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Poster 63

Design of innovative products for green agriculture: The Presspad project

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Abstract: The "Presspad - Extractive methods and products for green agriculture" project, funded by Tuscany's Rural Development Program 2014-2020, involved a Tuscan olive oil company and three research institutions. Its goals were to produce aqueous extracts and essential oils from urban and agricultural pruning waste of Mediterranean plants, rich in antioxidants, antimicrobial, and repellent properties, and to develop natural products for preventing and treating olive tree diseases. Innovative products were tested on olive trees to assess their protective effects. The project promoted circular use of waste and by-products from olive oil production and green maintenance, fostering sustainable agriculture solutions.

Keywords: green agriculture; polyphenols; antimicrobial activity; natural extracts; HPLC-DAD-MS analysis

1. Introduction

Green agriculture, also known as sustainable agriculture, is an approach to agriculture that aims to meet the needs of the present while preserving and enhancing the environment for future generations. It encompasses a variety of practices that focus on minimizing environmental impact, promoting biodiversity, and ensuring the long-term viability of agricultural systems. One of the key principles of green agriculture is soil health. Healthy soils are essential for productive and sustainable farming. In this context, the "PRESSPAD - Extractive methods and products for green agriculture" project was born, which aims to design antimicrobial and repellent products for the olive oil industry. The extra-virgin olive oil (EVOO) sector in Tuscany involves approximately 74.000 hectares, 15 million plants, 36.000 EVOO companies; the regional production of EVOO stands at 150.000 quintals per year (around 7% of Italian production) for a value of 130 million euros, equal to 5% of the total value of Tuscan agricultural production. The strength of the Tuscan EVOO sector is represented by the high quality of production recognized and appreciated all over the world thanks to the Protected Designations of Origin (PDO) and the Protected Geographical Indications (PGI) (www.regione.toscana.it). The focus of the PRESSPAD project is linked to the protection of the Tuscan EVOO supply chain, as in recent years, the olive trees were affected by numerous pathogens that damage the olives, producing less and low-quality EVOO and causes economic losses. To meet the needs of EVOO companies, the PRESSPAD project consortium was established to develop products based on natural extracts to replace the synthetic ones allowed in organic farming. The use of bio-pesticides in agriculture is in line with market trends and European policies that promote healthy, safe and sustainable nutrition. The project took place in Seggiano where the Seggianese cultivar is present together with the Leccino and Frantoio cultivar that are the most recognized varieties of EVOO on the international market. The PRESSPAD project was financed by the Tuscany Region for the Rural Development Program 2014-2020 GAL F.A.R. Maremma and the project consortium is made up of one Tuscan EVOO company and three Italian research centers such as University of Florence (PHYTOLAB laboratory- Department of Statistics, Computer Science and Applications), CRISBA laboratory (Istituto Superiore "Leopoldo II di Lorena" di Grosseto) and INSTM (National Inter-University Consortium for the Science and Technology of Materials). The coordinator and principal investigator of the project is Manni Oil S.r.l. company specialized in high quality PGI Tuscan EVOO production. The starting aim of the

project was the optimization of extraction methods of plants, rich in antioxidant, antimicrobial and repellent compounds, present in the area involved by the project (i.e. phillyrea, olive trees, lentisk, ailanthus, eucalyptus, oregano, mentha and thyme), preferring those of the Mediterranean bush. The second objective was to design, according to the extract's composition of natural active compounds, innovative green products for the prevention and treatment of the most widespread pathologies of olive trees. Moreover, the innovative products were tested in vitro and in vivo and the qualitative analysis of the EVOOs was performed. Research and development activities for the design of products for green agriculture can be linked to increasing the quality of soil and food and therefore to human well-being.

2. Materials and Methods

2.1. Materials

Pistacia lentiscus L. (LEN), Phyllirea angustifolia L. (PHY), Olea europaea L. (OLIV), Salvia officinalis L. (SAL), Inula viscosa L. (INU), paradise tree (Ailanthus altissima Mill.) (AIL), Thymbra capitata L. (THY), Origanum vulgare L. (ORE), Mentha spicata L. (MEN) and Eucalyptus globulus L. (EUC) were selected.

2.2. Extraction methods

A preliminary lab-scale extraction was carried out testing different solvents such as water and hydroalcoholic solution (70:30 EtOH:H_2O) with different amounts of raw material, temperature and extraction times. The solutions obtained were pre-filtered through a medium-mesh plastic filter than filtered at low pressure by filter paper. The obtained extracts were analyzed by HPLC- DAD-MS to monitor the yields of active compounds and to optimize the extraction protocols. Afterwards the method was scaled-up in a semi-industrial setting using a rapid extractor Naviglio, with a 200L inox steel chamber that houses a microporous bag (50 µm mesh) containing raw vegetable material. A pneumatic semi-automatic piston that produces up to 200 bar of pressure completes the extraction. LEN, PHY and OLIV were extracted individually, whereas SAL, INU and AIL were combined in a blend (MIX EXT) due to the scarce availability of these species with respect to the volume of the extraction chamber in the semi-industrial equipment used for the up-scaling process. The semi-industrial extractions were then performed in the Naviglio extractor using 35-40 kg of dried raw material, in 200L water at 95°C. The essential oils of THY, ORE, MEN and EUC were extracted by steam distillation. A preliminary lab-scale phase of experimental extractions was carried out. The extracts obtained were analyzed by gas chromatography to optimize the extraction protocol. The method was scaled-up in a semi-industrial scale extractor, with a maximum volume of 600L, temperature up to 105°C and pressure of 1.2-1.3bar. For each extract, 80 kg of raw material was used with 30- 35L of solvent. All the obtained extracts were used for *in vitro* and *in vivo* tests.

2.3. Analysis of natural extracts and EVOOs

The qualitative and quantitative analysis of the active compounds present in aqueous extracts was performed by using a HP-1260 liquid chromatograph equipped with a DAD detector (Agilent-Technologies, Palo Alto, USA). The HPLC system was interfaced with an Agilent MS system equipped with an ESI source (Agilent Corp, Santa Clara, CA, USA). Analyzes were acquired in full-scan mode, and the mass range was set to m/z 100–2000 in both positive and negative modes. Mass spectrometer operating conditions were: gas temperature 350 °C at a flow rate of 10.0 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C and capillary voltage 3500 V. The fragmentor was set at 120 eV. For the extracts of LEN and the mixed vegetal species (INU, AIL, SAL) a Luna, C18 250×4.60 mm, 5µm column (Phenomenex), operating at 26°C was used. The eluents were H2O (adjusted to pH 3.2 with HCOOH) and CH3CN. A four- step linear solvent gradient starting from 100% H2O up to 100% CH3CN was performed with a flow rate of 0.8mL/min over a 55-min period, as previously described (Campo et al., 2016). For the extracts of PHY and OLIV an analytical column Lichrosorb RP18 250 × 4.60 mm i.d, 5 µm (Merck Darmstadt, Germany) was used. The eluents were H2O adjusted to pH 3.2 with HCOOH and CH3CN. A four-step linear solvent gradient was used, starting from 100% H2O up to 100% CH3CN, for 88 min at a flow rate of 0.8 mL/min (Romani et al., 2020). The compounds found in the extracts were identified by comparing retention times and UV/Vis spectra with those of the authentic standards available. The quantification of the individual compound was directly performed by HPLC-DAD using five-point regression curves built with the specific standards. Curves with an r2>0.9998 were considered. Calibration was performed at the wavelength of the maximum UV-Vis absorbance, by applying the correction of molecular weights. In particular, gallic acid and its derivatives were calibrated at 280 nm using gallic acid as a reference; flavonoids were calibrated at 350 nm using quercetin 3-O glucoside and myricetin as references; phenolic acids were calibrated at 280 nm using hydroxytyrosol and oleuropein as references; hydroxycinnamic derivatives were calibrated at 330 nm using caffeic acid as reference. The determinations of the polyphenol contents were carried out in triplicate; the results are given as means and the standard error was <5%. The essential oils of THY, ORE, MEN and EUC were subjected to gas chromatographic analysis as described by Ieri et al., 2019. Briefly, the analysis was performed with an Agilent 5975C

MSD instrument equipped with a multifunction injection system (Gerstel MPS-2) equipped for automatic sampling. Analytical separation was achieved with an Agilent DB InnoWAX 50m, 0.20 µm id, 0.40 µm df column. Chromatographic conditions: initial temperature 40 °C for 0.5 min, then 6 °C/min up to 260 °C. This temperature is maintained for 1 min. The tentative identification of the compounds was obtained by comparing the mass spectrum of each peak with those present in the Database (NIST Mass Spectral Library). For each chromatogram the peaks were integrated using the TIC (Total ion current) areas. Quality parameters, such as acidity, peroxides and total polyphenols, were evaluated on all EVOOs using the CDR-Oxitester instrument (CDR srl, Florence, Italy) as described in Romani et al. 2020.

2.4. In vitro test

The in vitro test with water-based extracts was carried out using the poisoned food technique as described in Romani et al., (2021). The essential oils can be degraded with the sterilization in autoclave. For these substances, the Potato Dextrose Agar (PDA) was prepared and sterilized, then, during the cooling, when the temperature of the molten substrate was around 50°C, the essential oils were added to it and vigorously shaken. The doses (w/v) used are the following: LEN 1.5g/L; PHY 0.5g/L; OLIV 1.5g/L; extract mix composed by INU, AIL and SAL, (MIX EXT) 0.5g/L; salts mix (composed by Zn, Fe, Mn, Cu) 0.4g/L; terpineol (TERP) 0.25g/L; EUC 0.1g/L, MEN 0.1g/L; ORE 0.1g/L; THY 0.1g/L; prototype of the product (P1) 5g/L. In a second test, carried out with the same method, the innovative product was tested alone in different doses (0, 0.5, 1.0, 2.5, 5.0g/L w/v) to establish the optimal use dose.

2.5. In vivo test

The *in vivo* test was carried out on within the Manni Oil S.r.l. farm (Seggiano, GR), on 40 olive trees divided into plots where different treatments were executed. The innovative formulation was tested ad such (liquid) as a leaves treatment (Prototype 1, P1) and absorbed on biochar as a root treatment (Prototype 2, P2). P1 was also tested on leaves and roots at the same time. All treatments described were compared with control olive trees (no treatment). The monitoring and data collection were made twice, in August and October, measuring the incidence of peacock spot (*Spilocacea oleaginea*) and olive fruit fly (*Bactrocera oleae*) by visual analysis, and mean weight of the olives. Table 1 shows the treatment protocol carried out in the field.

Table 1. Treatment scheme

Name of treatment	Prototype	Part of plant
P2R	Prototype 2	Root
P1L	Prototype 1	Leaves
P1LR	Prototype 1	Leaves and root
Control (CNT)	None	None

3. Results

The first step of the project was the optimization of extraction methods of selected species, listed in the "Materials and Methods" section. The selection of these species, within the flora present in the area, was done according to scientific studies reported in literature and to the previous results obtained by testing analogous extracts in different conditions, in greenhouse experimentations (Boufissiou et al., 2024, Varo et al., 2017, Goussous et al., 2010). The parameters of the extraction processes were optimized by monitoring the yields of active compounds and their stability in the extraction conditions through HPLC-DAD-MS or gas- chromatographic analysis. Afterwards, an in-depth chemical characterization of the contents of active secondary metabolites were performed by liquid chromatography and gas chromatography, respectively for the aqueous extracts and the essential oils. Table 2 shows the qualitative and quantitative analysis by HPLC-DAD-MS of the aqueous extracts of LEN, PHY, OLIV and MIX EXT. The detected molecules were identified and quantified individually using specific analytical standards and reported in the table divided into polyphenolic subclasses. The analysis showed the presence of different bioactive molecules belonging to the subclasses of flavonoids, tannins, phenolic acids and hydroxycinnamic derivatives. These compounds are known for their bio-stimulant, antimicrobial and repellent activities and for their ability to defend the plant from insects and pathogenic microorganisms and assist it in responding to external stresses of biotic or abiotic origin. The innovative formulation, intended for the in vitro and vivo tests on olive trees, was designed according to the reported project results.

Table 2. Qualitative and quantitative HPLC-DAD-MS analysis of the extracts by phenolic subclasses; amounts in mg/mL of extract. (standard deviation < 5%). (ND: not detected). LEN: Pistacia lentiscus L., PHY: Phyllirea angustifolia L., OLIV: Olea europaea L. MIX EXT: Mix of extracts of Salvia officinalis L., Inula viscosa L., and Ailanthus altissima Mill.

Compounds	LEN	PHY	OLIV	MIX EXT
Tannins	1.73±0.05	ND	ND	2.03±0.06
Flavonoids	0.28±0.01	3.66±0.11	0.83±0.02	0.59±0.02
Phenolic Acids	1.44±0.04	2.41±0.07	1.35±0.04	0.59±0.02
Hydroxycinnamic derivatives	ND	2.91±0.09	0.28±0.01	ND
Total polyphenols	3.46±0.10	8.98±0.27	2.46±0.07	3.21±0.10

Even the chemical composition of EUC, MEN, THY and ORE essential oils was evaluated. Gas chromatography (GC) combined with mass spectrometry (MS) constitutes the ideal tool for characterizing and quantifying volatile secondary metabolites (VOCs) of plants. GC data evidenced amounts of 1,8-cineole (eucalyptol) greater than 83% in eucalyptus essential oil. Mentha essential oil showed high amounts of carvone (27%), Caryophyllene (11%) and Dihydrocarvone isomers (10.6%) for a total of volatile compounds equal to 80.9% W/V. ORE and THY essential oils showed high quantities of carvacrol, 90% and 53%, respectively.

3.1. Formulation of innovative agronomic products

Two innovative products (P1 and P2) for green agriculture were formulated starting from the results obtained from chemical analyses and according to literature and previous results (Tegli et al., 2016; Simone et al., 2020). The P1 product is a liquid formulation and was applied on leaves and root of the olive trees, while the P2 product was designed starting from P1 absorbed on biochar and was applied only on the root part. Table 3 shows the percentages of each ingredient used to obtain the products.

Table 3. Composition of innovative products for green agriculture

Ingredients	(%)
Pistacia lentiscus	30
Phyllirea angustifolia	5
Olea europaea	3
Mix extracts (Salvia officinalis, Inula viscosa, Ailanthus altissima)	10
Thymbra capitata essential oil	1.5
Origanum vulgare essential oil	3
Mentha essential oil	1
Eucalyptus essential oil	2
Co-formulants	7
Mix of salts	7.5
Water	30

3.2. In vitro test

Table 4 shows the activity of P1 and the individual ingredients. OLIV added as a natural preservative due to its antioxidant and antiradical activities. The MIX SAL showed inhibition against Alternaria and Arthrinium. The best results among single extracts were obtained with THY and ORE essential oils, with higher inhibition values compared

to the other essential oils and extracts. Their effects are quite promising since the dose used was very low. P1 showed very interesting results with inhibition values of 100% or little lower (minimum of 98%) against all the pathogens used.

Table 4: *In vitro* tests results. (standard deviation < 5%). LEN: Pistacia lentiscus L., PHY: Phyllirea angustifolia L., OLIV: Olea europaea L. MIX EXT: Mix of extracts of Salvia officinalis L., Inula viscosa L., and Ailanthus altissima Mill., MIX SAL: mix of salts, TERP: terpineol, EUC: Eucalyptus globulus L. essential oil, MEN: Mentha spicata L. essential oil, ORE: Origanum vulgare L essential oil, THY: Thymbra capitata L. essential oil, P1: prototype product 1

	LEN	**PHY**	**OLIV**	**MIX EXT**	**MIX SAL**	**TERP**	**EUC**	**MEN**	**ORE**	**THY**	**P1**
Alternaria sp.	1.85 ± 1.89	1.88 ±3.35	0.0 ±0.00	0.20 ±1.98	55.68 ±1.56	26.88 ±10.8	3.60 ±4.59	10.3 ±3.25	66.55 ±3.90	66.05 ±2.47	100.0 ±0.00
Artrinium marii	0.87 ±1.06	8.44 ±4.84	0.0 ±0.00	0.22 ±0.53	49.46 ±14.60	40.80 ±6.69	13.20 ±11.83	1.30 ±1.64	98.48 ±3.71	99.57 ±1.06	100 ±0.00
Fusarium solani	2.03 ±2.67	1.99 ±2.22	0.0 ±0.00	2.02 ±2.22	4.62 ±2.42	6.37 ±4.57	2.25 ±3.27	2.82 ±3.15	36.05 ±18.31	47.04 ±15.04	98.27 ±15.54
Collectotrichum acutatum	3.07 ±1.82	1.87 ±1.24	0.0 ±0.00	1.87 ±1.68	1.85 ±2.49	6.40 ±2.15	4.94 ±1.98	7.71 ±2.28	34.89 ±4.92	35.85 ±5.54	98.94 ±1.64
Fomitiporia mediterranea	-3.01 ±4.53	-4.24 ±4.32	0.0 ±0.00	-5.15 ±3.07	19.76 ±6.0	6.93 ±2.82	-0.41 ±6.34	5.10 ±4.68	32.31 ±8.22	28.93 ±3.60	100.0 ±0.00
Verticillium dahliae	4.56 ±7.17	0,90 ±2.08	0.0 ±0.00	1.23 ±0.31	4.02 ±5.39	9.02 ±4.39	-1.63 ±2.93	1.60 ±2.93	69.55 ±9.31	68.89 ±4.78	100 ±0.00

In the second trial, it can be confirmed the efficacy of P1 at the maximum dose (5g/L), that made a complete inhibition against all pathogens except F. solani, that was inhibited by the 90% (Table 5). The 100% efficacy with the lower dose (2.5%) was maintained only against A. marii and V. dahliae, while it oscillated between 78.3% and 92.6% against the other fungi. At 1g/L the inhibition was between 48.0% and 92.85%, while at the lowest dose it was between 4.3% and 45.4%. Following these results, the dose chosen for the field trials was 5g/L w/v.

Table 5. Dose and efficacy tests

	0.5%	**1.0%**	**2.5%**	**5.0%**
Alternaria sp.	28.35 ±0.99	74.46 ±6.14	92.64 ±0.75	100.0 ±0.00
Artrinium marii	45.45 ±1.30	91.77 ±0.75	100.0 ±0,00	100.0 ±0.00
Fusarium solani	4.33 ±1.98	48.05 ±0.00	78.35 ±0.75	90.91 ±0,00
Collectotrichum acutatum	40.48 ±0.99	65.37 ±0.75	79.65 ±0.75	100.0 ±0.00
Fomitiporia mediterranea	41.13 ±0.75	65.37 ±2.09	82.03 ±4.42	100.0 ±0.00
Verticillium dahliae	15.63 ±2.32	92.86 ±0.77	100.0 ±0.00	100.0 ±0.00

3.3. In vivo test

At the first monitoring 100% of olive trees were infected with S. oleaginea. Then the symptom severity was evaluated through the randomic collection of leaves at two different times in August and October. It was observed that the severity was lower on the treated plants (7%) compared to the control thesis (19.6%). For the olive fruit fly monitoring, the data collection was made 4 times. At the first monitoring (T0) the drupes weren't formed yet. The second time, in July, before any treatment, 17% of fruits were punctured. After two treatments (carried out in July and September), the monitoring and fruit harvest were done twice (in August and October). In the first sampling, the punctured fruits were 46% in the treated group, while they were 39% in the control group. This result can be explained after the weighing: the treated group produced larger fruits compared to the untreated, thus, they were preferentially attacked by the olive fruit fly. In the second sampling the fruits weight in the treated and untreated groups was comparable, and the percentage of damaged fruits was higher in the untreated group. This allows us to state that the tested prototypes have some repellent effect against the olive fruit fly. Finally, during the harvest, the mean productivity of each plant was measured. The treated groups produced average 11.05 kg olives each plant compared to 8.87kg of the control group, which can be translated into a higher productivity of 20-28%. This could be the result of a bio-stimulant effect and the reduced damage by olive fruit fly.

3.4. Qualitative analysis of EVOOs

A further activity carried out concerned the evaluation of the quality of the EVOOs obtained from treated olive trees and control. Four EVOOs were obtained, three from the experimental areas (P2R, P1L, P1LR) and one from the control area (CNT). The EVOOs analysis of acidity, peroxides and total polyphenols are shown in Table 6. There are no significant differences between the results found for treated samples and the control regarding the parameters of acidity and peroxides of EVOO that are according to legal limits defined by RegUE. 61/2011 (acidity ≤0.8% of oleic acid and peroxides ≤ 20 mEq O_2/kg) (RegUE. 61/2011). The total polyphenols content (expressed as mg of tyrosol/kg oil) was evaluated to assess the EVOOs functional quality. The EVOO obtained from olive trees treated with P1 (P1LR) on both the leaves and roots shows a higher content of polyphenols (802 mg/kg). All EVOOs showed a high value of total polyphenols, and also in this case no significant difference was reported between the control and the oils from treated olive trees. According to the results it is hypothesized that all EVOOs could obtain the EFSA EU 432/2012 health claim relating to the protection of blood lipids from oxidation (RegUE432/2012).

Table 6. Qualitative analysis of EVOOs. (standard deviation < 5%). P2R: prototype product 2 on roots, P1L: prototype product 1on leaves, P1LR: prototype product 1on leaves and roots, CNR: control

Parameters	P2R oil	P1L oil	P1LR oil	CNT oil
Acidity (% oleic acid)	0,12	0,07	0,15	0,13
Peroxides (mEq/Kg O_2)	11,11	10,18	10,88	16,02
Total polyphenols (mg/kg tyrosol)	751	743	802	769

4. Conclusions

In this project, environmentally friendly semi-industrial processes were optimized to obtain natural and environmentally friendly extracts with biostimulant, antimicrobial and repellent activity. The selected raw materials were phillyrea, olive tree, lentisk, ailanthus, eucalyptus, oregano, mentha and thyme. The obtained extracts were chemically characterized for their content of bioactive compounds and used as ingredients for the formulation of an innovative blend for organic cultivation of Tuscan olive trees. In vitro tests were performed to assess the antimicrobial activities of the extracts and the blend against Olea europaea L. pathogens. The innovative formulation was tested in vivo in liquid form for application by nebulization on the foliage and adsorbed on biochar for root application. The chemical analyses confirmed the composition of the formulation in hydrosoluble and volatile active compounds and the results of in vitro and in vivo tests showed the ability of the new formulation to enhance the resistance of olive trees in the presence of pathogens and parasites. Moreover, it was demonstrated that the applied treatments are also able to maintain the EVOOs quality unchanged and improve the productivity of olive trees. The project involved an area in the province of Grosseto with an olive-growing and agricultural vocation in general, represented in particular by the Municipality of Seggiano, where the Seggianese variety is typically bringing an increase in the market at a national and international level of quality organic EVOOs with local brand. The Presspad project linked to the European Green Deal strategy for sustainability by suggesting the use of innovative natural products as an eco-friendly alternative to synthetic pesticides. By adopting sustainable and innovative practices, agri-food companies can help mitigate the impacts of climate change, preserve natural resources, and create a more resilient and equitable food system for future generations.

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