The power of grape extracts: antimicrobial and antioxidant properties to prevent the use of antibiotics in farmed animals: 101036768

D2.2

Due date of deliverable: 31/03/2022

Actual submission date: 28/03/2022

Horizon 2020
European Union Funding
for Research & Innovation

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101036768

TABLE OF CONTENTS

PROJECT INFORMATION

Project full title: The power of grape extracts: antimicrobial and antioxidant properties to prevent the use of antibiotics in farmed animals

Acronym: NeoGiANT

Call: H2020-LC-GD-2020-4

Topic: LC-GD-6-1-2020

Start date: 1st October 2021

Duration: 48 months

List of participants:

No.	Acronym	Participant organisation name	Country
1 (Coord)	USC	Universidade de Santiago de Compostela	Spain
2	MRI	Moredun Research Institute	United Kingdom
3	IBPRS	Instytut Biotechnologii Przemysłu Rolno-Spożywczego im. prof. Wacława Dąbrowskiego	Poland
4	VRI	Veterinary Research Institute	Czech Republic
5	MATE	Nemzeti Agrárkutatási és Innovációs Központ	Hungary
6	FUB	Freie Universität Berlin	Germany
7	FCUP	Universidade do Porto – Faculdade de Ciências	Portugal
8	ULL	Universidad de La Laguna	Spain
9	UNE	Asociación Española de normalización	Spain
10	JU	Jihočeská Univerzita	Czech Republic
11	CONICET	Consejo Nacional de Investigaciones Científicas y Técnicas	Argentina
12	ASAJA	Asociación Agraria de Jóvenes Agricultores	Spain
13	ATM	Anitom S.L	Belgium
14	i-GRAPE	i-GRAPE	Spain
15	CTA	Contactica S.L	Spain
16	NUS	Nutrition Science	Belgium
17	CZV	CZ VACCINES	Spain
18	LBE	LIFEBIOENCAPSULATION SL	Spain
19	BIAN	BIANOR BIOTECH	Spain
20	MAGA	MAGAPOR S.L.	Spain

DELIVERABLE DETAILS

Document Number:	D2.2
Document Title:	Deep chemical characterization report of bioactive compounds of grape extracts
Dissemination level	PU – Public
Period:	PR1
WP:	WP2
Task:	Task 2.2
Author:	UNIVERSITY OF SANTIAGO DE COMPOSTELA (USC)
Abstract:	This document corresponds to the Deliverable 2.2 Deep chemical characterization report of bioactive compounds of grape extracts. It covers the quantification of individual polyphenols and unwanted compounds (fungicides) in the first extracts at lab-scale employing advanced analytical tools.

Version	Date	Change
V1	22/03/2022	Initial version

Disclaimer

The views and opinions expressed in this document reflect only the authors' views, and not necessarily those of the European Commission.

1 BACKGROUND

The first bioactive extracts were obtained at lab-scale from the raw material (grape marc) in different solvents and their hydro-organic mixtures, as it is described in D2.1.

The selected solvents are shown in ***Table D2.2.1***. All of the extracts will be analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in two ways: first, to assure the absence of unwanted compounds such as fungicides; and second, to guarantee the presence of the bioactive compounds, identifying and quantifying each individual polyphenol.

Table D2.2.1. Tested organic solvents and their mixtures to obtain the firsts extracts at lab-scale

TESTED ORGANIC SOLVENTS TO OBTAIN FIRST EXTRACTS AT LAB-SCALE	
SOLVENT	**PROPORTION**
Ethyl lactate/water	50:50 (v/v)
Ethanol/water	50:50 (v/v)
Propylene glycol/water	50:50 (v/v)
Dimethylformamide	100%
Dimethylsulfoxide	100%
Acetone	100%
Dichloromethane[a]	100%

[a] Not characterized due to incompatibility of the solvent with the LC-MS/MS instrument

2 CONFIMING THE ABSENCE OF UNWANTED COMPOUNDS IN THE OBTAINED EXTRACTS

In previous experiments, the concentration of fungicides was determined in the raw material (grape marc) showing that it was well below the Maximum Residues Levels (MRLs) established by the EU legislation for wine grapes (see results in **D2.1.**), therefore demonstrating that the raw material can be safely employed to produce extracts. Despite this result and to a greater extent, to assure that the obtained extracts are "free" of unwanted compounds such as fungicides, the first extracts obtained at lab-scale employing the selected solvents and their hydro-organic mixtures were also analyzed by LC-MS/MS. Solvent details and analytical results are depicted in ***Table D2.2.1.***

Table D2.2.1. Target fungicides, CAS identification number, and concentration (mg L^{-1}) found in the first extracts at lab-scale

FUNGICIDE	CAS	CONCENTRATION (mg L^{-1})					
		DMF	DMSO	Acetone	Ethanol/H_2O	Ethyl lactate/H_2O	Propileneglycol/ H_2O
Metalaxyl	57837-19-1	0.024 ± 0.001	0.012 ± 0.001	0.025 ± 0.001	0.024 ± 0.001	0.019 ± 0.003	0.022 ± 0.001
Cyprodinil	121552-61-2	0.17 ± 0.01	0.203 ± 0.001	0.39 ± 0.02	0.053 ± 0.004	0.15 ± 0.01	0.022 ± 0.001
Tolylfuanid	731-27-1	n.d	n.d	n.d	n.d	n.d	n.d
Procymidone	32809-16-8	n.d	n.d	n.d	n.d	n.d	n.d
Folpet	133-07-3	n.d	n.d	n.d	n.d	n.d	n.d
Fludioxonil	131341-86-1	n.d	n.d	n.d	n.d	n.d	n.d
Kresoxim-methyl	143390-89-0	0.014 ± 0.001	0.0078 ± 0.0002	0.017 ± 0.002	0.009 ± 0.002	0.008 ± 0.001	0.002 ± 0.001
Iprovalicarb (E+Z)[a]	140923-17-7	0.034 ± 0.002	0.029 ± 0.001	0.069 ± 0.002	0.038 ± 0.001	0.024 ± 0.004	0.027 ± 0.001
Myclobutanil	88671-89-0	0.015 ± 0.001	0.0075 ± 0.0004	0.031 ± 0.002	0.015 ± 0.001	0.008 ± 0.003	0.009 ± 0.001
Trifloxystrobin	141517-21-7	n.d	n.d	n.d	n.d	n.d	n.d
Benalaxyl	71626-11-4	n.d	n.d	n.d	n.d	n.d	n.d
Fenhexamid	126833-17-8	0.17 ± 0.01	0.114 ± 0.002	0.28 ± 0.02	0.174 ± 0.001	0.14 ± 0.01	0.103 ± 0.007
Tebuconazole	80443-41-0	0.016 ± 0.001	0.013 ± 0.002	0.021 ± 0.001	0.013 ± 0.001	0.010 ± 0.004	n.d
Pyraclostrobin	175013-18-0	0.026 ± 0.002	0.0283 ± 0.0001	0.033 ± 0.002	0.019 ± 0.001	0.017 ± 0.004	n.d
Azoxystrobin	131860-33-8	n.d	n.d	n.d	n.d	n.d	n.d
Dimethomorph (E+Z)[a]	113210-97-2	0.45 ± 0.01	0.29 ± 0.02	0.48 ± 0.02	0.38 ± 0.01	0.32 ± 0.01	0.28 ± 0.03

[a] Sum of both isomers.

n.d: not detected (lower than the limits of detection, LODs, of the employed methodology that were in all cases lower than 0.0001 mg L^{-1}).

As can be seen in ***Table D2.2.1.***, several fungicides were detected in the extracts obtained at lab-scale at trace levels, being in all cases very well below than the established MRLs for wine grapes (i.e. for human consumption). Thus, demonstrating that the obtained extracts are safe to be used in further experiments and formulations.

3 INDIVIDUAL POLYPHENOLIC CONTENT

The lab-scale extracts were analyzed by LC-MS/MS to quantify the concentration of individual polyphenols. The 59 target compounds are shown in Annex I. They were selected based on the LIDSA group background and knowhow in analyzing these molecules in extracts rich in bioactive compounds. As an example, the chromatogram showing the detected compounds in the propyleneglycol/water extract is depicted in ***Figure D2.2.1***.

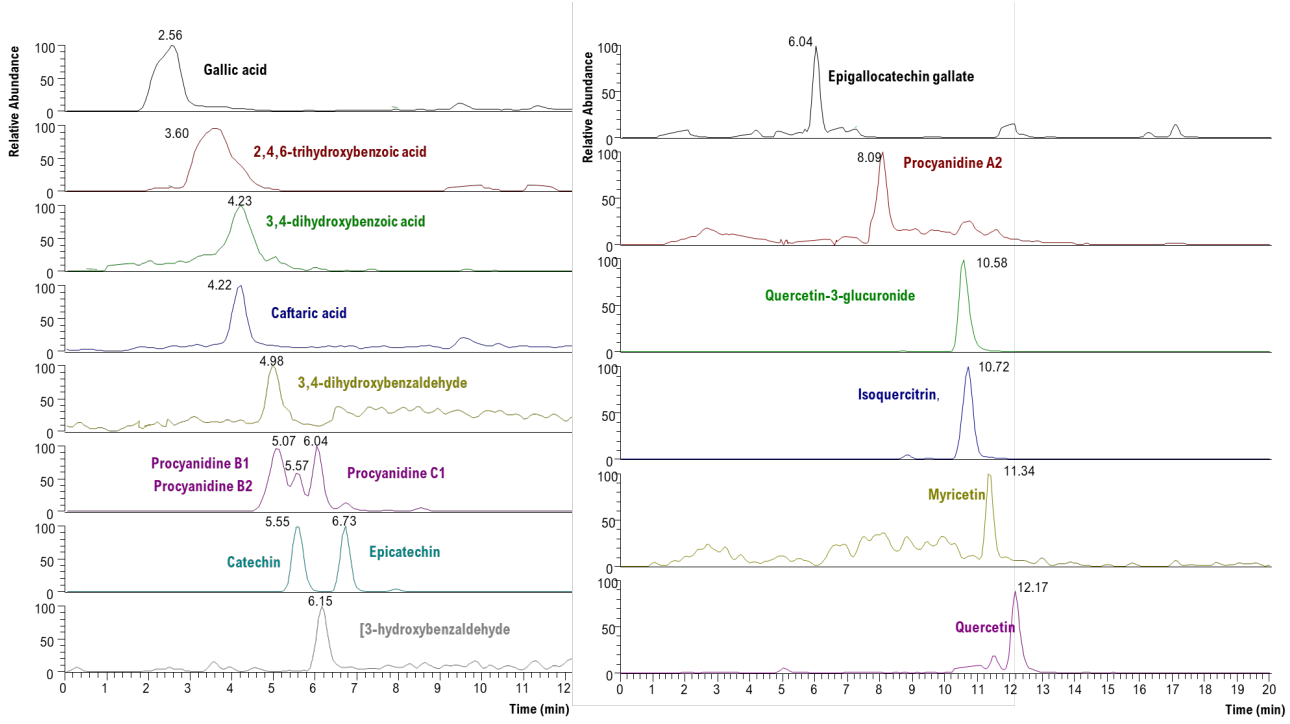

Figure D2.2.1. SRM (Selected Reaction Monitoring) extracted chromatogram for the detected polyphenols present in the propyleneglycol/water extract.

Individual quantification results in all the obtained extracts are summarized in ***Table D2.2.2.***

Table D2.2.2. Concentration, expressed as mg L^{-1}, of the bioactive compounds detected in the analyzed extracts.

Plant Polyphenols Group	Compound name	First extracts obtained at lab-scale					
		Ethyl lactate/H_2O	**Ethanol/H_20**	**Propileneglycol/ H_2O**	**Dimethyl formamide**	**Dimethyl sulfoxide**	**Acetone**
Hydroxycinnamic acids	Caftaric acid	0.34 ± 0.05	1.2 ± 0.3	1.5 ± 0.1	1.5 ± 0.1	2.8 ± 0.3	0.30 ± 0.02
Benzoic acids	Gallic acid	6.6 ± 0.2	10.31 ± 0.04	15.7 ± 0.3	11.5 ± 0.1	5.4 ± 0.3	2.6 ± 0.3
	2,4,6-trihydroxybenzoic acid	n.d	n.d	8 ± 1	0.614 ± 0.003	0.75 ± 0.02	n.d
	3,4-dihydroxybenzoic acid	n.d	0.27 ± 0.02	0.39 ± 0.03	0.29 ± 0.02	0.36 ± 0.02	n.d
Phenolic aldehydes	3-hydroxybenzaldehyde	0.11 ± 0.01	0.17 ± 0.03	0.110 ± 0.001	0.124 ± 0.001	0.12 ± 0.01	0.07 ± 0.01
	3,4-dihydroxybenzaldehyde		0.04 ± 0.02	0.014 ± 0.002	n.d	0.03 ± 0.02	n.d
∑ Non-Flavanoids		***7***	***12***	***26***	***14***	***9***	***3***
Flavan-3-ols	Catechin	23.2 ± 0.6	34.5 ± 0.4	13.5 ± 0.2	20 ± 1	24.2 ± 0.4	16 ± 2
	Epicatechin	7.0 ± 0.4	19.2 ± 0.3	8.2 ± 0.1	11.8 ± 0.1	18 ± 1	4.5 ± 0.1
	Epigallocatechin gallate	n.d	n.d	10 ± 1	0.070 ± 0.003	0.10 ± 0.03	0.05 ± 0.02
	Epicatechin gallate	51.0 ± 0.1	29 ± 1	n.d	44 ± 1	51 ± 1	45 ± 1
∑ Flavan-3-ols		***81***	***83***	***32***	***32***	***93***	***66***
Flavan-3-ols oligomeric derivatives	Procyanidine B1+B2+C1	18 ± 1	38.0 ± 0.1	31.0 ± 0.1	58 ± 2	52.6 ± 0.5	28.3 ± 0.3
	Procyanidine A2	0.22 ± 0.03	0.39 ± 0.02	0.44 ± 0.04	0.20 ± 0.05	0.1 ± 0.01	
∑ Procyanidines		***18***	***38***	***31***	***58***	***53***	***28***
Flavonols	Quercetin	5.0 ± 0.01	22.2 ± 0.3	5.2 ± 0.2	2.54 ± 0.03	1.7 ± 0.1	6.0 ± 0.2
	Quercetin-3-glucoside	15.51 ± 0.01	17.1 ± 0.2	6.7 ± 0.1	21 ± 1	15.4 ± 0.3	36 ± 1
	Quercetin-3-glucuronide	9.77 ± 0.02	11.62 ± 0.04	10.2 ± 0.2	10.1 ± 0.2	9.1 ± 0.1	16.9 ± 0.4
	Rutin	0.40 ± 0.02	0.19 ± 0.02	n.d	0.7 ± 0.2	0.72 ± 0.05	0.775 ± 0.003
	Myricetin	0.143 ± 0.004	2.07 ± 0.05	0.21 ± 0.04	0.146 ± 0.001	0.13 ± 0.01	0.25 ± 0.01
	Kaempferol	0.07 ± 0.01	0.44 ± 0.03	n.d	n.d	0.12 ± 0.01	0.46 ± 0.02
∑ Flavonols		***31***	***54***	***22***	***34***	***27***	***60***
∑ Bioactive Polyphenols		**137**	**187**	**111**	**138**	**182**	**157**

n.d: not detected (lower than the limits of detection, LODs, of the employed methodology that were in all cases lower than 0.005 mg L^{-1}).

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101036768

As can be seen in ***Table D2.2.2***, 18 of the 59 target polyphenols were detected in the analyzed extracts.

The summatory of bioactive compounds (∑) is similar in all extracts, ranging between 111-187 mg L^{-1}. However, as it is expected, the profile for each plant phenolic group is slightly different depending on the solvent employed to obtain the extracts (see ***Figure D2.2.2***.).

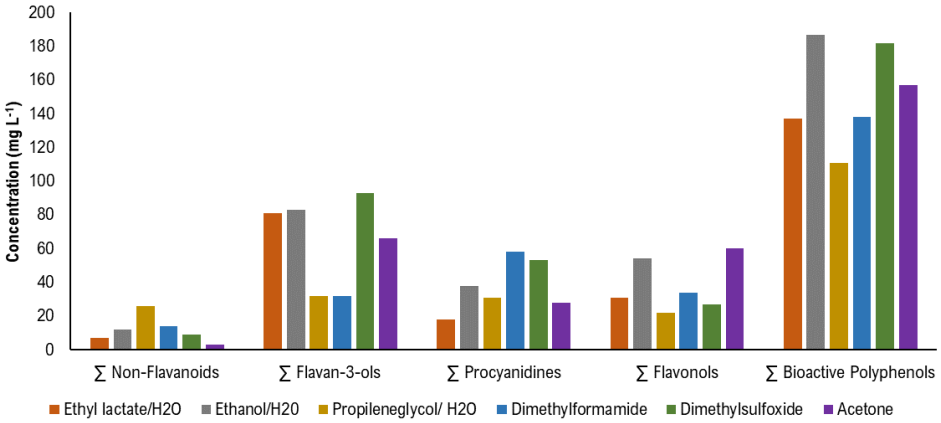

Figure D2.2.2. Profile of the different polyphenolic plant group depending on the extract.

These results demonstrate the versatility of the obtained extracts which can be selected according to the formulation requirements and/or the further applications to be designed, showing that the employed solvent to obtain extracts is crucial to modulate the polarity profile of the bioactive compounds present in the ready-to-use extracts.

4 CONCLUSIONS

- ✓ All grape marc extracts obtained in the different GRAS solvents and their hydro-organic mixtures (ethyl lactate/water, ethanol/water, propyleneglycol/water, dimethylformamide, dimethylsulfoxide and acetone) complies with the MRLs for fungicide residues, being safe for further use.
- ✓ A high number of polyphenolic compounds were quantified in the different extracts, highlighting the presence of flavan-3-ols.
- ✓ Although the ∑ bioactive compounds are similar in all extracts, with values between 111-187 mg L^{-1}, depending on the employed solvent, the profile for each phenolic group is slightly different, demonstrating the versatility of the obtained extracts.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101036768

5 ANNEX 1

The target polyphenols and their CAS identification numbers are shown in the Table presented below.

Compound	CAS
Gallic acid	149-91-7
2,4-dihydroxybenzoic acid	89-86-1
(+)-gallocatechin	970-73-0
3,4-dihydroxybenzoic acid	89-86-1
2,4,6-trihydrobenzoic acid	83-30-7
Caftaric acid	67879-58-7
Procyanidine B1	20315-25-7
3,4-dihydroxybenzaldehyde	139-85-5
2,5-dihydroxybenzoic acid	490-79-9
4-hydroxybenzoic acid	99-96-7
Catechin	18829-70-4
2,6-dihydroxybenzoic acid	89-86-1
3,5-dihydroxybenzoic acid	99-10-5
Procyanidine B2	29106-49-8
2,5-dihydroxybenzaldehyde	1194-98-5
3-hydroxybenzoic acid	99-06-9
3-hydroxybenzaldehyde	100-83-4
4-hydroxybenzaldehyde	123-08-0
Procyanidine C1	37064-30-5
Chlorogenic acid	327-97-9
3,4-dimetoxybenzoic acid	93-07-2
Caffeic Acid	331-39-5
Epigallocatechin gallate	989-51-5
Epicatechin	35323-91-2
Procyanidine A1	103883-03-0
Gallocatechin gallate	84650-60-2
4-hydroxycinnamic acid	7400-08-0

Epicatechin gallate	1257-08-5
7-Hydroxycoumarin	93-35-6
Procyanidine A2	41743-41-3
Orientine	28608-75-5
Trans-ferulic acid	537-98-4
3-4-dimethoxybenzaldehyde	120-14-9
Robinetin	490-31-3
4-methoxybenzaldehyde	123-11-5
Quercetin-3-glucuronide	22688-79-5
Quercetin-3-rutinoside	153-18-4
Quercetin-3-glucoside	482-35-9
Myricetin	529-44-2
Fisetin	528-48-3
3-4-5-trimethoxycinnamic acid	90-50-6
3-5-dimethoxybenzaldehyde	7311-34-4
Quercetine	117-39-5
Butein	487-52-5
Kaempferol	520-18-3
Apigenin	520-36-5
Chrysin	480-40-0
Vanillic acid	121-34-6
Isoharmnetin	480-19-3
Luteolin	491-70-3
Naringerin	480-41-1
Taxifolin	480-18-2
Ellagic acid	476-66-4
Rosmarinic acid	20283-92-5
Astragalin	480-10-4
p-coumaric acid	501-98-4
Epsilon viniferin	62218-08-0
Ampelopsin	27200-12-0
Resveratrol	501-36-0