PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII cycles, PON)

Name of PhD student: .....Eleonora Mari.....

Title of PhD research: Green economy: the potential role of the hope plants, from flowers to leaves.

Name of PhD supervisor: .....Maria Grazia Ortore.....
Research lab name: .....Laboratorio di Biofisica Molecolare.....

Mesi in IMPRESA (Italia): .....0.....
Mesi in IMPRESA (Estero, se previsti): .....0.....

Cycle:
[ ] XXXVI
[X] XXXVII

PhD Curriculum:
[ ] Marine biology and ecology
[X] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[X] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate: .....

ABSTRACT:
The main idea of this project is to exploit the bioactive compounds extracted from the waste of the hop plant: the leaves, which are not used for commercial aims and are often burned after the harvest. It is hence crucial to understand how to optimize the extraction of bioactive compounds and to make possible their use in different kind of products. The biomass of the hop plant contains several principles that have been shown to have antibacterial and antiviral properties, and other compounds able to interfere with the amyloid fibrillation process, the cause of the most important neurodegenerative diseases.
Hop biomass has hence several possible applications ranging from pharmaceutical up to nutraceutical industries, because hop waste can be transformed into natural additives for food preservation and for pharmacological aims.

Part 1. Scientific case of the PhD Research

BACKGROUND: This project needs an integrated approach which ranges from biophysics to microbiology. The potential of the hop plant is wide, and an extensive experimental work is required to test its efficiency. Recent studies have shown that hops are rich in bioactive compounds with antibacterial, antioxidant, anti-inflammatory and antiviral properties[1]. Beneficial effects have also recently been demonstrated in Alzheimer's disease (AD)[2], the most common cause of dementia, although the mechanism of action of hops compounds is still far from being understood[3]. Currently the most investigated bioactive compounds are xanthohumol, humulone and lupulon[1].

SCIENTIFIC AIMS: The objectives of this research will be to investigate functional groups present in hops extracts, whose biological activity has not been evaluated. We will monitor the influence of saponins, xanthohumol, α-humulene, and the whole extract on amyloid aggregation and the antimicrobial capacities for future application in the food and health issues. We will investigate human insulin and amyloid beta peptide, proteins involved in human diseases.[4] Because hop extracts are obtained from agricultural waste, the aim is to reuse and develop a circular and green protocol.

WORKPLAN AND RESEARCH ACTIVITIES:

WP 1. Production of the extracts from the leaves of the hop plant.

Methods. First, we sampled leaves of different hop varieties (Cascade, Centennial, Comet, Chinook, Mercur) and at two ripening times of the plant. The leaves were very accurately cleaned, lyophilized, and frozen at a temperature of -20°C. Subsequently the leaves were grinded using a pestle and mortar[5]. The extraction of bioactive compounds from the hop powder was performed using an 80% ethanol solution (80% ethanol-20% milli q water), and a dilution factor of 1:20 (1g of hop's powder in 20 ml of solvent). The extraction was performed using a stirrer at room temperature for two hours. The suspension was centrifuged (5000 rpm, 10 min) and filtered with a filter paper. The extract is stored at -10°C.

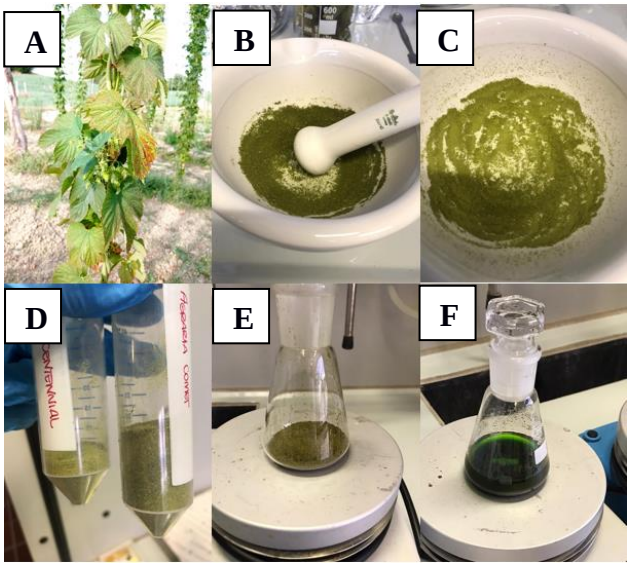

Figure 1. **A**: Hop plants from the field located in Sant'Angelo in Pontano (MC), 6th September. **B-C**: grinding phase of the lyophilized leaves, using a pestle and mortar. **D-E**: powder obtained from the hop leaves. **F**: extraction phase using the 80% ethanol solution.

Expected/Obtained Results.

Until now we have not determined meaningful differences in the antioxidant properties of the extract of hop leaves collected in the two ripening times. On the other side, a comparison between different hop species' extracts efficiency is still in progress.

WP 2. Study of the effect of saponins (contained in hops) on the amyloid aggregation of human insulin

Methods. First, we optimized the human insulin aggregation protocol. The amyloid aggregation was performed with commercial human insulin (Sigma Aldrich) at a concentration of 0,5 mg/ml in phosphate buffer 50mM, pH 7.4, and 37°C under stirring. Solution conditions are comparable to those

in vivo. We optimized the protocol by choosing stirring rates and test tubes to obtain a good repeatability for the aggregation process. Saponins were used in a molar ratio [insulin]: [saponins] =1:1. To monitor the formation of β-structures (in absence and in presence of saponins) as a function of time we performed UV-Visible absorption spectrophotometry measurements, using Congo Red as an amyloid specific dye[6]. To study the overall structural features and size of aggregates, we performed Synchrotron Small Angle X- ray Scattering and Dynamic Light Scattering experiments. The final products resulting from the aggregation processes of human insulin after 24 hours were analyzed by DLS and SAXS. Also, several time-steps from the aggregation pattern were monitored by SAXS at the Elettra synchrotron.

Expected/Obtained Results. Experimental data show that saponins interfere with the amyloid aggregation, proving that these induce a high inhibition on the formation of insulin fibrils.

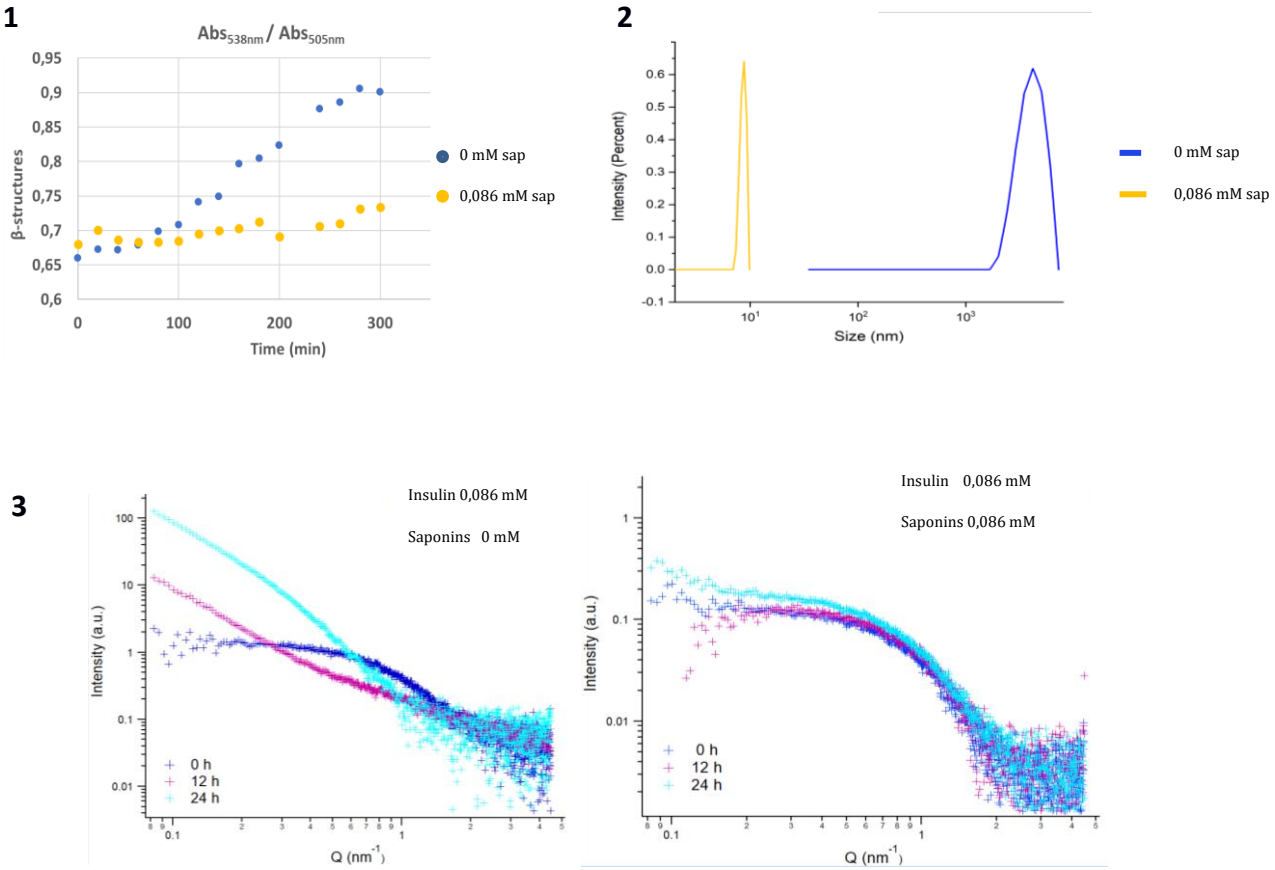

Figure 2. **[1] Absorption Spectroscopy** data. Graphic representation of the quantitative analysis through the ratio between the absorption peaks at λ equal to 538 nm and to 505 nm, in absence and presence of saponins (molar ratio insulin-saponins 1:1). **[2] DLS** data obtained for the final state of insulin kinetics, in absence and presence of saponins, showing the size of the structures present in the sample. **[3] SAXS** data obtained for human insulin in phosphate buffer 50 mM, pH 7,4 at 37° C in gentle agitation, in absence [left] and presence [right] of saponins (molar ratio insulin-saponins 1:1), as a function of time.

We will conduct further studies with other bioactive compounds of hop biomass (xantumol, α-humulene) in order to compare the effects on the amyloid aggregation, and to evidence possible synergies between different compounds.

WP 3. Study of the effect of hop extract (contained in hops) on the amyloid aggregation of human insulin

Methods. We monitored the increase of β-structures, as a function of time, in presence of the hop extract, by Absorption Spectroscopy, as in WP2. The experimental conditions for insulin aggregation were the same investigated in WP2. Until now, just three kinetics were monitored: one in the absence of hop extract, one in the presence of hop extract at a concentration of 0.5 mg/ml, and a third in the presence of hop extract at a concentration of 1 mg/ml.

Expected/Obtained Results.

Our experimental results show that hop extract produces a slowing down of the kinetics and a reduction in the final number of fibrils.

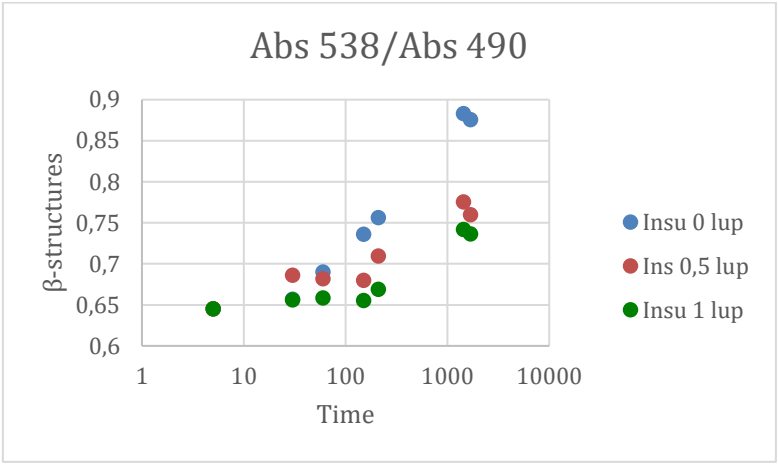

Figure 3. Absorption Spectroscopy data. Graphic representation of the quantitative analysis through the ratio between the absorption peaks at λ equal to 538 nm and to 490 nm, in absence and in presence of hop extract at two concentration (0,5 mg/ml and 1 mg/ml)

WP 4. Recombinant production of the β-amyloid protein.

Methods. The production of the β-amyloid protein was obtained using Pet-Sac-Abeta (M1-42), plasmid purchased from the Addgene company. E. coli BL21 derived cells from the Thermo-Fisher company were used for the expression of the target protein in bacteria. Obtained the transformation, a pre-culture was carried out in 10 ml of the LB medium (Luria Bertani) with 10µl of Ampicillin and 10µl of Chloramphenicol. The cells were grown o/n at 37°C under agitation. The next day the pre-culture was inoculated in a 2l flask in 1l of LB with 1 ml Ampicillin and 1ml Chloramphenicol. This culture was incubated at 37°C at 220 rpm; reached an optical density of 0,4-0,6 has been added IPTG (Isopropyl-β-D-1- thiogalactopyranoside) and the culture was incubated at 20°C o/n under agitation. The next day pellets were obtained from the cultures and frozen at -80°C. Subsequently the pellets were resuspended in Tris EDTA- urea buffer, centrifugated and sonicated many times. After that dialysis was performed, to eliminate the urea.

Expected/Obtained Results. Finally, the protein was purified and concentrated by means of special concentrators. The protein had been measured by SAXS at Elettra Synchrotron, data analysis is in progress.

- REFERENCES

1. Bogdanova K. et al. (2018). Antibiofilm activity of bioactive hop compounds humulone, lupulone and xanthohumol toward susceptible and resistant staphylococci. *Research in microbiology*, *169*(3), 127–134. https://doi.org/10.1016/j.resmic.2017.12.005

2. Huang X et al. (2018) The Prenylflavonoid Xanthohumol Reduces Alzheimer-Like Changes and Modulates Multiple Pathogenic Molecular Pathways in the Neuro2a/APP_{swe} Cell Model of AD. *Front. Pharmacol.* 9:199. doi: 10.3389/fphar.2018.00199

3. Palmioli, A. et al. (2022). Alzheimer's Disease Prevention through Natural Compounds: Cell-Free, *In Vitro*, and *In Vivo* Dissection of Hop (*Humulus lupulus* L.) Multitarget Activity. *ACS chemical neuroscience*, 10.1021/acschemneuro.2c00444. Advance online publication. https://doi.org/10.1021/acschemneuro.2c00444

4. Chiti, F., & Dobson, C. M. (2006). Protein misfolding, functional amyloid, and human disease. *Annual review of biochemistry*, *75*, 333–366. https://doi.org/10.1146/annurev.biochem.75.101304.123901

5. Knez Hrnčič, M., Španinger, E., Košir, I. J., Knez, Ž., & Bren, U. (2019). Hop Compounds: Extraction Techniques, Chemical Analyses, Antioxidative, Antimicrobial, and Anticarcinogenic Effects. *Nutrients*, *11*(2), 257. https://doi.org/10.3390/nu11020257

6. Mari E.et al. *Trehalose Effect on The Aggregation of Model Proteins into Amyloid Fibrils* Life 2020**,** 10,60; doi: 10.3390/life10050060

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

Courses:

1. Attended course of Technology transfer and innovation, Prof Donato Iacobucci.

2. Attended course of 'Introduzione all'ambiente LaTeX per la redazione di documenti scientifici', Prof. Francesco Spinozzi.

3. Attended course of 'Theory and application of complex network', Prof.ssa Maria Grazia Ortore.

Seminars:

1. 23/03/22 'Caratteristiche biofisiche dei sistemi viventi (e come guardarli)', Prof. R. Bizzarri

2. 09/05/22 'Cenni di psicologia dell'emergenza. Casi studio relativi ad emergenza riconducibili a differenti scenari di rischio.", Dott.ssa Dorotea Ricci

3. 07/06/22 'The resolution revolution in cryo-electron-microscopy in structural biology and in life sciences' Prof. Martino Bolognesi

4. 'Current threats to research ethics and how to cope with them', Dott. Marco Seeber

List of periods spent abroad

1. From 27 to 29 April on mission at the European Synchrotron Radiation Facility, in Grenoble (Fr)

List of conferences/workshops attended and of contributions eventually presented

1. From 11 to 14 September 2022 participation at the XXVI Congresso Nazionale della Società Italiana di Biofisica Pura e Applicata, in San Miniato (PI) and poster presentation on the results obtained 'Effect of bioactive compounds from hop plant on the aggregation of human insulin in amyloid aggregation'

2. From 13 to 14 September 2022 partecipation at the Second DiSVA-MaSBiC Annual Symposium - Protein Structure and Function in Biology, Medicine and Nanotechnology, in Ancona (AN) and poster presentation on results obtained 'Effect of natural compounds in amiloyd aggregation'.

Part 3. PhD student information on publications

If not yet published, please indicate the publication status (submitted, accepted, in preparation...)

List of publications on international journals

Mari E., Galeazzi R., Ortore M.G. "Effect of Saponins on the aggregation of Human Insulin in amyloid fibrils" *in preparation.*

List of publications on conference proceedings

List of other publications (books, book chapters, patents)

[Date]

17/11/'22

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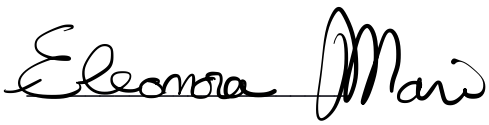

Supervisor signature