NeoGiANT

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

D3.3

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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

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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:	D3.3
Document Title:	MIC and IC50 for *Toxoplasma gondii* using the extracts
Dissemination level	PU – Public
Period:	PR1
WP:	WP3
Task:	Task 3.3
Author:	Moredun Research Institute
Abstract:	The assay evaluated the effect of different dilutions of the grape marc extracts based on different solvents: Ethanol, Ethyl lactate and Propylene glycol on the growth of *Toxoplasma gondii*. *T. gondii* tachyzoites were culture in four dilutions of the extracts (1:100, 1:150, 1:200 and 1:250) for three days. Parasite multiplication in Vero cells were estimated by DNA measuring using qPCR analysis. These experiments showed statistical differences displaying the effect resulting from the extract exposure. The IC50 was 1/200 for PG, and 1/250 for EL and Et. However, the MIC could not be determined due to the cytotoxicity of the extract in Vero Cells, required for in vitro culture of *T. gondii*.

Version	Date	Change
1	07/Jul/22	

Disclaimer

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

1 METHODOLOGY

1.1 Toxoplasma gondii culture and DNA extraction

Toxoplasma gondii tachyzoites (stage of *Toxoplasma* that rapidly multiply in tissue cells) were cultured within Vero cell monolayers using medium with different dilutions of extracts (1/100, 1/150, 1/200, 1/250). The extracts assayed were Ethyl lactate 50:50 water (EL), Propylene glycol 50:50 water (P), Ethanol 50:50 water (E). After 4 days, the parasites were harvested by scraping the infected cell monolayers, releasing the tachyzoites into the supernatant. DNA from these tachyzoites was extracted after separation from the tissue cells using the Promega wizard kit and stored at 4ºC for qPCR analyses.

1.2 Quantitative PCR conditions

The following table shows the qPCR conditions at which the samples were processed using the Lightcycler 480 (Roche)

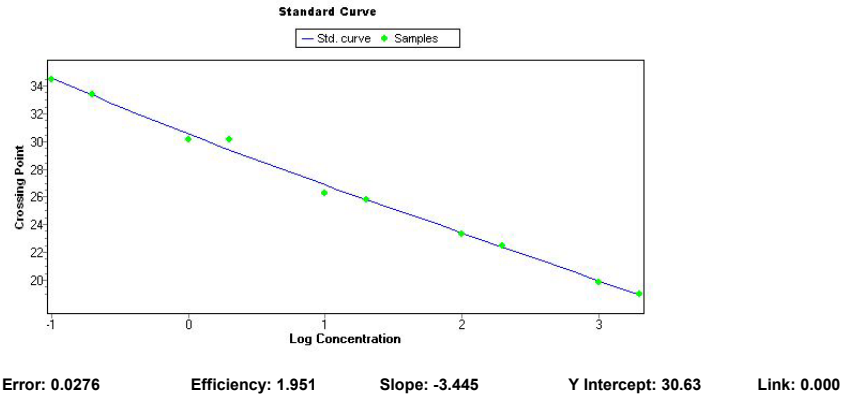
Program

	segment	temp.	hold time	slope	aquisition
Denaturation	1	95C	10 min	20 C/s	none
Amplification	1	95C	10 s	20 C/s	none
45x	2	58C	20 s	20 C/s	none
	3	72C	20 s	20 C/s	single
Cooling	1	40C	5 s	20 C/s	none

Table 1. qPCR conditions used to quantify *T. gondii* DNA

1.3 PCR standardization

A standard curve was produced using standard concentrations ranging from 2000 pg to 0.1 pg of *T. gondii* DNA per reaction and statistical analyses were performed. Every different concentration was run in duplicate. DNA samples used were *T. gondii* 529bp repeat DNA fragment.



Error: 0.0276 **Efficiency: 1.951** **Slope: -3.445** **Y Intercept: 30.63** **Link: 0.000**

Figure 2. Standard curve for qPCR. Standardisation were set up using standard concentration ranging from 2000 pg to 0.1 pg of *T. gondii* DNA

1.4 Statistical analysis

A Student´s T test was used for statistical analyses of the obtained data

2 RESULTS

The following tables and figure show the results obtained for the qPCR of *T. gondii* DNA after tachyzoite exposure to different concentrations of the three assayed extracts, including the statistical analyses of data. Experiments 1 and 2 are biological replicates.

Table 2. Results of qPCR analysis for ethanol-extract (NG01E22ET050RM7CO1L01). Data expressed as DNA concentration (pg) and statistically analysed by Student´s T test for each experiment and for the combination of both.

Dilution	Exp	Ct	**DNA (pg) Conc**	Mean DNA Conc (pg)	STDev DNA (pg)	T-Test (p=) Exp 1	T-Test (p=) Exp 2	T-Test (p=) Comb Data
1:100	1	15.15	24800	25100	424	0.010		**≥0.001**
		15.12	25400					
	2	14.91	29200	28250	1344		0.002	
		15.01	27300					
1:150	1	14.15	48400	43550	6859	0.016		**≥0.001**
		14.49	38700					
	2	14.01	53500	52500	1414		0.003	
		14.06	51500					
1:200	1	14.58	36400	36600	283	0.012		**≥0.001**
		14.56	36800					
	2	14.96	28200	27550	919		0.005	
		15.03	26900					
1:250	1	13.73	64400	61950	3465	0.002		**≥0.001**
		13.84	59500					
	2	14.17	48000	45650	3323		0.001	
		14.32	43300					
Control	1	12.59	137000	135000	2828			
		12.64	133000					
	2	12.46	150000	148000	2828			
		12.51	146000					

Exp – Experiment number
CP – Crossing point (Cycle at which a sample is considered positive)
DNA Conc – Amount of *T. gondii* DNA (pg) in each sample
STDev – Standard deviation (pg) between replicates
T-Test – Results from analysis of extract concentration against appropriate experimental control
T-Test Comb. Data - Results from analysis of combined extracts data (Exp. 1 & 2) compared to the combined data from appropriate controls.

Table 3. Results of qPCR analysis for Ethyl lactate-extract (NG01E22LE050RM7CO1L01). Data expressed as DNA concentration (pg) and statistically analysed by Student´s T test for each experiment and for the combination of both.

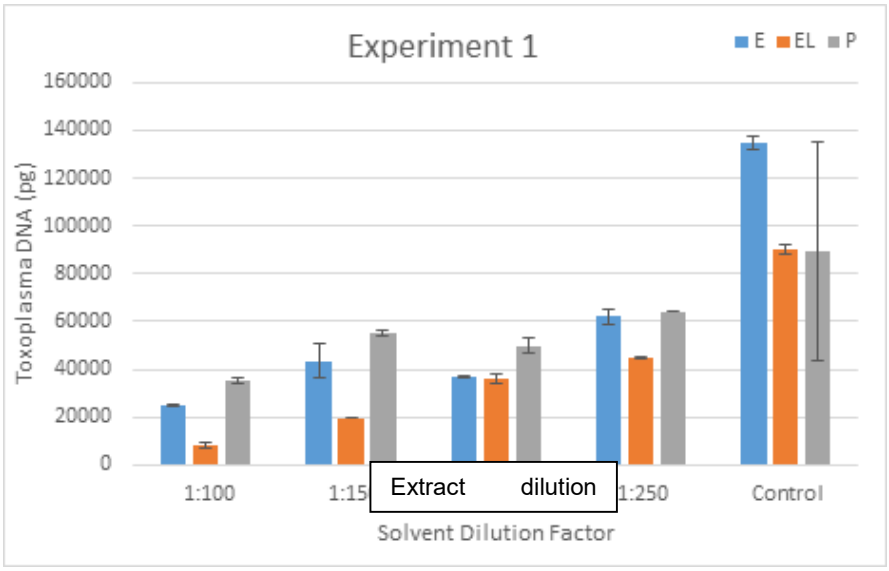
Dilution	Exp	CP	DNA Conc (pg)	Mean DNA Conc (pg)	STDev DNA (pg)	T-Test (p=) Exp 1	T-Test (p=) Exp 2	T-Test (p=) Comb Data
1:100	1	16.71	8740	8090	919	0.003		**0.006**
		16.95	7440					
	2	15.87	15400	14450	1344		0.017	
		16.06	13500					
1:150	1	15.5	19700	19600	141	0.012		**0.008**
		15.51	19500					
	2	14.77	32000	31100	1273		0.021	
		14.86	30200					
1:200	1	14.65	34800	36000	1697	0.001		**0.014**
		14.55	37200					
	2	14.09	50600	51150	778		0.029	
		14.05	51700					
1:250	1	14.27	44900	44650	354	0.016		**0.020**
		14.28	44400					
	2	13.95	55600	55200	566		0.031	
		13.97	54800					
Control	1	13.2	91300	89900	1980			
		13.25	88500					
	2	12.49	147000	142500	6364			
		12.59	138000					

Exp – Experiment number
CP – Crossing point (Cycle at which a sample is considered positive)
DNA Conc – Amount of *T. gondii* DNA (pg) in each sample
STDev – Standard deviation (pg) between replicates
T-Test – Results from analysis of extract concentration against appropriate experimental control
T-Test Comb. Data - Results from analysis of combined extracts data (Exp. 1 & 2) compared to the combined data from appropriate controls.

Table 4. Results of qPCR analysis for Propylene glycol-extract (NG01E22PG050RM7CO2L01). Data expressed as DNA concentration (pg) and statistically analysed by Student´s T test for each experiment and for the combination of both (comb data).

Dilution	Exp	CP	DNA Conc (pg)	Mean DNA Conc (pg)	STDev DNA (pg)	T-Test (p=) Exp 1	T-Test (p=) Exp 2	T-Test (p=) Comb Data
1:100	1	14.58	36300	35450	1202	0.35		**0.023**
		14.65	34600					
	2	14.71	33400	37850	6293		0.043	
		14.35	42300					
1:150	1	13.94	56000	55000	1414	0.48		**0.026**
		13.99	54000					
	2	14.71	33300	31800	2121		0.004	
		14.86	30300					
1:200	1	14.05	52000	49900	2969	0.44		**0.032**
		14.17	47800					
	2	14.52	38000	38500	707		0.0004	
		14.48	39000					
1:250	1			64100	#DIV/0!	#DIV/0!		0.436
		13.73	64100					
	2	13.29	86500	87800	1838		0.045	
		13.24	89100					
Control	1	12.77	122000	89450	46032			
		13.91	56900					
	2	13.1	97700	97000	989			
		13.12	96300					

Exp – Experiment number
CP – Crossing point (Cycle at which a sample is considered positive)
DNA Conc – Amount of *T. gondii* DNA (pg) in each sample
STDev – Standard deviation (pg) between replicates
T-Test – Results from analysis of extract concentration against appropriate experimental control
T-Test Comb. Data - Results from analysis of combined extracts data (Exp. 1 & 2) compared to the combined data from appropriate controls.



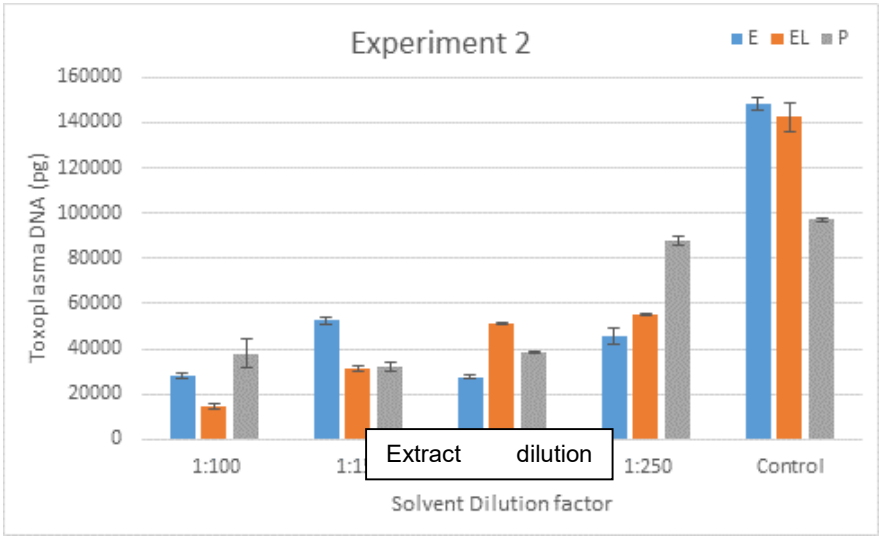


Figure 2. Comparative analysis of the different loads of *Toxoplasma gondii* DNA resulting from a culture with different dilutions of each extract placed in two separated experiments (1 and 2). The graph showed the concentration of DNA on pg. The results were represented in bars showing the Mean±SE (Error bars are the STDev of each pair of duplicate wells) for each dilution (1:100, 1:150, 1:200, 1:250 and 0-control).

3 CONCLUSION

All the extracts in any solvent reduced *T. gondii* tachyzoite multiplication in Vero cells. Differences were statistically significant in both duplicates for extracts in Ethanol (E) and Ethyl Lactate (EL), at all of the concentrations tested (1:100, 1:150, 1:200 and 1:250).

For extract in Propylene glycol (P), statistically significant differences were only seen in experiment 2 for all of the dilutions tested.

The values of IC50 are:

- **1/200** for P
- **1/250** for EL and E

The MIC could not be determined because of the cytotoxicity of the extract in Vero Cells. *T. gondii* requires Vero cells for *in vitro* culture. This is the reason why any substance that affects the host cells also triggers the death of the parasite. Therefore, the extracts were tested using the higher concentrations that let a normal growth of the Vero cells. Other approaches are currently being assayed in order to provide MIC values.