3.Cytotoxicity tests

3.1 Cell lines

The cell lines used in this study were: 1. A-375 human melanoma cells (ATCC CRL-1619), American Tissue Culture Collection (Manassas, VA, USA) 2. HT-29 human colon cancer cells (ATCC HTB-38), American Tissue Culture Collection (Manassas, VA, USA) 3. NCTC-2544 human keratinocyte skin cells (ICLC HL97002). Interlab Cell Line Collection (ICLC), Italy.

3.2 Cytotoxicity assay

The cell viability assay was determined by the MTT reduction assay (Mosmann, 1983) based on the color reaction of the mitochondrial dehydrogenase of living cells with MTT. The cytotoxic or antiproliferative effect of each extract was expressed as IC50 (Inhibitory dose that inhibits 50% of cell growth).

Cells were cultured in RPMI (HeLa) or DMEM media (HT29) supplemented with 10% fetal bovine serum (FBS) at 37ºC under a humidified atmosphere of 95% air and 5% CO_2. *Seeding density:* HeLa (2000 cells/well) and HT29 (6000 cells/well). They were seeded in 96-well plates, 18 h before addition.

Experimental procedure: Cells were treated with lichen extracts in final doses ranging from 0.1 mg/mL to 100 mg/mL during 72 hours. At the end of the incubation period, 10 mL of a 5 mg/mL of filtered MTT solution in PBS and 240 mL of PBS were added to the wells. 4 h later formazan crystals were dissolved (using 150 mL DMSO) and spectrophotometrically measured at 590 nm using a VictorX5 plate reader.

According to the standards of National Cancer Institute (NCI), a crude extract may be considered as active for an **$IC_{50} \leq 30\mu g/ml$** (Itharat et al., 2004).

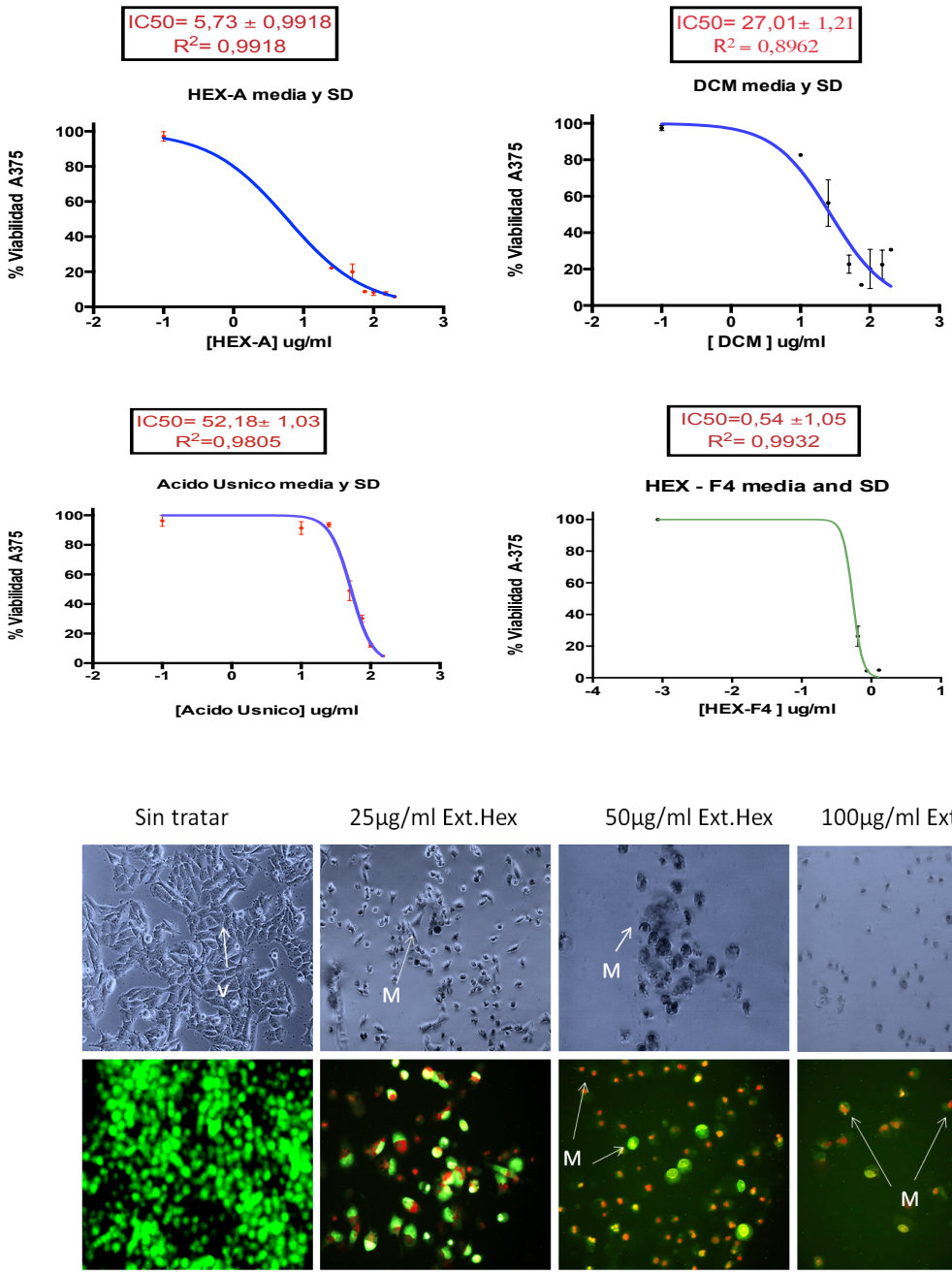
The results we obtained on the melanoma lines were the following:

A375	IC50µg/ml	R^2
Extract HEX-Media y SD	5,73 ± 1,19	0,9918
A375	IC50 µg/ml	R^2
Extract DCM-Media y SD	27,02 ± 1,21	0,8962
A375	IC50 µg/ml	R^2
Extract MEOH-Media y SD	293,3 ± 1,17	0,7343

A375	IC50 µg/ml	R^2
Usnic Ac Media y SD	52,18 ± 1,03	0.9805

A375	IC50 µg/ml	R^2
F4 (Hex) Media y SD	0,5403 ± 1,05	0.9932

The fraction F4 of the hexane extract was chosen to add to the melanoma cells (A-375) because it is the only fraction of the hexane extract that has sterols and a high percentage of linoleic acid, unsaturated fatty acid with cytotoxic activity (Yang et al., 2015) and also does not contain usnic acid.



The melanoma cells (A-375) were stained with AT in the upper panels and with NA / IP in the lower panels. As can be seen in both stains, when the concentration of extract increases the number of living cells decreases. In the upper panels dead cells are dark blue and in the bottom panels they are either red or orange.

4.Determination of the antioxidant activity in the methanolic extract.

The antioxidant activity was determined on the methanolic extract by means of a DPPH solution following the method previously published by (Fukumoto and Mazza, 2000), using BHT (2,6-di-t-butyl-4-methylphenol) as a reference. All reactions were carried out at room temperature protected from light and in triplicate. Subsequently, the discoloration of the DPPH radical was measured spectrophotometrically with a Victor X5 plate reader (Perkin Elmer) at 520 nm.

The antioxidant activity is expressed as the concentration of antioxidant compounds capable of inhibiting 50% of the DPPH radical. (IC50).

The obtained results can be seen in the following table.

	IC50 (mg/ml)	IC50 (µg/ml)
MeOH Extrac	0,13075	130,75 ± 4,3
BHT	0,01059	10,59 ± 3,7

A plant extract with an IC50 with values smaller than 30 µl / ml is considered to have a high antioxidant potential (Ramos et al, 2003). Therefore, we observe that our extract does not have a high antioxidant activity. In addition, if we compare it with the BHT pattern, we can see that it is 10 times more powerful and occurs at lower concentrations.

5. Determination of Total Phenols on the methanolic extract

The content determination of total phenols was performed on the methanolic extract using the Folin-Ciocalteu reagent according to the method (Slinkard and Singleton, 1977) and taking gallic acid as a reference.

Total Phenols	mg GA/g Extracto
Ex MeOH sin DERV	68,61 ± 0,01
Ex MeOH-SP1 (Agua100%)	64,28 ± 0,02
Ex MeOH-SP3 (MeOH100%)	67,15 ± 0,02
Ex MeOH-SP4 (AcO Et 100%)	66,92 ± 0,03

From the obtained results, we see that the methanolic extract has phenolic compounds in significant amounts. However, if we compare these data with the antioxidant activity of said extract, there is no good correlation and a higher antioxidant activity can be expected.

The DPPH method is fast, simple and cheap but it can give conflicting results because this test can be influenced by various factors such as solvent polarity or by the pH of the mixture obtained with the extract (Saito and Kawabata, 2005),

6. Insecticidal activity

The insecticidal tests were carried out exclusively against ***Ceratitis capitata Wiedmann*** diptera in the CEQA insectarium where the colonies of insects are maintained at the conditions of temperature (27 ± 2ºC) and humidity (50-60%) and adequate light.

Entomotoxicity tests were performed by ingestion of the three extracts and of the single-acid acid previously dissolved and mixed with the food (protein hydrolyzate and sucrose).

Each treatment was carried out in triplicate on 10 flies (5 males and 5 females) 2-3 days old (virgins). The ingestion period of the treated food was 4 days. Once this period was over, the treated food was replaced by normal food.

The initial dose of the trial was 50,000 ppm, which is equivalent to 50 mg / g of diet for the extracts and the single acid. Three repetitions were performed per extract, with one control, for which two repetitions were performed.

Mortality was observed daily, for 10 days, and the degree of toxicity was expressed as the average of% mortality, after 10 days. The eggs were followed up to see if it affected fertility (number of eggs laid per female and day) and fertility (% of eggs hatching) on the planned days.

The results obtained can be seen in the following table.

• *Ceratitis capitata*	% Mortality	% Control	% Hatching eggs
Extracto Hexánico	**23 ± 3,2**	**10 ± 1,1**	**≥ 50 %**
Extracto Diclorometánico	**9,2 ± 2,3**	**4,6 ± 1,3**	**≥ 50 %**
Extracto Metanólico	**3 ± 2,2**	**6,3 ± 1,2**	**-**
Ácido úsnico puro	**39 ± 4,23**	**10 ± 1,3**	**-**

Regarding mortality we see:

The **hexane extract** has a mortality of 23 ± 3.2 as of the sixth day and extract dichloromethane of 9.2 ± 2.3 from the seventh day although both mortalities are low if we compare them with the controls. The methanolic extract does not seem to affect even the mortality of flies. The mortality of ***pure usnic acid*** in flies is 39 ± 4.23% from the 6th day of ingestion.

Regarding the hatching of eggs:

We see that hexane and dichloromethane extracts from the 2nd day of egg collection 50% of these do not hatch, and this effect is maintained until the sixth day of egg collection. However, as of the seventh day, they already hatch almost 100% again. Pure usnic acid and methanolic extract we see that they do not affect the hatching of eggs.

We conclude that our extracts do not have an insecticidal activity of interest.

Conclusions

1. The phytochemical study of the extracts shows that the most abundant metabolite is usnic acid, present in decreasing proportions in hexane, dichloromethane and methanolic extracts.

2. We have identified by CG-MS other metabolites such as triterpenes, highlighting **3 α, 5-cycle - 5α - ergosta - 6.8 (14), 22 - triene** present in fraction F1 of the hexane extract and **Ergosta -5, 22 -dien -3- ol (3β, 22Є, 24S)** in hexane and dichloromethane extracts, in fractions F3 and F4 respectively. Of the fatty acids found, the linoleic acid present in the hexane and methanolic extract in similar amounts stands out for its cytotoxic activity.

3. Regarding the cytotoxic activity, the greatest activity has been obtained with the hexane and dichloromethane extract, considering that its extracts have cytotoxic activity against the skin cell lines A-375 and NCTC-5524, while against HT- 29 the activity of the extracts is antiproliferative. In vitro cytotoxic activity of the methanolic extract is low or zero in the three cell lines tested. Activity of commercial usnic acid is antiproliferative in all three extracts.

4. Hexane and dichloromethane extracts have greater cytotoxic activity than commercial usnic acid. Therefore, that this activity could be due to other metabolites present in the extracts. The high cytotoxic activity that appears in some fractions containing terpenes without usnic acid, suggests a direct relationship between the presence of these compounds and their cytotoxic activity.

5. There is no good correlation between the presence of phenolic compounds in our extracts and the antioxidant activity obtained by the DPPH method.

6. Regarding the insecticidal activity, we have determined that the hexane extract has shown a low insecticidal activity against Ceratitis capitata, when compared with commercial usnic acid.

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