Supercritical fluid extraction of heather (*Calluna vulgaris*) and evaluation of anti-hepatitis C virus activity of the extracts

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ABSTRACT

Heather (*Calluna vulgaris*) leaves contain good amounts of ursolic and oleanolic acid, which have been reported to present antiviral activity against hepatitis C virus (HCV). In this work, the supercritical fluid extraction of heather was studied with the target of assessing a potential anti-HCV activity of the extracts owing to their triterpenic acid content. Supercritical extraction assays were carried out exploring the pressure range of 20-50 MPa, temperatures of 40-70 °C and 0-15% of ethanol cosolvent. The content of oleanolic and ursolic acid in the extracts were determined, and different samples were screened for cellular cytotoxicity and virus inhibition using a HCV cell culture infection system. Antiviral activity was observed in most extracts. In general, superior anti-HCV activity was observed for higher content of oleanolic and ursolic acids in the extracts.

Keywords: supercritical fluid extraction; *Calluna vulgaris* L.; heather; hepatitis C virus; ursolic acid; oleanolic acid.

1. INTRODUCTION

For many centuries plants have been used for medicinal purposes, many have led to the discovery of important molecules such as aspirin, morphine and codeine. Heather (*Calluna vulgaris* L.) has been used as an herbal remedy against kidney and urinary infections as well as rheumatism. Several phytochemicals with antioxidant, anti-inflammatory and anticancer activities have been identified in heather (Filip et al., 2012; Orhan et al., 2007; Saaby et al., 2009; Simon et al., 1992). Among these compounds, triterpenoids, in the form of free acids or aglycones of saponins, have gained more attention, and the number of papers describing their biological effects has increased sharply during the last decade (Banno et al., 2004; Checker et al., 2012; Ikeda et al., 2008; Liu, 1995; Yan et al., 2010).

Heather has also been reported to contain high concentrations of ursolic and oleanolic acid (around 40 mg/g and 10 mg/g dry matter, respectively) (Jalal et al., 1982; Zhao, 2011). Ursolic acid (3-hydroxy-urs-12-ene-28-oic acid) is a pentacyclic triterpenoid carboxylic acid which is well known because of its antioxidant, anti-inflammatory, and anticancer activities, combined with a relatively low toxicity(Checker et al., 2012; Ikeda et al., 2008). Oleanolic acid (3b-hydroxyolean-12-ene-28-oic acid) has been shown to have similar biological activities as ursolic acid (Liu, 1995; Yan et al., 2010). Recently, both of these compounds have been reported to have antiviral activity against hepatitis C virus (HCV) by inhibiting the NS5B RNA-dependent RNA Polymerase (Kong et al., 2013).

Approximately 2.8% of world population is infected with HCV (Mohd Hanafiah et al., 2013). Current standard of care treatment includes pegylated interferon alpha plus ribavirin, combined with the new directly antiviral agents (DAA) such as telaprevir, boceprevir and the recently approved polymerase inhibitor, sofosbuvir (Gilead, 2013).

Most currently available anti-HCV compounds target later stages of the viral lifecyle, such as viral RNA replication, and are intended for use in chronically infected patients. Therefore, the discovery of novel compounds to block HCV cell entry is an area of intense research, with the aim of restricting universal reinfection of the donor liver by circulating virions in the setting of liver transplantation for HCV-associated end stage liver disease. The entry step of the HCV lifecycle is critical for initiation, maintenance, and dissemination of viral infection *in vivo*, and represents an attractive target for therapeutic intervention (Fofana et al., 2014). In the past few years, various natural compounds have been described to have direct or indirect antiviral activities against HCV (Calland et al., 2012; Lindenbach et al., 2005; Reiss et al., 2011).

Supercritical fluid extraction (SFE) using carbon dioxide (CO_2) is progressively replacing the organic solvent extraction, particular in the processing of natural matter. Several applications, such as the extraction of hops and the removal of caffeine from coffee beans, are well known processes performed on an industrial scale, certainly due to its advantages in comparison with conventional solid-liquid extraction. Supercritical CO_2 is capable of extracting a wide range of diverse compounds, from non-polar or moderately polar compounds to more polar substances by combining CO_2 with a cosolvent, such as methanol, ethanol, acetone, water, diethyl ether, among others. Yet, ethanol may be the better choice in SFE of nutraceuticals and food ingredients because of its lower toxicity.

To our knowledge, the only information available concerning the supercritical CO_2 extraction of heather was reported by Hunt (Hunt, 2006) and Zhao(Zhao, 2011) in their respective PhD theses developed in the University of York, UK. In these works, the extraction of the aerial part of dry plants with different harvest time was investigated, and the higher yields obtained were in the range 3.9 - 4.5 % (depending on

plant harvest time) and were produced at 50°C, 35 MPa and employing 10 % of ethanol as CO_2 cosolvent. Ursolic acid and oleanolic acids were identified in these extracts, together with other high valued triterpenoids, such as α-amyrin, β-amyrin, taraxerone and taraxerol.

In this work the SFE of heather was investigated in the temperature range of 40-70°C, pressures of 20-50 MPa and using 0-15 % ethanol cosolvent. The effect of process conditions on extraction yield and content of the triterpenic acids identified, namely ursolic acid and oleanolic acid, was evaluated. Additionally, antiviral activities of each of the extracts were tested against HCV.

2. MATERIAL AND METHODS

2.1 Samples and Reagents.

Standards, chemicals and reagents: oleanolic acid (≥99%) and ursolic acid (≥98%) were purchased from Extrasynthese (Genay Cedex, France). Acetonitrile was HPLC grade from LabScan (Gliwice, Poland) and ethanol absolute was purchased from PANREAC (Barcelona, Spain).

Heather sample consisted of dry leaves and flowers (8.4 % w/w water content) purchased from an herbalist's producer (Murcia, Spain). The vegetal matter was ground in a cooled mill and sieved to sizes between 400 and 600 μm.

2.2 Supercritical Fluid Extraction (SFE).

The extractions were carried out in a pilot-plant scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising of a 2 L cylinder extraction cell with automatic control of temperature and pressure. For each experiment, the cell was filled with 0.5 kg of plant raw material. The extraction assays were performed at temperatures in the range of 40-70°C and pressures of 20-50 MPa. Table 1 show the conditions employed in each experiment.

Extracts 11a and 11b in Table 1 were obtained by fractionation of the extract using a depressurization cascade system comprised by two separators (S1 and S2). Fractionation was accomplished by maintaining S1 at 30 MPa while S2 was set at the recirculation CO_2 pressure (6 MPa). Extraction 13 was performed in two steps: the first step (Extract 13a) comprised the extraction with pure supercritical CO_2 (25 MPa, 50°C, 1.5 h), and the second step (Extract 13b) was carried out using ethanol as co-solvent (30 MPa, 50°C, 2.5 h). In all other extractions the extract was collected in S1 by depressurization up to 6 MPa. Ethanol was used to wash out the collector vessels and ensure a complete recovery of the material precipitated in the cell. Ethanol was eliminated by evaporation and the homogeneous solid samples obtained were kept at 4°C in the dark until analysis.

2.3 Chemical Analysis.

Identification and quantification of triterpenic acids was accomplished employing a HPLC model Varian ProStar (Varian, Palo Alto, CA, USA) equipped with a Col KROMAPHASE C18 column (Scharlab, Barcelona, Spain) of 25 mm × 4.6 mm and 5 mm particle size. The analytical method used comprises of an isocratic gradient of 30 min using a mixture of acetonitrile and water (90:10 % vol) as mobile phase. The flow rate was constant at 1 mL/min and the injection volume was 20 µL. The detection of compounds was carried out at a wavelength of 210 nm. The quantification of triterpenic acids was accomplished by calibration curves with commercial standards of ursolic, oleanolic and betulinic acids; straight lines were obtained with linear regressions higher than 0.999.

2.4 Cells, Virus, Extract Dilutions.

Huh7.5 cells were grown in complete Dulbecco's modification of Eagle's medium (DMEM) (Invitrogen) with 10% fetal calf serum (FCS), as previously

described in Perales et al (Perales et al., 2013). The HCV cell culture (HCVcc) virus derived from the plasmid, Jc1FLAG2(p7-nsGluc2A) or the monocistronic luciferase reporter virus JcR2a were used to infect Huh7.5 cells, a viral stocks had been previously generated as described (Perales et al., 2013; Reiss et al., 2011).

Supercritical heather extractions, oleanolic acid and ursolic acid were diluted in absolute ethanol to 50 mg/ml and stored at -80ºC, for further dilutions, the extracts were diluted in complete DMEM (Fan et al., 2011).

2.5 Cytotoxicity.

To test for cytotoxicity Huh7.5 cells were plated to semiconfluency and incubated at 37°C, 5% CO_2 with each extract in 5-fold dilutions at concentrations 0, 0.64, 3.2, 16, 80, 400, 2,000 and 10,000 μg/ml. After 48 hours, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was added to each well at a final concentration of 500 μg/ml and incubated for a further 3 hours, the media was then removed and 100 μl of dimethyl sulfoxide (DMSO) (Sigma) was added to each well, the optical density was measured at a wavelength of 550 nm. The 50% cytotoxicity concentration (CC_{50}) was then calculated using the optical density percentage compared to untreated cells. Standard deviations were calculated from 4 replicates.

2.6 HCV inhibition assay.

Huh7.5 cells were plated in 96-well plates to semiconfluency ($6.4x10^3$ per well) and infected with tissue culture infective dose (TCID) of 192 (approximately MOI 0.03) of HCVcc, 5 hours later, each extract was added to the wells in 2-fold dilutions at concentrations 0, 6.25, 12.5, 25, 50, 100, 200 and 400μg/ml and incubated for 48 hours. The cells were then washed with PBS, fixed with methanol and stained for HCV NS5A antibodies (Lindenbach et al., 2005). Foci forming units were counted and compared to

wells without extract. Percentages were used to calculate HCV inhibition. Standard deviations were calculated from 4 replicates.

For the HCV entry assay, a monocistronic luciferase reporter virus JcR2a was used(Anggakusuma et al., 2013). Huh7.5 cells were inoculated with JcR2a virus in the presence of increasing concentration of the extracts. The green tea molecule Epigallocatechin-3-gallate (EGCG) was used as positive control(Ciesek et al., 2011). The inoculum was removed 4 h later and then monolayers were washed and overlaid with fresh medium containing no inhibitors. Infected cells were lysed 3 days later and Renilla luciferase activity was determined as described(Ciesek et al., 2011).

3 RESULTS

3.1 Extraction Yield and Recovery of Triterpenic Acids.

The extraction yield (g extract / g heather) obtained in the different experiments are given in Table 1. Extraction 9 was carried out at similar extraction conditions than those employed by Zhao (2011)(Zhao, 2011) and similar yields were obtained.

Figure 1 shows the variation of extraction yield with temperature (Figure 1a), pressure (Figure 1b) and amount of ethanol cosolvent (Figure 1c). As observed, the most significant effect on extraction yield is produced by the addition of ethanol as cosolvent of the supercritical CO_2 solvent. With respect to pure CO_2, around a 4-fold increase (from 2.16 to 7.31 %) of yield is produced when using ethanol as CO_2 cosolvent.

Additionally, the increase of extraction temperature from 40°C to 60°C favors the recovery of phytochemicals from heather, but a further increase of temperature has the opposite effect.

Extractions 4 and 11 in Table 1 were obtained using the same extraction temperature and pressure, but slightly higher CO_2/plant ratio in extraction 4 (24 kg/kg vs 21.6 kg/kg) and also slightly higher extraction yield was obtained (2.45 vs 2.20 %). Nevertheless, fractionation of the extract in extraction 11 produced two samples with noticeably different yields: extraction 11a (0.26 % yield, S1 separator) and extraction 11b (1.94 % yield, S2 separator).

Alternatively, extraction 13 was carried out in two steps, using different extraction conditions in each step. Comparing both steps it can be clearly deduced the key effect of ethanol as CO_2 cosolvent, since 5.28 % yield was obtained in the second step when 2.65 % of phytochemicals were already extracted in the first step.

The concentration (mg acid / g extract) of ursolic and oleanolic determined by HPLC in all supercritical extracts produced is shown in Table 2. The higher concentrations of the triterpenic acids were obtained when at least 10 % ethanol was employed as cosolvent (Extracts 9, 10, 12 and 13b) with values in the range of 90-220 mg triterpenic acids / g extract (1.1-2.3 % w/w). In all other experiments no ethanol was employed, and triterpenic acids were present in concentrations lower than 30 mg/g, but as a result of the on-line fractionation procedure, 116 mg/g of triterpenic acids was obtained in the first fraction of extraction 11 (Ext. 11a) without using ethanol, although very low extraction yield was achieved in this fraction (0.26 %).

The highest concentration of triterpenic acids was obtained in extract 10 (82.87 mg/g of oleanolic acid and 141.45 mg/g of ursolic acid) which was produced at moderate pressure (30 MPa) and with the higher ethanol content utilized in this work (15 %). Taking into account the concentration of these triterpenic acids in the dry matter reported in the literature(Jalal et al., 1982; Zhao, 2011), this represents a 3.5 and 8.3

fold increase for, respectively, ursolic and oleanolic acid. Furthermore, this extraction was the one with the higher yield (7.31 %).

Additionally, it could be observed from Table 2 that the ratio of oleanolic acid / ursolic acid resulted in values considerably lower than 1 (from 0.14 to 0.43), in the case of extractions with pure CO_2. Unfortunately, no solubility data was found in the literature to support this observed behavior. Nevertheless, this seems to be a reasonable tendency, since ursolic acid is present in heather in higher concentrations than oleanolic acid (oleanolic acid / ursolic acid ratio in heather ≈ 0.25). On the contrary, when ethanol is utilized, this ratio becomes very close or greater than 1 and higher concentrations of oleanolic acid with respect to ursolic acid were obtained (oleanolic acid / ursolic acid ratio up to 1.42 in extraction 13b). This result could be related to the higher solubility of oleanolic acid in ethanol in comparison with ursolic acid solubility(Fan et al., 2011), and is showing a great effect of ethanol as cosolvent in the supercritical extraction, tuning selectivity towards the extraction of oleanolic acid.

3.2 Anti-HCV Activity of the Extracts.

Previously, Kong et al(Kong et al., 2013) reported that oleanolic and ursolic acid were able to inhibit the HCV polymerase activity. Considering the high content of oleanolic and ursolic acids found in heather, the anti-HCV activity of heather supercritical extracts produced in this work was evaluated and compared.

All 15 extracts with different contents of oleanolic and ursolic acid, were screened for cellular cytotoxicity and HCV inhibition using the HCV cell culture system (see materials and methods). Moreover, both oleanolic and ursolic compounds were also tested in parallel as controls.

The system uses a full length HCV infectious clone that is able to undergo the complete replication cycle in vitro. Cytotoxicity was observed in all extracts at

concentrations more than 200 µg/ml and in most extracts at 100 µg/mg. Nevertheless, in all cases there was reduction in the viral infectivity in a dose dependent manner at concentrations where cytotoxicity was not observed. In particular, extracts 9 and 13b showed more striking reductions in their infectivity, which coincided with higher levels of oleanolic and ursolic acid (see Figure 2A and Table 2).

To investigate specifically the early steps (viral entry) of the viral life cycle, a luciferase reporter HCV was used. Human hepatoma cells were incubated for 4 hours in the presence of the extracts and virus before washing and replacing with fresh media (Figure 2B). The green tea molecule Epigallocatechin-3-gallate (EGCG) was used as positive control, as we have previously shown that ECGC inhibits HCV infectivity by blocking the virus entry process(Ciesek et al., 2011). No cytotoxicity was observed at concentrations of 125 µg/ml or less. All extracts showed some inhibition in the HCV infectivity in a dose-dependent manner and 6/15 inhibited at least 50% HCV entry. Again high connection was observed with the inhibition of HCV entry and the concentration of oleanolic and ursolic acid.

To determine whether there was a correlation between the concentration of oleanolic acid or ursolic acid and HCV inhibition, infectivity (% of negative control) was represented as a function of the actual amount of oleanolic or ursolic acid utilized in the assays, and calculated as the concentration of each compound for each extract dilution used in the viral entry assay or the complete replication assay. These results are depicted in Figure 3; only extracts which do not exhibit cytotoxicity were included in the representation. As can be observed in Figure 3 a certain dependence of the antiviral effect can be attributed to the presence of both ursolic and oleanolic acids, suggesting both these compounds may have anti-HCV properties. Oleanolic and ursolic acid concentrations correlated more with inhibition of viral entry (Figures 3A and C) (R^2=

0.7948 and 0.3732 respectively) compared to the inhibition of the full viral lifecycle (Figures 3B and C) (oleanolic acid R^2= 0.2598 and ursolic acid R^2= 0.0821).

4 CONCLUSIONS.

Supercritical fluid extraction produced heather extracts with high concentration of triterpenic (oleanolic and ursolic) acids. In this respect, the use of ethanol as cosolvent was crucial. Concentrations up to 80 mg/g of oleanolic acid and 140 mg/g of ursolic acid were attained using 15% ethanol cosolvent, which are significantly higher than those obtained without cosolvent (2-12 mg/g of oleanolic acid and 10-20 mg/g of ursolic acid) despite the extraction pressure (20-50 MPa) or temperature (40-70 °C) applied. Furthermore, the use of ethanol as cosolvent turned the selectivity of the supercritical solvent to favor the extraction of oleanolic acid, despite the lower amount of this triterpenic acid in the vegetal raw matter.

The extracts showed antiviral activity in a complete life cycle assay for HCV and interestingly also when only presented during viral entry. The extracts with higher concentration of ursolic and oleanolic acid showed higher inhibition, and certain dependence of the anti-HCV activity and the presence of ursolic and oleanolic acid in the extracts was observed. Further studies are required to elucidate the mode of action against that human important virus.

5 ACKNOWLEDGEMENTS

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366 **Table 1.** SFE conditions and yields obtained in the extraction of heather leaves.

Extract		Pressure (MPa)	Temperature (ºC)	Time (h)	CO_2 flow (g/ min)	Co-solvent (% ethanol)	Yield (%)
1		20	50	3	60	0	1.17
2		30	50	3	60	0	2.16
3		40	50	3	60	0	2.32
4		50	50	3	60	0	2.45
5		40	40	3	60	0	2.04
6		40	60	3	60	0	3.35
7		40	70	3	60	0	2.40
8		30	50	3	60	5	3.66
9		30	50	3	60	10	4.68
10		30	50	3	60	15	7.31
11a	S1	50	50	4	50	0	0.26
11b	S2						1.94
12		30	50	4	50	10	1.71
13a	First step	25	50	1.5	50	0	2.65
13b	Second step	30	50	2.5	50	10	5.28

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369 **Table 2.** Content of triterpenic acids identified in heather SFE extracts.

Extract		Oleanolic acid (mg/g extract)	Ursolic acid (mg/g extract)
1		0.53	16.02
2		2.36	16.92
3		3.55	15.49
4		5.66	18.86
5		2.49	17.85
6		5.90	14.31
7		9.19	21.59
8		12.41	11.33
9		69.75	55.57
10		82.87	141.45
11	S1	13.50	98.56
	S2	2.53	2.95
12		54.04	38.67
13	First step	1.44	2.19
	Second step	73.14	51.62

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FIGURE LEGENDS

Figure 1. Extraction yields of heather SFE as a function of (a) extraction pressure (50°C, no cosolvent), (b) extraction temperature (40 MPa, no cosolvent) and (c) ethanol cosolvent added to supercritical CO_2 (50°C and 30 MPa). Solid lines: experimental trend.

Figure 2. Anti-HCV activity of Heather extracts. (A) Huh7.5 cells were infected with HCVcc at a MOI of 0.03, 5 hours later; each extract was added to the wells in 2-fold dilutions at concentrations 0, 6.25, 12.5, 25, 50, 100μg/ml and incubated for 48 hours. The cells stained for HCV NS5A antibodies and foci forming units were counted and compared to wells without extract (see materials and methods). Percentages were used to calculate HCV inhibition. Standard deviations were calculated from 4 replicates. (*) indicate when cytotoxicity was observed. (B) Huh7.5 cells were inoculated with HCV (JcR2a virus) in the presence of increasing concentration of the extracts. The green tea molecule Epigallocatechin-3-gallate (EGCG) was used as positive control [23]. The inoculum was removed 4 h later and then monolayers were washed and overlaid with fresh medium containing no inhibitors. Infected cells were lysed 3 days later and Renilla luciferase activity was determined as described [23].

Figure 3. Anti-HCV activity of Oleanolic and Ursolic acids. Concentrations of oleanolic acid (3A, 3B) or ursolic acid (3C, 3D) are plotted against HCV inhibition, for either the 4 h entry assay (3A, 3C) or 48 h full lifecycle assays (3B, 3D). Linear regression analyses are represented in the box at the top right corner of each graph.

Figure(s)

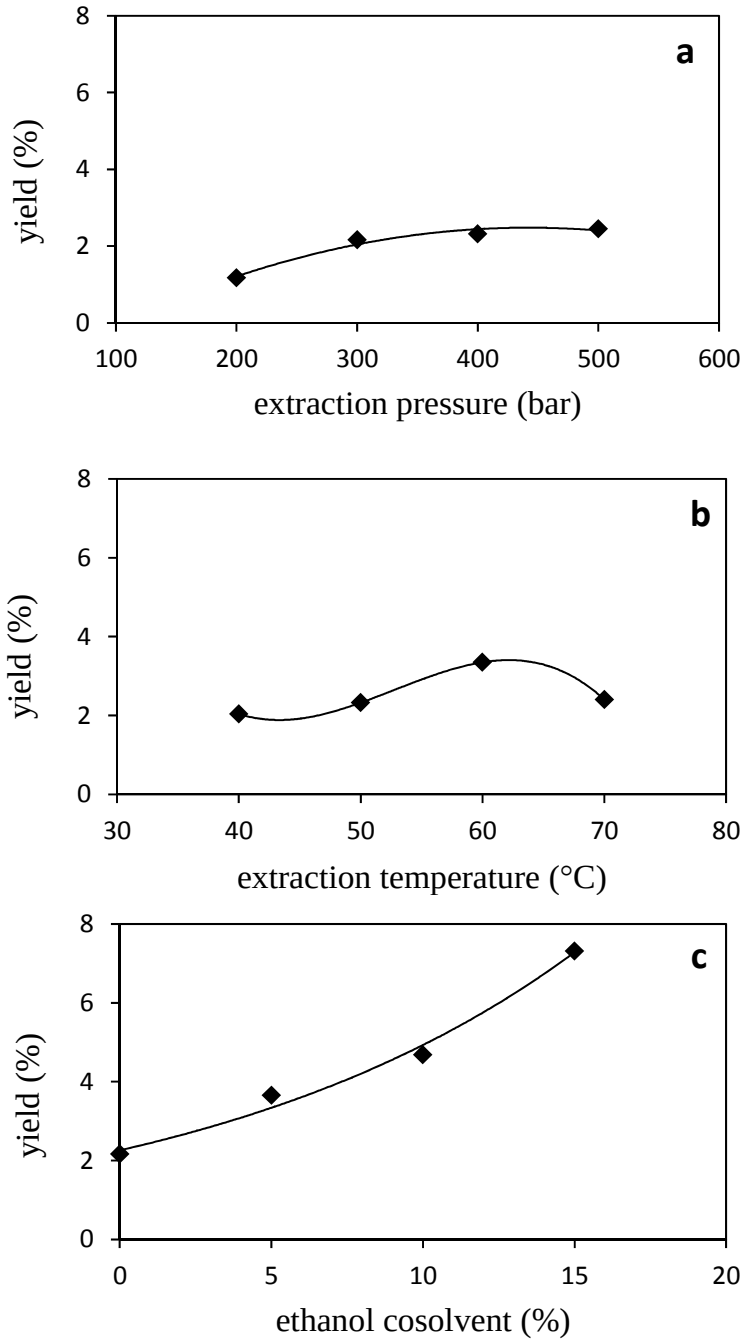
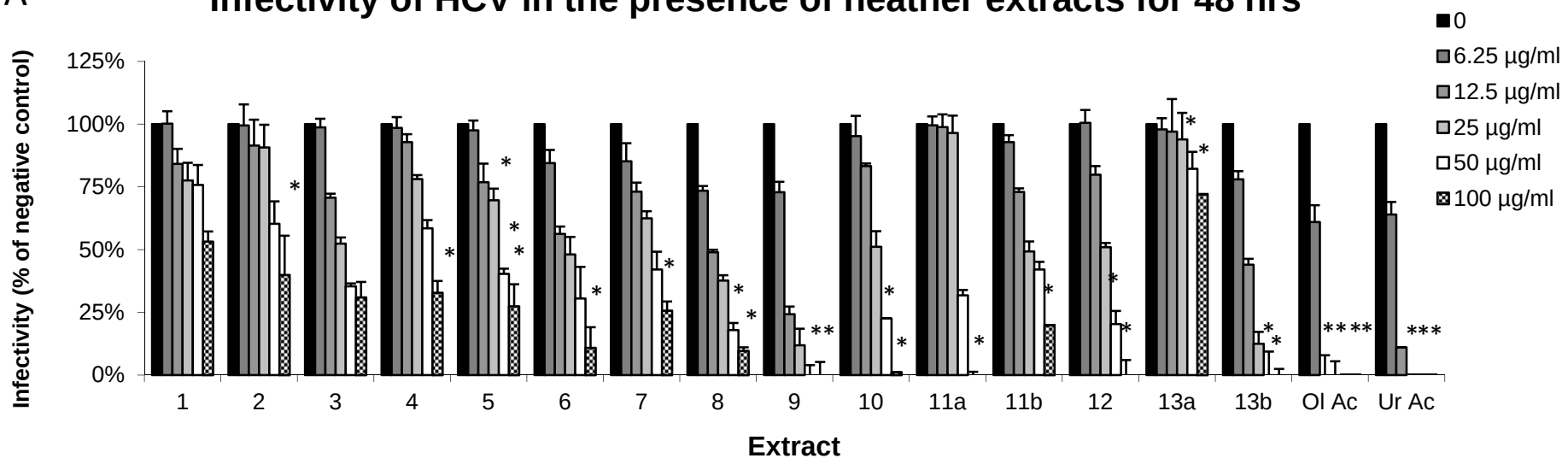


Figure 1.

A


Infectivity of HCV in the presence of heather extracts for 48 hrs
Infectivity (% of negative control)
125%
100%
75%
50%
25%
0%
1
2
3
4
5
6
7
8
9
10
11a
11b
12
13a
13b
Ol Ac
Ur Ac
Extract
0
6.25 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml

B

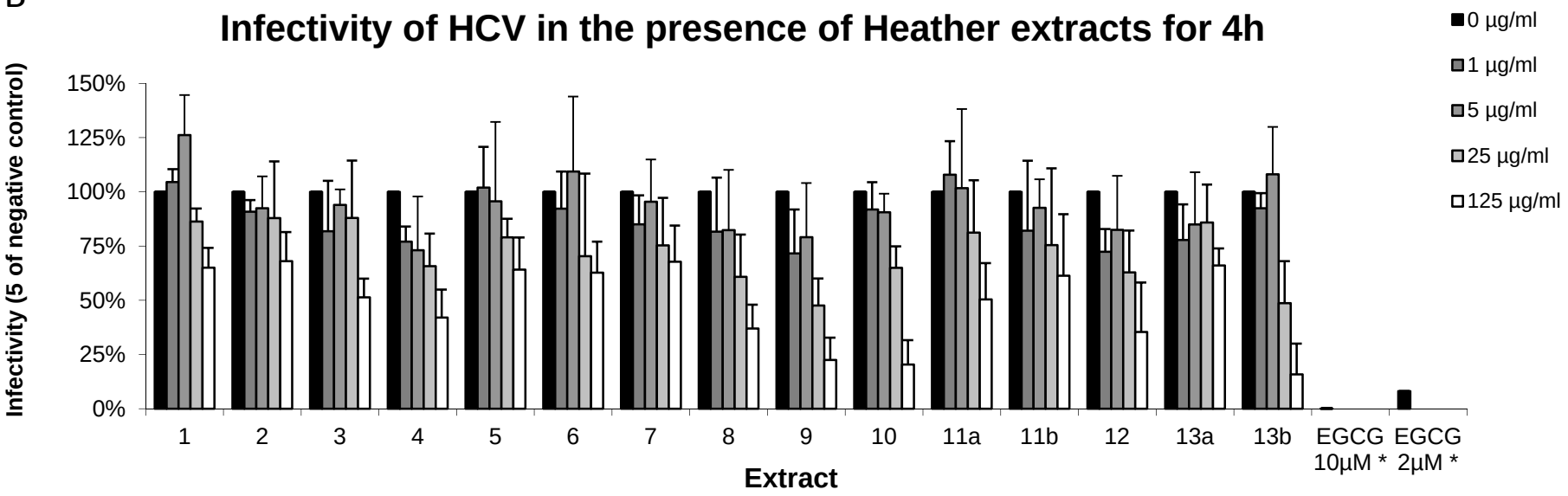

Infectivity of HCV in the presence of Heather extracts for 4h
Infectivity (5 of negative control)
150%
125%
100%
75%
50%
25%
0%
1
2
3
4
5
6
7
8
9
10
11a
11b
12
13a
13b
EGCG 10µM *
EGCG 2µM *
Extract
0 µg/ml
1 µg/ml
5 µg/ml
25 µg/ml
125 µg/ml

Figure 2

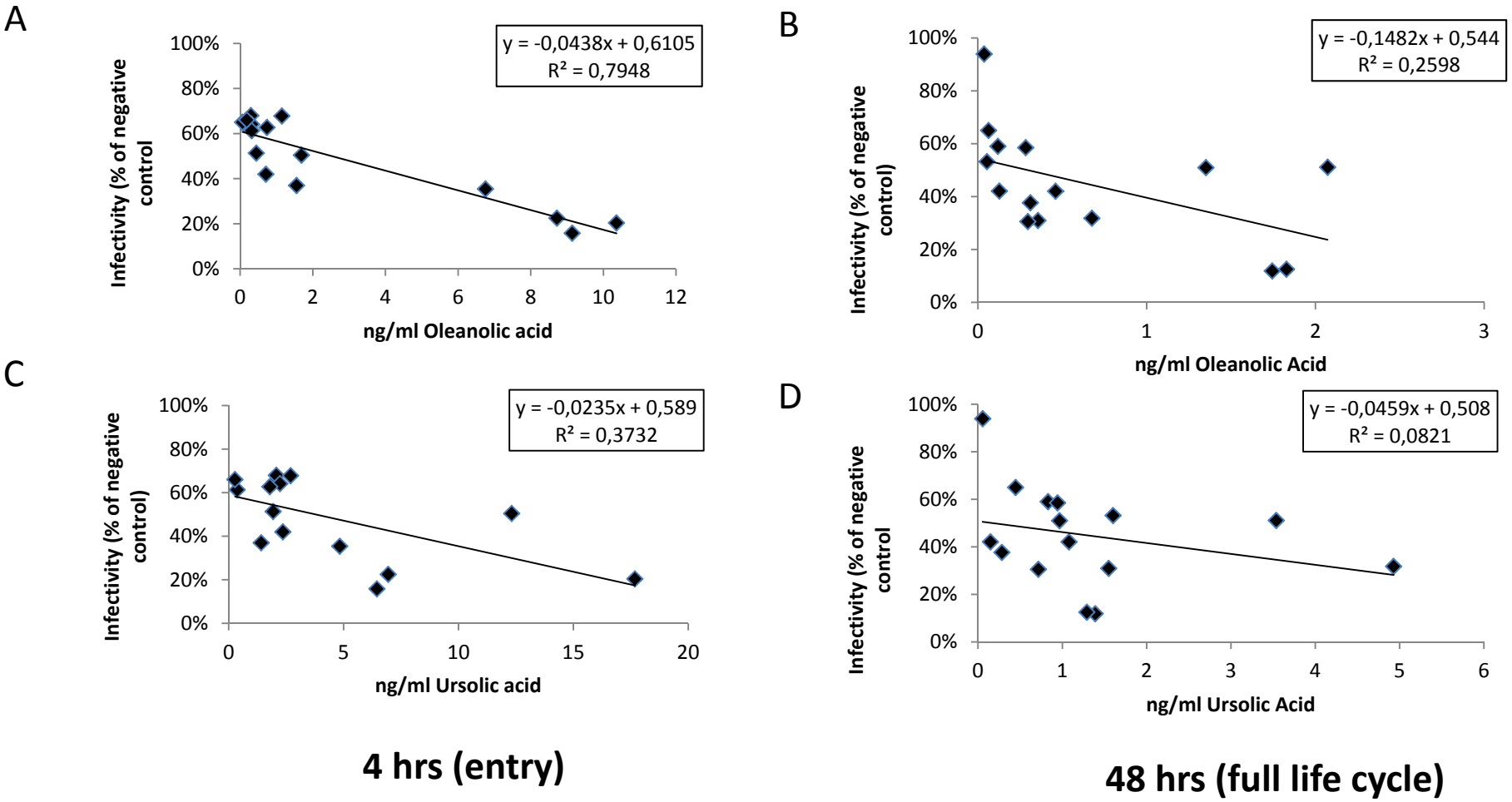

A
y = -0,0438x + 0,6105
R² = 0,7948
Infectivity (% of negative control)
ng/ml Oleanolic acid
B
y = -0,1482x + 0,544
R² = 0,2598
Infectivity (% of negative control)
ng/ml Oleanolic Acid
C
y = -0,0235x + 0,589
R² = 0,3732
Infectivity (% of negative control)
ng/ml Ursolic acid
D
y = -0,0459x + 0,508
R² = 0,0821
Infectivity (% of negative control)
ng/ml Ursolic Acid
4 hrs (entry)
48 hrs (full life cycle)

Figure 3