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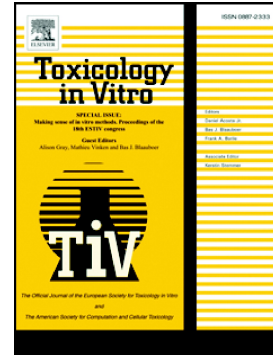
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The origin of *in-vitro* estrogen-like activity in oregano herb extracts

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Global market of herbs has been struggling with food adulteration issues. A number of assays have been developed to aid the detection of the tampered samples and ensure high quality of the marketed products. However, herbs are marketed not only for their culinary applications but also as remedies due to high levels of biologically active constituents. Nevertheless, there is no information in the literature about the influence of herbs adulteration on the biological activity of the final product. Current study aims at assessing the influence of oregano adulteration on its in-vitro estrogen-like activity. High responses in a mammalian reporter gene assay have been detected in pure and adulterated samples, translating to 21 – 7,409 ng of 17β-estradiol equivalents per gram of oregano. The origin of those responses was assessed by combining fractionation and UHPLC-HRMS. Three flavones were proposed as the most active extract constituents i.e. luteolin-glucoside, luteolin- and apigenin-glucuronides all of which have been previously identified in other herbal extracts with estrogenic activity. This study underlines challenges of biological activity assessment in complex herbal extracts as well as the need for further assessment of such supplement administrations in the case of postmenopausal women and breast cancer patients undergoing hormone therapy.

Highlights:

- Oregano extracts exhibit estrogen-like activity *in-vitro*
- Conjugated flavonoids are the origin of the detected biological activity
- Combining bio- and chemical assays is a fast tool for detecting bioactive compounds

Keywords: oregano, endocrine disruption, reporter gene assay, mass spectrometry, estrogens, flavonoids

'Declarations of interest: none'

1. Introduction

The endocrine system regulates various bodily functions essential for growth, development and reproduction with biological responses triggered solely by endogenous hormones (McCabe, 2018). However, other compounds present in our environment are also capable of doing so and disrupting our endocrine system by mimicking or blocking natural hormones or their pathways (UNEP, 2012). Consequently, exposure to endocrine disruptors (EDs) may lead to detrimental health effects including sexual precocity, hormone related cancers, reproductive tract abnormalities and infertility (Diamanti-Kandarakis and Gore, 2012). EDs consist of various groups of chemicals including compounds of a natural origin such as phytoestrogens or mycotoxins as well as a wide range of man-made chemicals such as pesticides, brominated flame retardants (BfRs), UV filters and phthalates (Darbre, 2015). Food is a major route of exposure to EDs for the human population and naturally occurring EDs such as endogenous steroid hormones in food of animal origin or phytoestrogens in plants contribute vastly to this exposure (Thomson, 2009). Flavonoids are a class of phytochemicals encompassing over 10,000 compounds (George et al., 2017) ubiquitously found in plants and herbs (Justesen and Knuthsen, 2001). They have long been a subject of considerable scientific interest due to their numerous biological activities including anticarcinogenic, neuroprotective, anti-inflammatory, and antihypertensive characteristics (George et al., 2017; Gross, 2004; Kocic et al., 2013; Nabavi et al., 2015a; Nabavi et al., 2015b). Nevertheless, flavonoids, which are structurally similar to estrogens, are able to bind to the estrogen receptor (ER) and trigger agonistic and/or antagonistic responses (Bovee et al., 2008; Cederroth and Nef, 2009; Mueller and Korach, 2001; Pilsakova et al., 2010).

Oregano encompasses at least 60 species among various botanical families but the most significant are *Verbenaceae* and *Lamiaceae* families (Calpouzos, 1954). Oregano has been used as a fragrant culinary herb due to a high essential oil content but also as a folk remedy to treat airways infections, digestive distress and inflammation-related illnesses (Pascual et al., 2001) due to its anti-inflammatory and antioxidant properties attributed to its polyphenols content (Goncalves et al., 2017; Leyva-Lopez et al., 2016). The herbs and spices market is estimated to be worth 2.97 billion dollars world-wide (Marieschi et al., 2009). As such it is prone to fraud, with cases of substitution or dilution with cheaper alternatives already having been reported with some cases threatening consumers well-being by introducing substitutes with toxic constituents (Moore et al., 2012; Wielogorska et al., 2018). Due to an inherent need to protect both consumers health and the market an array of methods available to detect adulteration have been developed recently including FTIR, DNA barcoding or GC/LC-(HR)MS assays (Black et al., 2016; Ellis et al., 2015; Parveen et al., 2016; Xie et al., 2006). However, there is still little information regarding changes in biological activities in adulterated products, especially in the case of herbs and herbal remedies containing high levels of biologically active compounds

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(Justesen and Knuthsen, 2001; Xu et al., 2013). Numerous studies quote flavones, such as luteolin and apigenin, to have DNA protective, antipoliferative properties (George et al., 2017), while showing estrogenic activity (Xu et al., 2013). As such they are of great clinical interest (Romano et al., 2013) and despite their low bioavailability and metabolic stability (Martinez-Perez et al., 2016), flavones containing remedies are being assessed for suitability in menopausal estrogen deficiency symptoms treatment (Rahte et al., 2013; Seo et al., 2006), or in breast cancer prevention (Martinez-Perez et al., 2016). Nevertheless, the literature reports that the activity of pure compounds varies significantly due to experimental design (Marino and Galluzzo, 2008; Ramos, 2007) with flavones rich plant extracts presenting an even bigger challenge due to varied, complementary and/or overlapping biological activities that flavones may exert in mixtures (Marino and Galluzzo, 2008; Xu et al., 2013). What is more, less attention is paid to flavones' conjugated forms which are frequently detected in herbal extracts including oregano, thyme, agrimony, sage and yarrow (Garritano et al., 2005; Innocenti et al., 2007; Rahte et al., 2013) with reported *in vitro* estrogenic activities detected in their extracts being shown to be the main constituents responsible for estrogenic responses (Gutiérrez-Grijalva et al., 2017; Innocenti et al., 2007; Lee et al., 2012; Nagy et al., 2011; Rahte et al., 2013). Also, some studies cite increased cancer cell proliferation resulting from herbal extract exposures (Lee et al., 2012) or even the antagonisation of antiproliferative effects of tamoxifen which may have detrimental effects on the endocrine therapy administered in hormone dependant breast cancer patients (Seo et al., 2006).

Therefore, the purpose of this study is to assess the hormonal load of both pure and adulterated oregano and to assess if adulteration has any impact on the extracts' hormonal load. RGAs have been shown to be an efficient tool in screening for hormonal activities in food, nevertheless, these assays lack information about activity origin (Wielogorska et al., 2014). Thus, to shed more light on the source of detected biological activity, two analytical platforms were combined for that purpose i.e. estrogen responsive MMV-Luc reporter gene assay (RGA) and an untargeted UPLC-QToF-HRMS analysis which enabled a more detailed investigation of the chemical identities of the targeted compounds.

2. Materials and Methods

2.1. Samples origin and preparation

A set of ten samples which have been previously characterized (Wielogorska et al., 2018) were employed in the study. Five pure oregano samples supplied with full provenance and traceability including *Origanum vulgare* (samples 10, 11, 12, 24) and *Origanum onites* (sample 28). Five adulterated samples were picked from samples screened in a previous study (Black et al., 2016) i.e. samples 39, 40, 61, 103 and 116 all adulterated with olive leaves with sample 61 additionally adulterated with myrtle leaves.

The samples were milled to a homogenous powder on a PM-100 Retsch Planetary Ball Mill (Retsch, Haan, Germany). 50 mg (+/- 1%) was weighed out on a Discovery DV215CD Analytical Balance (Ohaus Europe GmbH, Nanikon, Switzerland) into a 1.5 mL Eppendorf tube and 500 μL of ultra-pure water (18.2MΩ/cm)/LC-MS Chromasolv methanol (1:1, v/v) solution added (Merk Millipore, Billerica, USA and Sigma-Aldrich, St Louis, MO, USA respectively). Samples were then extracted by vortexing 10 min at 2,500 rpm with a DVX-2500 Multitube Vortexer (VWR International, Lutterworth, UK), sonicated for 10 min in camSonix C1274 sonicator (Camlab, Cambridge, UK) and centrifuged at 9,500 xg in a MIKRO 200R centrifuge (Hettich UK, Salford, UK). 300 μL of the resulting supernatant was transferred into a maximum recovery vial (Waters, Manchester, UK) and stored at -20°C until the day of the analysis.

2.2. Cell culture

Cell culture reagents were supplied by Life Technologies (Paisley, UK). The estrogen specific RGA cell line (MMV-Luc) was developed as previously described (Willemsen, Scippo et al. 2004). Briefly, this RGA cell line was generated from a human mammary gland cell line by stable transfection with the luciferase gene under the control of a steroid hormone inducible promoter. The cells were cultured in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C with 5% CO_2 and 95% humidity and routinely cultured in cell culture medium containing: Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Life Technologies, UK) , which is a weak estrogen, 10% (v/v) foetal bovine serum, 1% (v/v) penicillin–streptomycin and 1% (v/v) L-glutamine (Life Technologies, UK). Cells were subcultured at least three times prior to the RGA.

2.3. Reporter Gene Assay (RGA)

The estrogen specific, MMV-Luc cell line, was seeded at a concentration of 4 x 10^5 cells ng/mL, 100 μL well, into white walled, clear and flat bottomed 96-well plates (Greiner Bio-One, Fricken-Hausen, Germany) and incubated for 24h. On the following day, 17β-Estradiol (E$_2$) (Sigma-Aldrich, UK) standard curve and samples extracts were prepared in an assay media (1:200) in the range of 0.0005 – 10 ng mL^{-1} of media. The cells were incubated for 24h. The supernatant was discharged and the cells were washed with phosphate buffered saline (PBS) pH 7.1 prior to lysis with 25μL cell culture lysis buffer (Promega, Southampton, UK). Finally, 100 μL luciferase substrate (Promega, Southampton, UK) was injected into each well and luciferase activity was measured using a Mithras Multimode Reader (Berthold, Other, Germany). The responses of the cells were measured and compared with the negative control (H$_2$O: MeOH (1:1, v/v)).

2.4. Cytotoxicity assay

The MTT cell viability assay was performed to monitor oregano extracts for possible cytotoxicity. Cells were seeded in white walled, clear and flat bottomed 96-well plates (Greiner Bio-One, Fricken-

hausen, Germany) as for the RGA with the same test compound concentrations and incubated for 24h. The supernatant was discarded and the cells washed once with PBS pH 7.1. A 50 μL of thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, UK) solution (5 mg mL^{-1} stock in PBS pH 7.1, diluted 1:2.5 (v/v) in assay media) was added to each well and the cells incubated for 3h. Viable cells convert the soluble yellow MTT to insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. The supernatant was once again removed and 200 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) was added to dissolve the formazan crystals and the plate was incubated, with agitation, at 37°C for 10min. Optical density was measured in a Tecan Safire2 microplate reader (Tecan group Ltd., Männedorf, Switzerland) at 570nm with a reference filter at 630nm. Analytes were assessed in triplicate and in three independent exposures. Viability was calculated as the % absorbance of the sample when compared with the absorbance of the negative control.

2.5. UPLC-HRMS analysis

Analyses were performed on a Waters Acquity UPLC I-Class system (Milford, MA, USA) coupled to a Waters Xevo G2-S QToF mass spectrometer (Manchester, UK) equipped with an electrospray ionization source operating in positive ionisation mode with lock-spray interface for real time accurate mass correction. Instrument settings were as follows: source temperature was set at 120°C, cone gas flow at 50 L.h^{-1}, desolvation temperature at 450°C, and desolvation gas flow at 850 L.h^{-1}. The capillary voltage was set at 1.0 kV with source offset set to 60 (arbitrary unit). Mass spectra data were acquired in continuum using MS^{E} function (low energy: 4 eV; high energy: ramp from 15 to 30 eV) over the range m/z 50-1200 with a scan time of 0.08 s. A lock-mass solution of Leucine Enkephalin (1 ng μL^{-1}) in methanol/water containing 0.1% formic acid (1:1, v/v) was continuously infused into the MS via the lock-spray at a flow rate of 10 μL min^{-1}. A 10 μL aliquot of extracted oregano sample, diluted 1:1 with water to reach final MeOH content of 25% to facilitate chromatographic separation during reverse phase analysis, was injected onto an Acquity HSS T3 column (2.1 x 100 mm, 1.8 μm, Waters, Milford, MA, USA). The column oven temperature was set at 45°C and flow rate at 0.4mL min^{-1}. Mobile phase consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid. The gradient was set as follows: 2.0min of 99% (A) followed by a linear increase from 1% to 99% (B) over 16 min, isocratic cleaning step at 99% (B) for 0.5 min, then returned to initial conditions 99% (A) over 0.1min and column equilibration step at 99% (A) for 1.4min. Each sample was analysed in triplicate to assure reproducibility. Prior to all analyses 10 pooled conditioning samples (QCs) were injected. For quality control QCs were also injected at intervals of every 10 samples throughout the entire experiment to determine the chromatographic reproducibility of retention times and peak intensities (Spagou et al., 2011).

2.6. On-the-plate fractionation

Sample was manually fractionated post column employing an approach published previously [24]. Fractions of 200 μL were collected in a 96-well plate routinely used for the RGA analysis. A triplicate of a 200 μL aliquot of the mobile phase from the beginning, middle and end of the run were collected to on the plate to check for possible contamination and their average value was treated as a solvent blank. Additionally, E_2 curve was applied on the plate, diluted in 200 μL of the mobile phase (MeOH:H_2O (1:1, v/v) to account for possible input of the used solvents into estrogenic activity. The plate was then dried overnight in a biosafety cabinet to avoid microbiological contamination at 40°C. The next day the plate was seeded as for the RGA but with 200 μL of cells suspension at concentration 2 x 10^5. Plates were incubated for 48h and the estrogenic activity assessed.

2.7. Statistical analysis and quality control

For the RGA, dose–response curves were fitted with Graph Pad Prism software using the sigmoidal dose-response curve equation, Y = 100 / (1 + 10((LogEC50−X)*HS), where X is the logarithm of concentration, Y the normalized response, EC50 concentration yielding 50% increase in maximal response and HS the hill slope. Quality controls employed in the assay were similar to those outlined in the Organisation for Economic Co-operation and Development guideline regarding testing of chemicals for their estrogenic agonistic activity in the ER transactivation assays (OECD, 2009) and included monitoring performance standards such as: negative vehicle control, examining E2 calibration curves parameters such as values of EC_{50}, slope and coefficient of determination (used as measures of the assay's accuracy and sensitivity), fold induction values (the ratio of a response of negative control and the top E2 calibration point), as well as setting threshold of the coefficient of variation of each test point triplicate to a maximum of 15%. For the interpretation of data collected during the screening of samples parametric, paired, one tailed student's t-test was used to determine significant differences between negative control and tested samples. Data on cell viability was analysed by Microsoft Excel where statistically significant influence was also determined by parametric, paired, two tailed student's t-test was used to determine significant differences between negative control and tested samples. To compare group responses between adulterated and non-adulterated oregano samples, a nonparametric, two-tailed, t-test was performed with Kolmogov-Smirnov post-test. A confidence level of 95% was set as an acceptable criterion. Associated graphs were prepared in GraphPad Prism 5.01 (GraphPadSoftware, Inc., La Jolla, USA).

Raw LC-HRMS data were imported to Progenesis QI 2.0 software (Waters, Newcastle, UK). After data import, using a filter set at 1, the runs were aligned to the best pool sample selected with automatic peak picking settings. Processed spectral data were then exported to SIMCA 14 (Umetrics, Umea, Sweden) for multivariate analysis. To assess general quality of the acquired spectral data were Pareto scaled and PCA analysis and model assessment were performed. All QCs were found to be tightly clustered within the centre of each representative scores plot which indicates good

reproducibility throughout the analytical run. Next, data were grouped into respective classes prior to OPLS-DA. R2 (cumulative) and Q2 (cumulative) were used to determine the validity of the model with R2 (cum) employed as indicator of the variation described by all components in the model and Q2 as a measure of how accurately the model can predict class membership (Graham et al., 2013).

3. Results

3.1. Cytotoxicity

No samples presented any cytotoxicity while two of them increased ($p<0.05$) cells proliferation including one pure (S28) and one adulterated oregano (S61) sample, as presented in Figure 1a. Both increased cells proliferation by 17%. Similar findings were published previously, noting however that induction of MCF7 cells proliferation is concentration dependant with increase in cell number quoted at lower rather than higher extract concentration factors (Lee et al., 2012).

3.2. Reporter gene assay: Estrogenic activity

Each day of the analysis the following parameters were monitored: curve's slope, EC_{50} and goodness of fit. Mean slope value was 0.95 with inter-day repeatability value (RSD) of 8% while EC_{50} mean value was established to be equal to 0.05 ng/mL of E_2 with inter-day repeatability value (RSD) of 11%. Goodness of fit coefficient was higher than 0.985. All the samples presented responses statistically higher than negative control ($p<0.05$). The responses varied from 14% - 95% (Figure 1b) with corresponding EEQ ranging from 17.7 to 7409 ng EEQ g of oregano $^{-1}$ (Table 1). Group comparison (Figure 1c) of the responses obtained revealed slightly higher (15%) mean response in pure oregano samples group ($p=0.0001$), with the highest singular response associated with a pure *Origanum Onites* sample – S28. Due to non-linear curve shape for responses above 80% of the top calibration point, EEQ estimation for sample 28 resulted in high standard deviation value.

3.3. On-the-plate fractionation

In order to assign detected biological activity in sample 28 subsequent post-column fractionation was performed. Estrogenic responses obtained in each fraction with corresponding chromatogram section are presented in Figure 2. Biological activity observed in the obtained fractions varies from 0 to 100% with five most prominent responses in fractions 15-20, corresponding to analytes elution between minute 8 and 11. Interestingly, the summed estrogenic activity for the five most estrogenic fractions is almost three times higher than that detected in the non-fractionated extract. It may indicate that there are other active substances in the extract with anti-estrogenic activities or the actions are modulated when assessed as a mixture. Similar results were reported in sage extracts whereby crude extract constituents expressed lesser activity than the partially purified extract (Rahte et al., 2013).

3.4. Multivariate analysis and bioactive compounds selection

Initially all the features were included in the multivariate analysis to assess unsupervised trends among the samples. Generated PCA plot (Figure 3a), presents a clear separation between pure and adulterated samples as reported previously (Black et al., 2016). To help narrow down the compounds responsible for detected estrogenic response data matrix was cut down to contain only features detected in the most biologically active fractions i.e. collected between 8 and 11 minute. The samples were then grouped according to their biological activity bracket from 20 – 50% relative response (in 10% increments) and sample 28 representing 100% response. In the subsequently created supervised OPLS-DA model (Figure 3b) clear group separation was obtained. Five features which contributed most to the separation between lowest (G20) and highest (G100) biologically active group were determined during S-plot investigation (Figure 3c) with their chemical characterisation and proposed identification presented in Table 2. Compound identification was based on raw spectra analysis (Figure 4) and their comparison with existing literature. The two most active compounds in fraction 15 were identified as luteolin-7-O-glucoronide and luteolin-7-O-glucoside eluting at minute 8.41min and 8.49min. Acquired spectra for both compounds show in source fragmentation both glucuronide (-176m/z) and –glucoside (-162m/z) moiety, yielding a deconjugated luteolin peak at 287m/z. High energy spectra of both compounds show a small 153m/z peak in accordance with previously published data (Hossain et al., 2010; Pereira et al., 2013; Song et al., 2014). The most active compound in fraction 16 was apigenin-7-O-glucuronide, eluting at 9.05min. Similarly to luteolin, a low energy spectrum also represents glucuronide loss (-176m/z) with a free apigenin peak at 477m/z. A high energy spectrum shows further apigenin fragmentation, yielding a 153 m/z peak (Song et al., 2014). Both apigenin and apigenin-7-O-glucuronide have been reported as oregano constituents (Nagy et al., 2011). The last two analytes, present in fraction 20, were identified as 3-methoxyapigenin and quercetin-7-O-methyl. To our knowledge no spectra LC-MS spectra of those compounds are available in the literature to be compared with. However, another analyte co-eluting with luteolin methyl ether can be identified on the low energy spectrum i.e. wogonoside with molecular ion of 461m/z and deconjugated (-176m/z) wogonin peak at 285m/z which may also contribute to the noted response in the estrogen RGA. To correlate the compounds presence with the detected biological activity, their extracted peak areas were plotted in each sample (Figure 5) to compare with the estrogenic response pattern obtained during whole extract analysis in the RGA (Figure 1b). Both luteolin-7-glucuronide and glucoside as well as apigenin-7-glucuronide pattern resemble very closely the pattern obtained during RGA analysis. These compounds have previously been linked with estrogenic activity detected in herbal extracts with confirmed ER mediated response in *in vitro* bioassays by binding to both ERα and ERβ, mRNA expression of estrogen-responsive genes and induction of ER positive MCF7 cell line proliferation (Innocenti et al., 2007; Lee et al., 2012; Rahte et al., 2013). Flavonoids conjugates including glucosides and glucuronides can enter the cell through passive diffusion, however slower than aglycons, and/or their transport can be mediated by a number of membrane transport proteins including solute carriers, sodium-glucose linked and

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ATP-binding cassette transporters (Gonzales et al., 2015). Also, β-glucosidase and β-glucuronidase activity have been associated with subsequent metabolites deconjugation and aglycon release *in vitro* (Aragones et al., 2017; Gonzales et al., 2015; Menendez et al., 2011). However, the results vary among studies and cell lines assessed. Also, limited amount of data is available on the subject in breast cancer cell lines, with a sole study describing β-glucuronidase activity MCF cell lines (Yuan et al., 2012). In the present study luteolin-7-glucuronide and glucoside as well as apigenin-7-glucuronide are proposed as the most likely active extract constituents that contribute the most to the detected activity in the oregano samples assessed, however, further research is required to describe in more detail fraction composition and fully elucidate cellular metabolism to reliably confirm the origin of the observed bioactivity.

4. Conclusions

Results presented herein confirm that herbal extracts can exert potent estrogenic responses *in vitro* due to high content of flavonoids. For the first time however, estrogen-like activity is reported in oregano extracts. Adopted analytical approach allow for a fast screening for biological activity in herbal extracts, without the need for laborious separation and purification, providing invaluable structural information on the most active compounds. In this case, the detected biological activity was attributed to three compounds, i.e. luteolin-glucoside, luteolin-glucuronide and apigenin-glucuronide, which have been previously described as estrogenic constituents of agrimony, sage and yarrow extracts. Presented results are in agreement with other studies assessing estrogenicity of herbal extracts, pointing however to more complex interactions between extract constituents. What is more, possible cellular metabolism should be furtherly studied to confirm those findings. Even though estrogenic-like responses were detected in all of the assessed samples, significant variation was noted between the responses possibly due to differences in concentrations of the most potent constituents but also their interplay with other bioactive extract components (Gutiérrez-Grijalva et al., 2017). Controlling the biological response, even in binary mixtures of flavonoids was shown to be challenging, as even a five times concentration difference of one component may modulate the desired effect (Yang et al., 2015), with more disruption to be expected *in vivo* resulting from differences in bioavailability, metabolism as well as interactions with different pharmaceuticals or diet components. Flavones rich herbal extracts are also beneficial supplements, alleviating estrogen deficiency symptoms, however the presented results show that the activity among the extracts vary significantly, with some extracts inducing cells proliferation *in vitro*, hence more research is required to fully assess *in vivo* influence of such supplements, especially in the administration of both postmenopausal women and breast cancer patients undergoing hormone therapy.

Figure captions:

Figure 1 a) Viability of the estrogen responsive MMV-Luc RGA cell line following exposure to the assessed oregano samples. Results are expressed as a mean of a triplicate normalised to the solvent control $\pm$ SEM. *** - $p \leq 0.0001$; b) Estrogenic activity of tested oregano samples with all responses being statistically higher than the solvent control ($p > 0.05$). Results are expressed as a mean of a triplicate $\pm$ SEM; c) Group comparison of pure and adulterated sample's response in the RGA with whiskers from 5-95 percentile, *** - $p \leq 0.0001$.

Figure 2. A bar chart representing the responses obtained for each fraction of the QC in the MMV-Luc estrogenic RGA, expressed as the % of the maximal response of the top E_2 calibration point. A chromatogram obtained during LC-HRMS analysis of sample 28 is presented below with the focus on the biologically active fractions from minute 8 to 11.

Figure 3 a) PCA plot of unsupervised analysis of all the samples with clear separation between pure and adulterated samples (A=10, R2X=0.979, Q2=0.919); b) OPLS-DA plot of the features detected in the most estrogenic fractions, with samples grouped according the response in the RGA (A=4+3, R2X=0.938, R2Y=0.928, Q2=0.885; c) S-plot representing features contributing most to the G20 and G100 groups' separation, with five most prominent features highlighted.

Figure 4. Extracted chromatograms and low and high energy mass spectra of the proposed biological active compounds present in oregano extract. a) luteolin-7-O-glucuronide; b) luteolin-7-O-glucoside; c) apigenin-7-O-glucuronide; d) 3-methoxyapigenin; e) quercetin-7-O-methyl.

Figure 5. Peak areas of the selected compounds presented in pure (white bars) and adulterated (grey bars) oregano samples. Bars represent the mean peak area of triplicate injection $\pm$ SD. The bars represent samples in the following order 10, 11, 12, 24, 28 (pure), 39, 40, 61, 103, 116 (adulterated).

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Sample number	EEQ [ng g oregano $^{-1}$]	SD [ng g oregano $^{-1}$]
	Pure Oregano	
S10	63	2
S11	57	3
S12	109	12
S24	35	3
S28	7,409	10,923
	Adulterated Oregano	
S39	47	1
S40	22	2
S61	18	5
S103	111	17
S116	124	11

Table 1. Hormonal load of tested oregano extracts expressed as EEQ per g of sample.

Feature	Adduct	Proposed formula	mDa	PPM	DBE	Proposed ID	CAS
							101858-43-9
8.41_463.0880 m/z	[M+H]	C21H18O12	1.3	2.8	12.5	Luteolin-7-*O*-glucuronide	
8.49_449.1122 m/z	[M+H]	C21H20O11	-0.6	-1.3	11.5	Luteolin-7-*O*-glucoside	5373-11-5
9.05_447.0931 m/z	[M+H]	C21H18O11	-2.4	-5.4	12.5	Apigenin-7-*O*-glucuronide	29741-09-1
10.59_301.0719 m/z	[M+H]	C16H13O6	0.8	2.7	10.5	3-Methoxyapigenin	22697-65-0
10.72_331.0834 m/z	[M+H]	C17H15O7	1.6	4.8	10.5	Quercetin-7-*O*-methyl	98751-51-0

Table 2. Proposed IDs of the features responsible for the estrogenic activity selected during multivariate analysis.

Highlights:

- Oregano extracts exhibit estrogen-like activity *in-vitro*
- Conjugated flavonoids are the origin of the detected biological activity
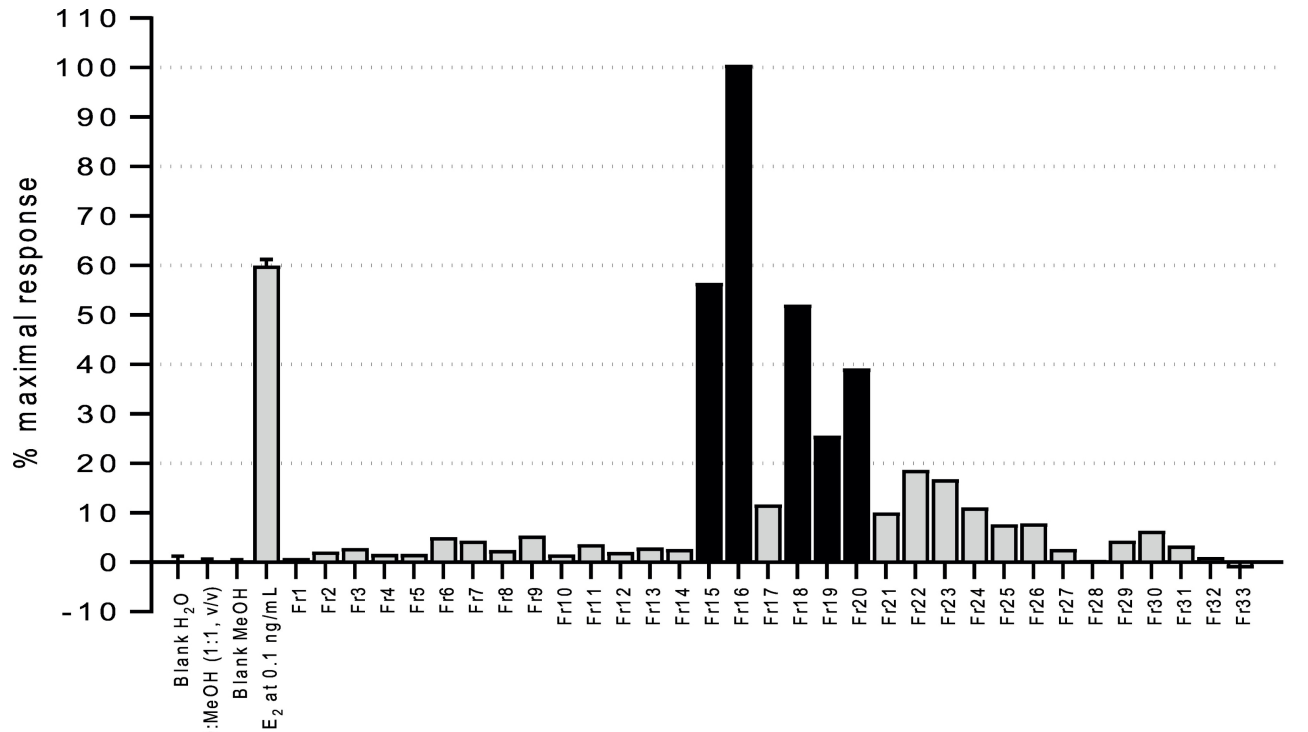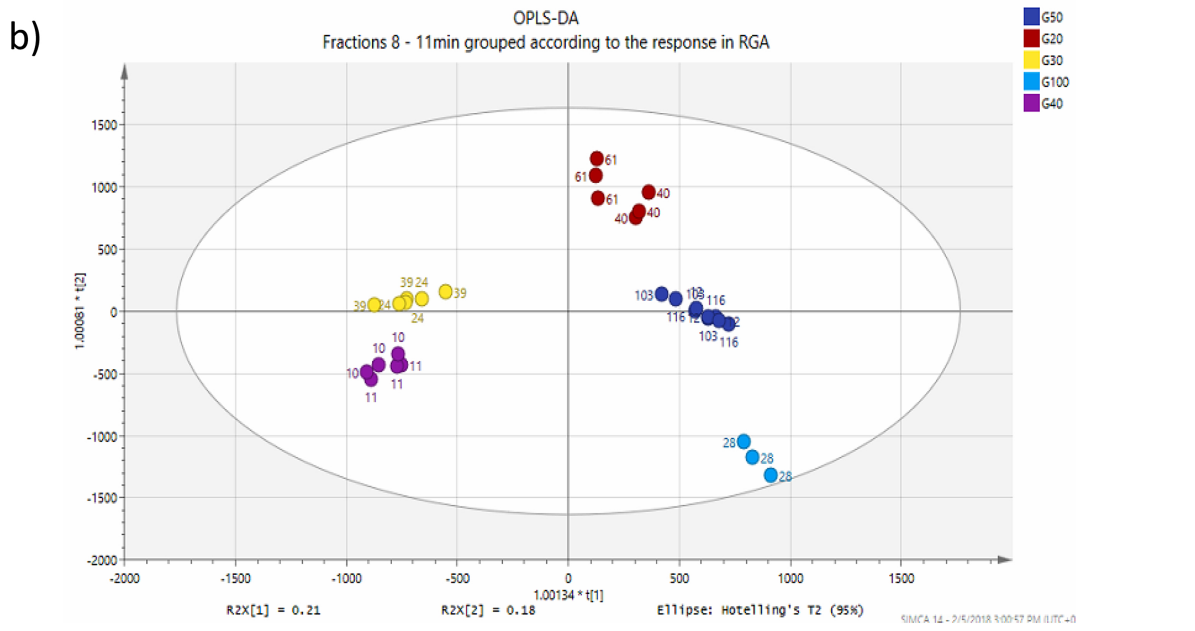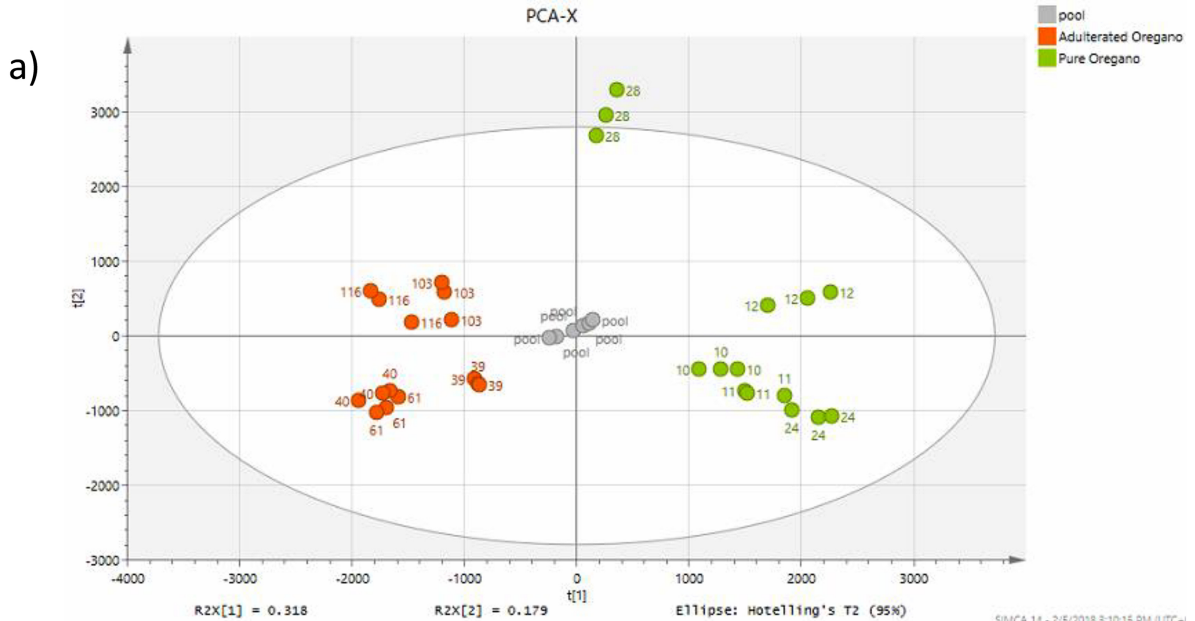- Combining bio- and chemical assays is a fast tool for detecting bioactive compounds
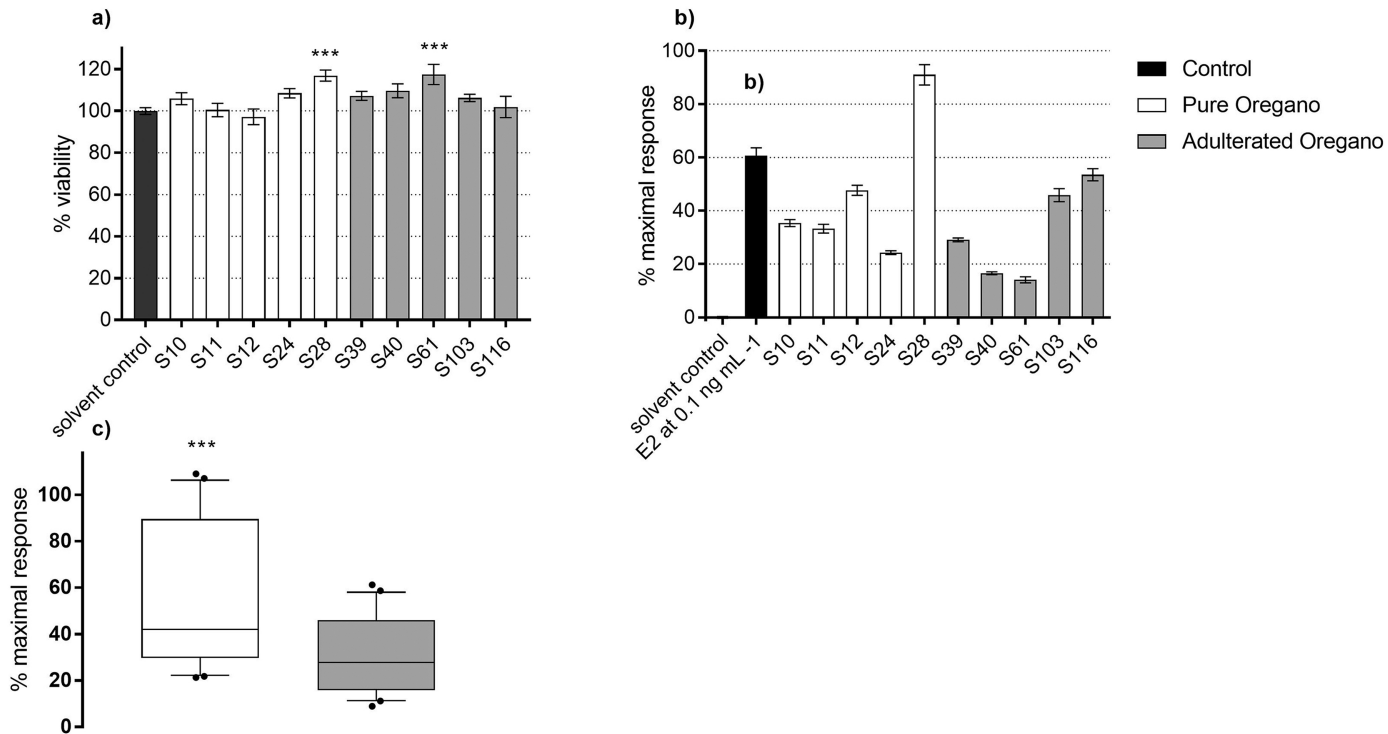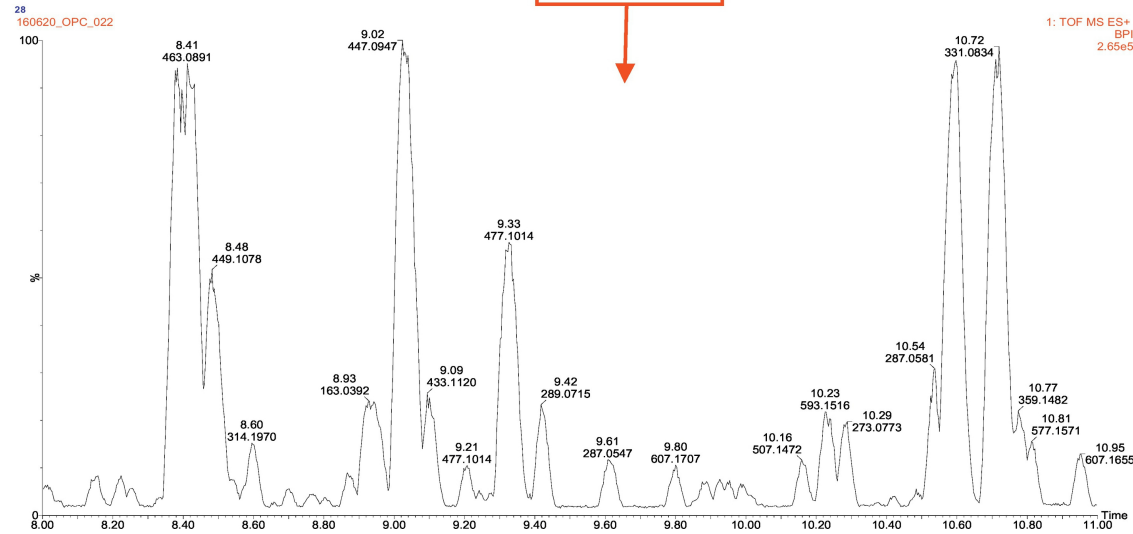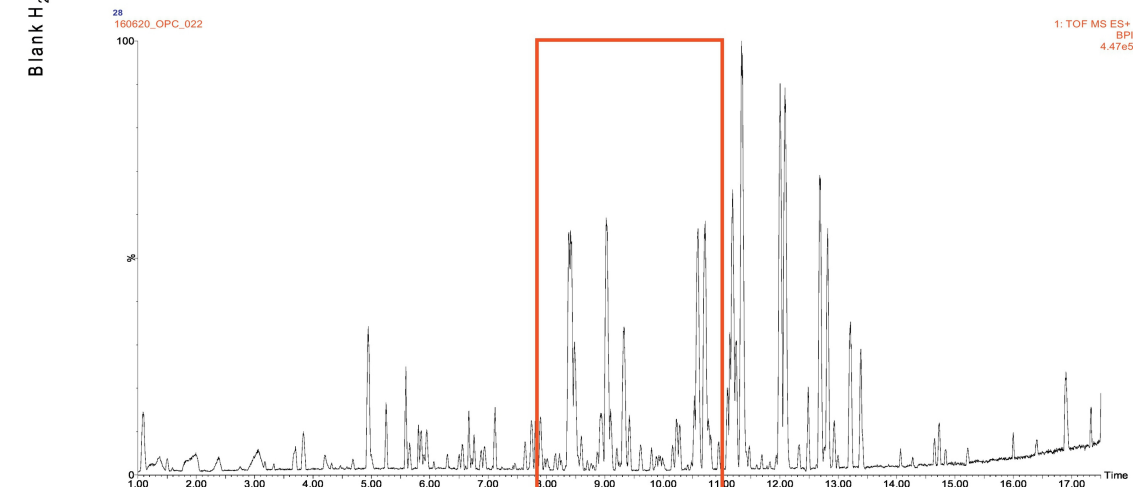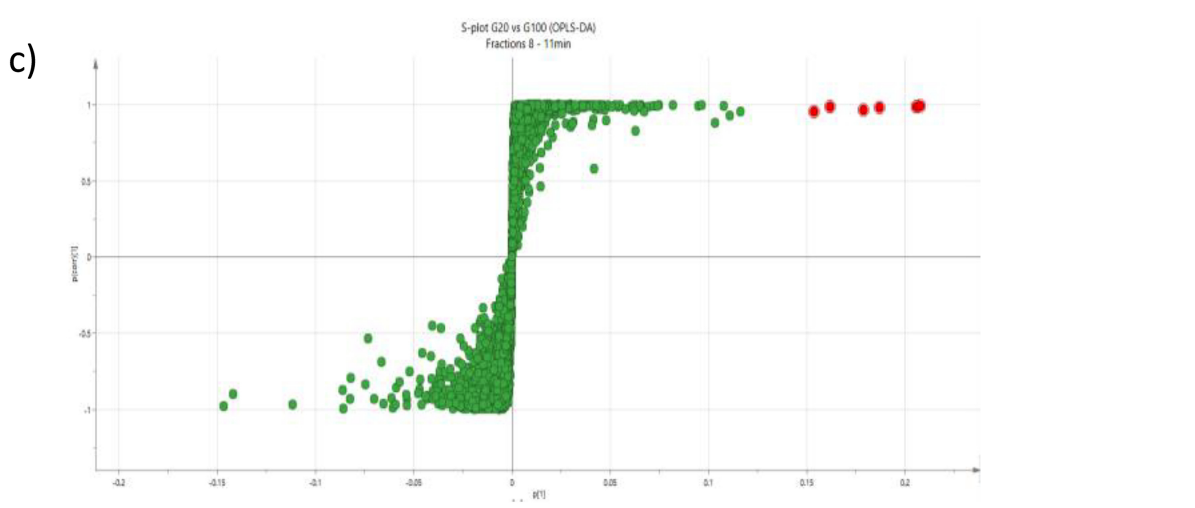
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5