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EURO-PLANT-ACT

EXPERIMENTAL ACTIVITIES GUIDE

Cooperation to implement innovative methods for the assessment of medicinal plants with central roles in pharmaceutics, agriculture and nutrition

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Project title: Cooperation to Implement Innovative Methods for the Assessment of Medicinal Plants with Central Roles in Pharmaceutics, Agriculture and Nutrition

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Guide addressed to the specialist from different area such as agriculture, biotechnology, food industry, pharmacy, nutrition presents the methodologies related to: (i) selection, cultivation, harvesting and characterization of organic medicinal plants, (ii) assessment of chemical composition and analysis of pharmacological properties, (iii) establishment of the pharmacokinetic safety profile and methods of valorization of medicinal plants and (iv) products derived therefrom (extracts, essential oils).

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Chapter 1. Methods of research on antifungal activity of essential oils against phytopathogenic fungi (P2 - UNIOS)

1.1. Introduction

Essential oils (EOs) are known as a biological approach to pest control. This method involves using organisms and products derived from them to directly or indirectly manage pest populations. Similarly, plant extracts have demonstrated antifungal properties against a broad spectrum of harmful fungi. These natural extracts offer environmentally friendly alternatives for protecting agricultural crops from diseases [Wilson et al., 1997].

An alternative approach to chemical plant protection against pests involves the utilization of various plant compounds and extracts, such as essential oils and their components [Kishore et al., 2007]. It is noteworthy that over 1,300 plant species have been identified for their ability to synthesize compounds with antimicrobial properties [Wilkins and Board, 1989]. Essential oils, in particular, exhibit complex compositions, often comprised of numerous components. Typically, the oil's composition is dominated by three main components, which account for approximately 90% of its overall volume, while the remaining constituents are found in smaller proportions [Dorman and Deans, 2000]. Efforts are made for essential oils and plant extracts to become an alternative for controlling plant pathogens because they are a source of biologically active compounds, which can lead to the development of new, safer ways to protect plants from disease-causing agents [Al-Reza et al., 2010; Veloz-Garcia et al., 2010].

EOs appear as liquid, volatile, limpid and colored mixtures of several aromatic compounds. They are obtained from all plant parts, mainly from herbs and spices. About 3000 EOs are known, 300 of which are commercially important, mainly used in the flavors and fragrances market but today there are numerous studies on their use in plant protection. In vitro tests indicate that they are very effective against phytopathogenic fungi.

In order to determine the antifungal activity, different tests are used, of which the volatile and the contact tests are distinguished. The oils are used in different concentrations.

1.2. Volatile test to evaluate the influence of essential oils *in vitro*

The experimental protocol is conducted according to the disk diffusion method described by Edris and Farrag (2003) and Silva *et al.* (2019).

Step-by-step protocol

1) in the middle of the Petri dishes (V = 65 mL) containing 15 mL of PDA medium (potato-dextrose agar), a circular section of mycelium (4 mm) (Figure 1.1) grown on PDA (mycelium age of 7 days) is placed with a sterile needle.

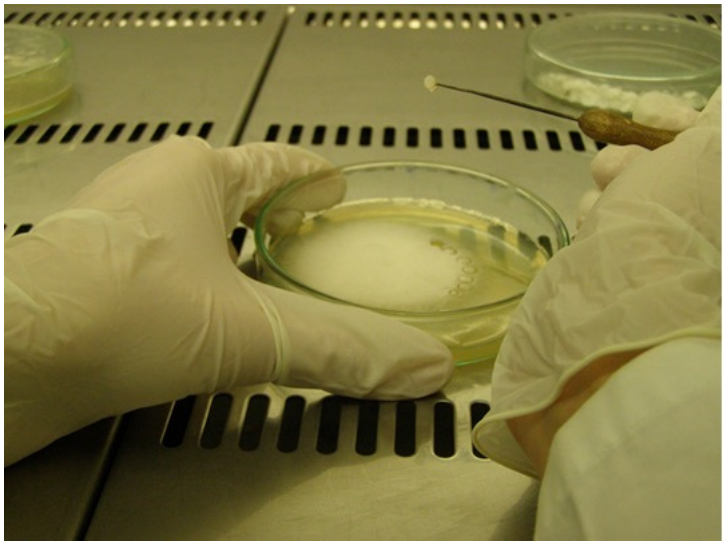

Figure 1.1. Circular section of mycelium.

2) a sterile filter paper with a diameter of 7 mm is placed in the lid of the Petri dish, and the essential oil is added (Figure 1.2) to it in order to obtain the experimentally predicted volume fraction of oil in the air.

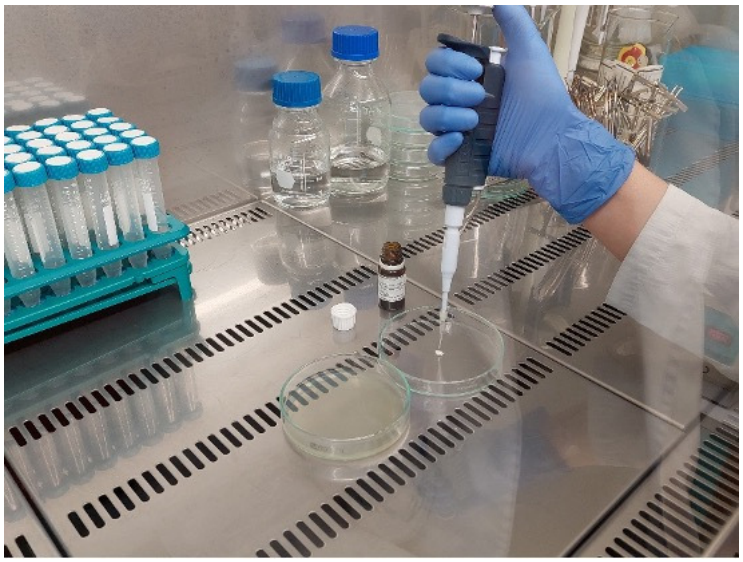

Figure 1.2. Application of essential oil.

3) the Petri dishes prepared in this way are closed with parafilm tape and stored in a thermostatic chamber at a temperature of 18-25°C (usually, the temperature depends on the type of fungal pathogen) and a light regime of 12 hours light / 12 hours dark (Figure 1.3).

Figure 1.3. Petri dishes stored in a climate chamber.

4) mycelium growth is measured after 72 and 168 hours (usually, depend on fungal species) (Figures 1.4 and 1.5). As control is used a filter paper soaked in an appropriate amount of sterile distilled water. After the last measurement of the diameter of the mycelium in the experiment with fungi which produce sclerotia e.g. *Sclerotina sclerotiorum*, the sclerotia are counted, their average mass determined, and after 4 months of storage at a temperature of 4 °C, their germination determined.

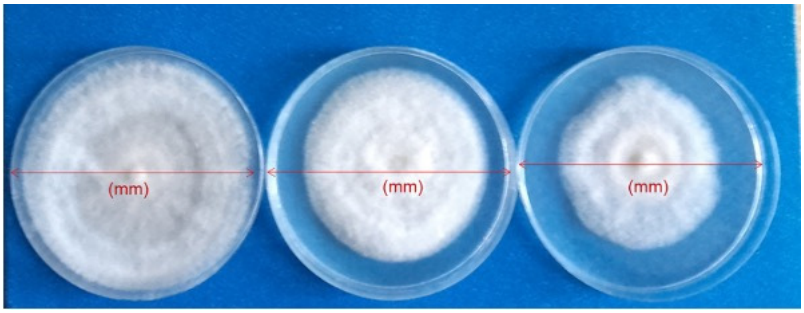

Figure 1.4. Mycelium growth is measured after 48, 72 and 168 hours.

Figure 1.5. The effect of different essential oils on the growth of the mycelium of the fungus *Neofusicoccum parvum.*

5) in the experiment with the fungus which produced conidia, after the last measurement of the mycelium diameter, the sporulation can be determined by preparing a suspension of conidia in 100 mL of sterile distilled water and using a hemocytometer to determine the concentration of conidia per ml of suspension for all variants of the experiment (Figure 1.6).

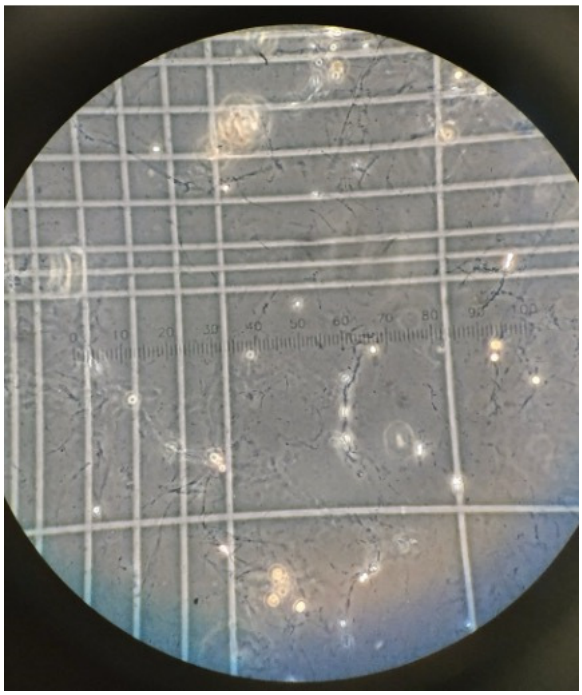

Figure 1.6. Spores counting using a hemocytometer.

6) in order to determine the germination of conidia from the prepared suspension for each variant of the experiment, 20 µL is taken and spread on the PDA substrate previously poured into Petri dishes with a diameter of 60 mm. Petri dishes are placed in a thermostatic chamber at a temperature of 18-25 °C and a light regime of 12 hours light / 12 hours darkness. After 24 hours, the number of germinated conidia is determined on a sample of 3 x 20 conidia. Conidia is considered germinated if the germ tube is equal to or greater than the length of the conidia (Figure 1.7).

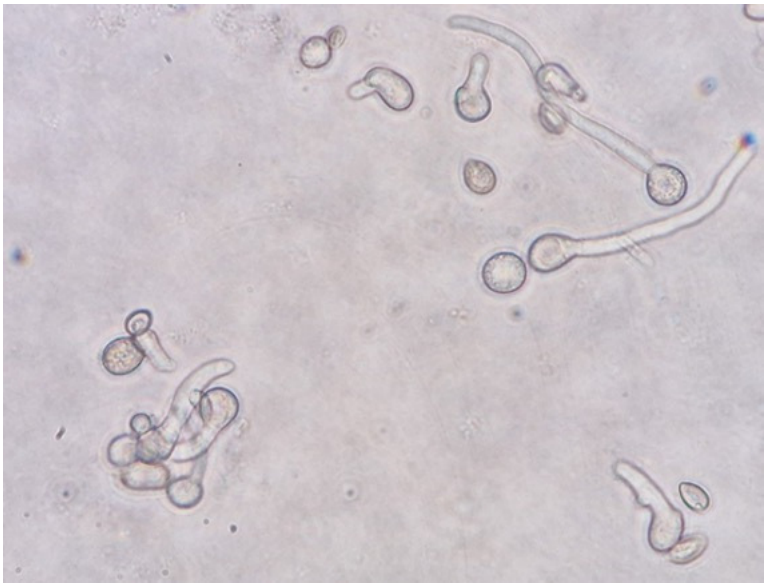

Figure 1.7. Germination of conidia.

7) for the fungi which form microsclerocia (e.g. *Sclerotium cepivora* and *Macrophomina phaseolina)*, after the last measurement of mycelial growth, a circular section with a diameter of 4 mm can be taken and the microsclerotia can be counted. The circular section taken to determine the number of microsclerotia must be taken at a distance of 10 mm from the edge of the disk placed in the middle of the Petri dish (Figure 1.8).

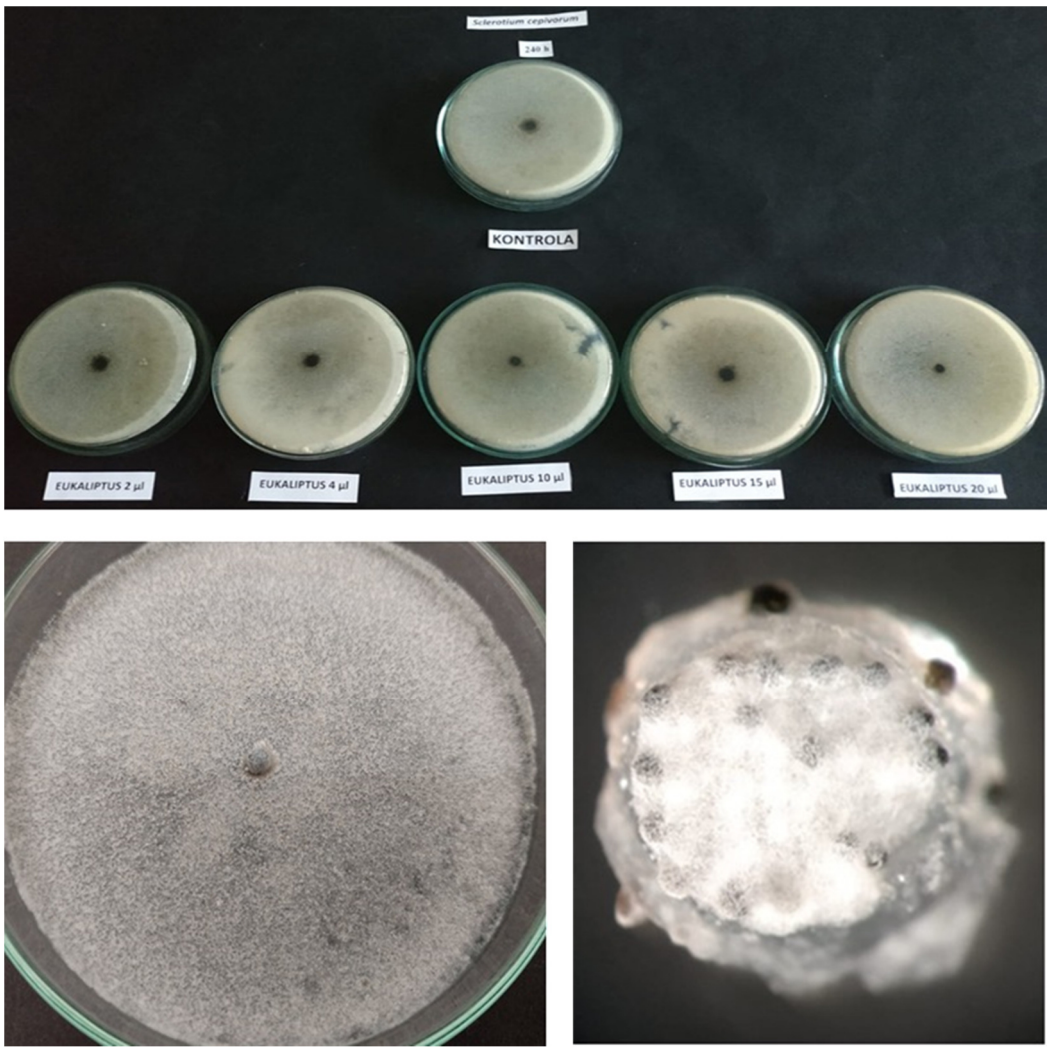

Figure 1.8. Influence of different essentials oils on *Sclerotium cepivora.*

1.3. Contact test for the assessment of essential oils effect *in vitro*

The procedure for the contact test is very similar to the volatile test with the difference that the oil is mixed into the PDA.

Step-by-step protocol

1) 10 mL of PDA is poured into Petri dishes with a diameter of 90 mm, into which the essential oil is previously mixed in order to obtain the experimentally predicted volume fraction of oil in the PDA substrate. The negative control is sterile distilled water.
2) the Petri dishes are closed with parafilm tape and stored in a thermostatic chamber at a temperature of 18-25°C, during which the growth of the mycelium of the fungus (mm) and the appearance of reproductive structure (e.g. *sclerotia* or *conidia*) are monitored.
3) mycelial growth is measured usually after 62 and 168 hours.

4) the mycelial growth inhibition is calculated using the formula of Wu *et al.* (2013):

I(%) = [(C-T)/(C-0.4)] × 100,

where:

I (%) - percent inhibition of mycelial growth on the tested compounds

C - mushroom growth diameter on pure PDA,

T - diameter of mushroom growth on treated PDA.

5) Based on the obtained data, EC50 values for each essential oil can be calculated. The EC50 values show which essential oils had the effect of reducing mycelial growth by 50% (Figure 1.9).

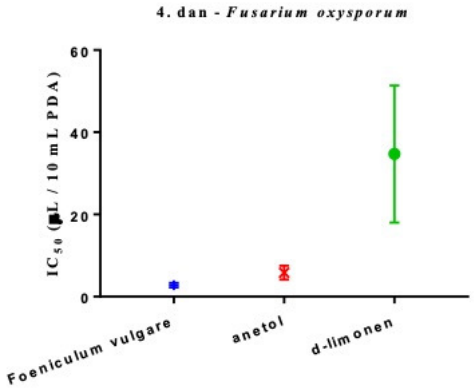

Figure 1.9. Comparison of EC50 of fennel essential oil (*Foeniculum vulgare*) and its components (anethole and d-limonene) for *Fusarium oxysporum* using analysis of variance (ANOVA) and Tukey test. Results are presented as 95% confidence limits for EC50.

Notes!!!

- In both variants of the experiment (contact and volatile) where the last measurement did not determine any mycelium growth, a circular section with mycelium is taken and transferred to a new Petri dish with PDA medium (without oil).
- The samples prepared in this way are kept for 48 hours (e.g. *Ssclerotinia sclerotiorum*, *Botrytis cinerea*, *Macrophomina phaseolina*) or 72 hours (e.g. *Sclerotium cepivora*) in a thermostat chamber at 25 °C.
- If the fungus has started to develop mycelium after incubation, the effect of the oil is fungistatic, and if there is no development of new mycelium, the effect of the oil is fungicidal.
- The lowest concentration (amount) of oil at which complete inhibition of mycelial growth was determined is designated as the minimum inhibitory concentration (MIC), and the lowest concentration (amount) of oil at which a fungicidal effect was determined is designated as the minimum fungicidal concentration (MFC).

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Chapter 2. Herbicidal effect of plant extracts and essential oils (P2 - UNIOS)

2.1. Introduction

Weed management in modern-day agriculture primarily relies on the use of synthetic herbicides due to their high efficiency, simple application and cost-effectiveness. Nevertheless, their improper and excessive application leads to occurrence of weed resistant populations, herbicide residues in food chain, and adverse effects on the environment and on human and animal health [Macías et al., 2003; Singh et al., 2003]. Additionally, frequent ban on active ingredients, lack of registered plant production products and restrictions in application of synthetic herbicides in organic agricultural systems as well as in protected areas requires a different approach in weed control.

Allelopathy is a biological phenomenon, defined as any direct or indirect, harmful or beneficial effect of one plant on the germination and growth of other through the production of allelochemicals that are released into the environment [Rice, 1984]. Allelopathy is considered as alternative tool for sustainable weed management. Allelopathic crops which possess strong herbicidal effect may be implemented as natural-based herbicides in form of plant extracts, powders and essential oils to reduce or inhibit germination and growth of weeds [Singh et al., 2003; Ravlić et al., 2016].

Medicinal plants from different botanical families, both cultivated and wild, represent a great source of bioactive compounds for the development of new, safe and biodegradable bioherbicides [Bhowmik and Inderjit, 2003; Fujii et al., 2003; Amini et al., 2016]. The bioactive plant secondary metabolites (allelochemicals) are present in various concentrations in all plants and plant parts [Alam et al., 2001]. Phytotoxic potential of plant extracts and essential oils depends on multiple factors, such as geographical origin, growing conditions, seasonal variation and plant growth stage as well as abiotic and biotic environmental factors which may increase the production of secondary metabolites in plants and enhance their inhibitory effect [Safdar et al., 2014; Sarić-Krsmanović et al., 2019; Medina-Villar et al., 2020; Appiah et al., 2022; Ravlić et al., 2022]. The activity is influenced by concentration, extraction method and whether plant material is fresh or dry, but also depends greatly on the target species as they differ in their sensitivity [Fujii et al., 2003; Norsworthy, 2003; Souza Filho et al., 2009; Ravlić et al., 2016; Ravlić et al., 2022].

Various tests and techniques are used for laboratory screening of plants in order to evaluate their herbicidal potential.

2.2. Herbicidal effect of plant extracts

For the preparation of plant extracts the first step is the collection of plant material. Proper botanical identification of plant species is crucial, and all collected plants are identified according to their morphological featured using dichotomous keys and atlases. Plant material without visible symptoms of diseases and physical damages is collected on different locations under various environmental factors and in different developmental stage of the plant.

Figure 2.1. Collection of plant material (*Solidago gigantea*) in flowering stage.

If various plant parts are tested, before the drying plant parts are separated (Figure 2.2).

Figure 2.2. Separation of fresh plant material to plant parts (*Oenothera biennis*).

Fresh biomass may be used for preparation of the extracts, or it may be dried. The material is shade dried for 24 to 72 h, and after that dried in oven up from 40 to 50 °C. Dry biomass is cut into small pieces, ground with electronic grinder into fine powder (Figure 2.3) and stored in paper bags in dry and cool place.

Figure 2.3. Plant material aspect after grind procedure.

Step-by-step protocol to prepare a water plant extract

Water extracts are prepared following the procedure of Norsworthy (2003) with some modifications.

1) fresh or dry plant biomass in the amount of 10 g is extracted in 100 mL of distilled water at room temperature 22 (±2) °C for 24 h (Figure 2.4). Alternatively, plant material may be extracted with hot water.

Figure 2.4. Preparation of water extracts.

2) the mixture is after that filtered through muslin cloth to remove debris and after that through filter paper to obtain 10% water extract. Extracts in various concentrations are obtained with further dilutions using distilled water. Concentrations used range from 1% to 10 %.

Step-by-step protocol to prepare an ethanolic plant extract

1) ethanolic extracts are obtained by maceration of plant powder in ethanol from 24 to 72 hours (Figure 2.5).

Figure 2.5. Preparation of ethanolic extracts – maceration of plant material in ethanol.

2) after the extraction, the extract is filtered, and the solvent is removed under pressure at lower temperatures to obtain crude ethanol extract (Figure 2.6). Different concentrations are further obtained by suspending crude extract in distilled water and Tween as dispersant [Silva et al., 2018]. Concentrations used range from 0.01% to 5 %.

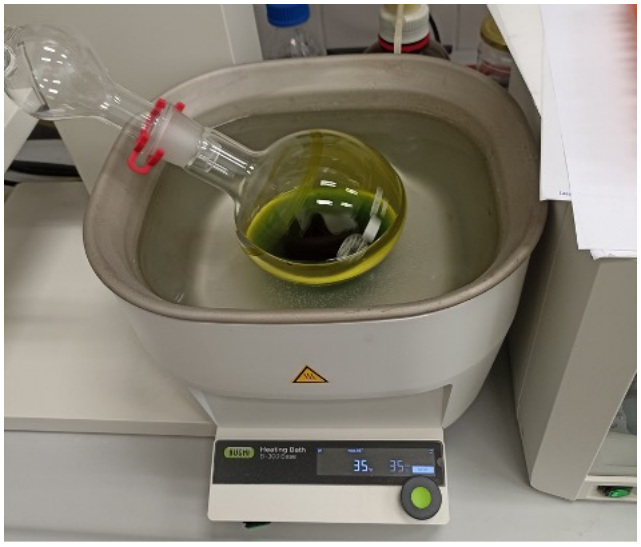

Figure 2.6. Preparation of ethanolic extracts – obtaining crude extract.

Step-by-step protocol to prepare a plant extract from weed seeds

1) seeds of weed species used in bioassay as test species are cleaned and dried at room temperature (Figure 2.7).

Figure 2.7. Cleaning and separating weed seeds prior to the experiment.

2) prior to the experiment, weed seed germination is assessed to determine whether there is a satisfactory germination percentage. Lettuce (Figure 2.8) or radish seeds are used when screening a large number of extracts, and extracts with the highest effect are further assessed on weed species.

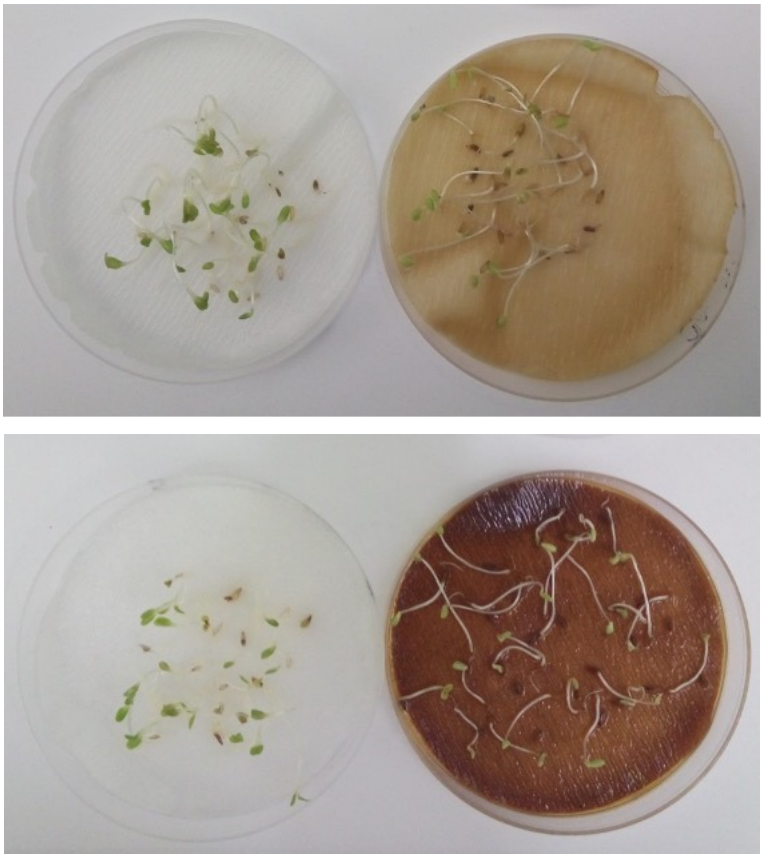

Figure 2.8. Screening of *Oenothera biennis* extracts from various plant parts (stem and leaf) using lettuce.

3) the effect of extracts is evaluated in Petri dish bioassay under controlled laboratory conditions. Weed seeds are placed in sterilized Petri dishes lined with filter paper. The filter paper is moistened with 4 to 6 mL of extract (depending on the test species) in each concentration, while distilled water is used in control (Figure 2.9, 2.10, 2.11). The seeds are incubated at alternating or constant temperatures and light/dark conditions optimal for germination and growth of each weed species.

Figure 2.9. Herbicidal effect of *Salvia pratensis* water extract on *Abutilon theophrasti.*

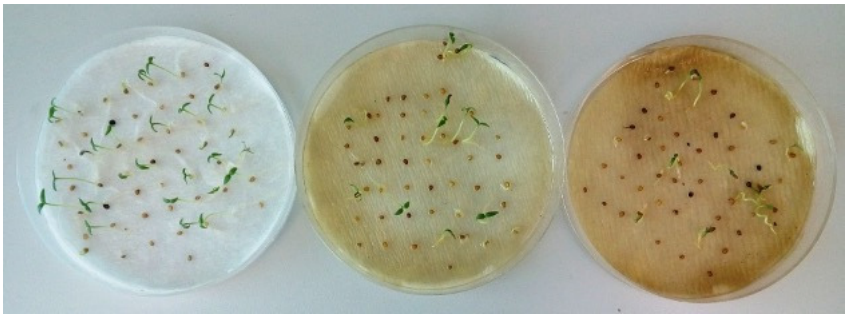

Figure 2.10. Herbicidal effect of various concentrations of *Chelidonium majus* extracts on *Solanum nigrum.*

Figure 2. 11. Herbicidal effect of various concentrations of basil water extracts on *Solanum nigrum.*

2.3. Herbicidal effect of essential oils

Herbicidal effect of essential oils on germination and growth of weed species is evaluated as volatile [Souza Filho et al., 2009] and direct contact effect [Sarić-Krsmanović et al., 2019].

Step-by-step protocol

1) essential oils are purchased or obtained from collected and shade dried plant material by hydro distillation for 2.5 h in a Clevenger type apparatus.
2) a solution of essential oils is prepared in different concentrations (from 0.1 to 1%) with distilled water and emulsified with Tween.
3) in the experiment with the volatile effect, the seeds of the weed species are placed in Petri dishes on filter paper moistened with water, while a solution of essential oil is applied to the filter paper attached on the upper side of the lid. In control, only distilled water is used.
4) in the experiment with direct contact, the seeds of the test species are placed in Petri dishes on filter paper moistened with a solution of essential oils. In the control treatment, the seeds of the test species are germinated without essential oils using only distilled water.

5) in both methods, the seeds are incubated at alternating or constant temperatures and light/dark conditions optimal for germination and growth of each weed species.
6) assessment of herbicidal potential of plant extracts and essential oils

For all the aforementioned methods, at the end of the incubation period, to determine herbicidal potential the following parameters are measured: germination, root and shoot length of seedlings and fresh and dry weight of seedlings.

- Germination percentage is calculated for each replication using the formula:
  - **G (germination) = (germinated seeds/total seeds) × 100.**
- The incubation period is different for each test species and is on average from 8 to 12 days.
- All collected data are analyzed statistically with ANOVA and the differences between treatment means of measured parameters for each weed specie are tested with the LSD test at probability level of 0.05.
- Plant extracts and essential oils are ranked according to their inhibition potential i.e. percent inhibition compared to the control treatment. The most promising materials are further tested in greenhouse experiments.

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Chapter 3. The use of medicinal plants as value-added ingredients in the industry of functional bakery and pastry products (P1 - USVT)

3.1. Introduction

Aromatic and medicinal plants include a very large number of plants that belong to different botanical families and have an annual, biennial or multi-year lifespan.

Medicinal plants:

- have been used for thousands of years in cuisine and are inexpensive, readily available, and healthy
- in meals are seen as an alternative to using synthetic chemicals
- are utilized in several medicinal formulations to both cure and prevent aliments
- are utilized in the food sector as natural antioxidants to prevent oxidation of lipids
- enhance the nutritional value of food, and provide taste to a variety of drinks

Medicinal plants can be added as such or in the form of extracts, essential oils in bakery products with the following purpose:

i) to improve the sensory properties of the products

ii) for an antioxidant effect determined by the polyphenolic active principles

iii) for an antimicrobial role due to the biologically active antifungal and antibacterial compounds found in medicinal plants

Plants contain phenolic compounds, glucosinolates, cyanogenic glycosides, oxylipins, and alkaloid.

Foods rich in secondary metabolites and bioactive compounds like flavonoids, alkaloids, and others are recommended for consumption by dietary guidelines to prevent stress, hypertension and cardiovascular diseases [Rivera et al., 2010].

Medicinal plants value from Lamiaceae family lays in the production of a wide range of secondary metabolites with potent antibacterial, antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer activities [Carović-Stanko et al., 2016].

In the following sections will be presented several examples of medicinal plants used in the bakery products for improving the sensory properties, but also for the antioxidant and antifungal effects.

3.2. Medicinal plants used to improve the taste, color and aroma of bakery products

Medicinal plants added in different forms of dough preparation improve the sensory properties, having positive or negative effects on its rheological properties (*Lavandula angustifolia, Cichorium intybus)* (Figure 3.1).

Figure 3.1. *Cichorium intybus* and *Lavandula angustifolia* – medicinal plants used in the bakery industry.

As a result of the wide range of use, they are used all year round, fresh or dry.

The tendency to use natural dyes to the detriment of artificial, synthetic ones is increasingly prevalent among consumers, and some medicinal plants, through their colour characteristics, can represent healthy alternatives to synthetic chemical dyes in the food industry, as follows:

Mint - a refreshing herb that can be used in cakes, cookies, creams, frostings and chocolates. Its cooling and slightly sweet flavour is perfect for desserts. Peppermint & spearmint are the most common varieties used in confectionery. It pairs well with chocolate and fruits like berries and citrus [Sik et al., 2023].

Basil - a herb with sweet, slightly peppery flavour that can be used in sweet baked goods like cakes, cupcakes, and ice cream, as well as in savory baked goods like focaccia bread or pizza. It pairs well with fruits like strawberries, peaches, and raspberries [Calderón Bravo et al., 2021].

Lavender **-** a fragrant herb that is often used in desserts like cookies, cakes, and ice cream. Its slightly sweet flavour adds a unique purple colour to baked goods. It pairs well with citrus flavors and adds a unique aroma to baked goods [Valková et al., 2021].

Coriander **-** a popular plant used in baking to add a fresh, citrusy flavor to foods. Dried coriander can also be added to the doughs, breadcrumbs or used to season savory pastries. Pairs well with chocolate and can be used to add a unique flavor to pastries, breads, crackers & biscuits [Sriti et al., 2019].

***Thyme* -** a herb that is often used in savory dishes like bread, pizza & focaccia, but it can also be used in sweet baked goods like shortbread cookies. Its earthy and slightly minty flavor is a great addition to buttery baked goods.

Sage - it has a slightly bitter, earthy flavor and is often used in savory baked goods like bread, biscuits & toppings. It can also be used in sweet baked goods like shortbread and scones [Bassiouny et al., 1990].

***Rosemary* -** a woody, aromatic herb that can be used in sweet baked goods like bread and biscuits. It can also add a unique flavor to baked goods, especially bread, quiches & tarts. Simply chop up some fresh rosemary leaves and mix them into your bread or pastry dough before baking [Valková et al., 2021].

***Oregano* -** a flavorful herb that works well in savory baked goods like pizza crust and focaccia. It can be chopped finely and mixed into the dough or used as a topping with salt and olive oil.

BIO FUNCTIONAL TPA MUFFINS based on whole wheat flour, lavender flowers and chicory root with high nutritional properties, low glycemic index, high biological potential and economically efficient, whose manufacturing technology can be implemented in bakery units (Figure 3.2).

Figure 3.2. BIO FUNCTIONAL TPA MUFFINS.

3.3. Medicinal plants as antioxidants agents in bakery products

The addition of 5% extracts of *Camellia sinensis, Asparagus racemosus* and *Curcuma longa* (Figure 3.3) increased the antioxidant capacity of the bread without altering the sensory properties. Antioxidant properties of green tea powder substituting some flour in sponge cakes was also reported by Sik *et al.* (2023).

Figure 3.3. *Camellia sinensis, Asparagus racemosus* and *Curcuma longa* – medicinal plants with antioxidant properties.

The gallic and tannic acids influenced the properties of gluten and produced stiffer and thicker films with lower vapor permeability.

The following sections comprise different bakery products recipes with medicinal plants added, offering a detailed description of the ingredients used and the appearance of the final product.

Bread with *Curcuma longa* L. (Figure 3.4)

Step-by-step protocol - Ingredients

- white bread flour
- salt
- turmeric powder
- fast-action yeast
- olive oil
- water.

Figure 3.4. Aspect of the bread with turmeric.

Notes!!!

- Turmeric (*Curcuma longa* L.) powder was used to substitute 0%, 2%, 4%, 6% and 8% of wheat flour for making turmeric wheat breads.
- A daily intake of 50 g or two slices of turmeric bread having 4% wheat flour substitution with turmeric powder can deliver approximately 4.6 mg of curcumin and 40.12 mg GAE of total phenolic compounds which can render additional health benefits to human body [Lim et al., 2011].
- The data on the exact recommended dosage of these phytochemicals is not available, however, different *in vitro* and *in vivo* studies have been carried out to analyze their biological effects.

Dietary Cake with Seabuckthorn (Hippophae rhamnoides) jelly (Figure 3.5)

Step-by-step protocol - Ingredients

- Whole wheat flour
- Milk
- Yeast
- Saccharine
- Butter
- Eggs
- Seabuckthorn (*Hippophae rhamnoides*) jelly.

Figure 3.5. Dietary Cake with Seabuckthorn jelly.

Sage sourdough bread (Figure 3.6)

Step-by-step protocol - Ingredients

- Water - 697 g (73%)
- Leaven - 191 g (20%)
- Total flour - 955 g (100%)
- Bread flour - 859 g (90%)
- Whole wheat flour - 95 g (10%)
- Roasted garlic - 29 g (3%)
- Sage - 10 g (1%)
- Salt - 19 g (2%)
- Total 1900 g (199%) [https://vituperio.com/roasted-garlic-sage-sourdough-bread/].

Figure 3.6. Sage sourdough bread.

Bread wheat, rye and chicory (Figure 3.7)

Step-by-step protocol - Ingredients

- Flour - 275 g
- Rye flour - 275 g
- Salt - 1.5 tsp.
- Sugar - 1.5 tbsp
- Yeast - 1.5 tsp.
- Vinegar - 1/2 tsp.
- Chicory - 1.5 tbsp
- Cumin - 2 tsp.
- Water - 400...420 ml
- Vegetable oil - 3 tbsp.

Figure 3.7. Bread wheat, rye and chicory.

Dandelion Bread (Figure 3.8)

Step-by-step protocol - Ingredients

- Dandelion Petals - ⅓ Cup
- Milk - 1 Cup
- Dry Active Yeast - 1 Tablespoon
- Warm Water - ½ Cup
- Honey - ¼ Cup
- Salt - 2 teaspoons
- Butter, softened - ½ Cup
- Eggs - 2
- Wheat Flour - 5 cups.

Figure 3.8. Dandelion Bread.

Bread with oregano (Figure 3.9)

Step-by-step protocol - Ingredients

- Wheat flour
- Active dry yeast or fresh yeast
- Sugar
- Extra virgin olive oil
- Honey
- Sea salt
- Fresh oregano [https://thegreekfoodie.com/greek-bread-with-oregano-and-olive-oil/].

Figure 3.9. Bread with oregano.

Notes!!!

- ❖ Data variation in parameters of bread making was observed when breads were prepared by incorporation of dried oregano at 1, 2, 3 and 4% level in flour [Dhillon and Kaur Amarjeet, 2013]
- ❖ Specific volume (4.72 cc/g) was best at 1% level and decreased to 4.22 cc/g at 4% level of oregano in the blend.
- ❖ Oregano also increased the loaf weight from 145 g at 1 % level to 149 g at 4 % level of oregano in the bread.
- ❖ At 2% level of oregano, water absorption was 72.24 % and it increased to 75 % at 4 % level.
- ❖ This showed that increased level of oregano had a detrimental effect on the specific volume of bread.

Buns with thyme (*Satureja hortensis* L.) (Figure 3.10)

Step-by-step protocol - Ingredients

- whole wheat flour - 4 cups (512 g)
- salt - 2 teaspoons (10 g)
- sugar - 2 teaspoons (8 g)
- dry yeast - 2 teaspoons (8 g)
- minced fresh thyme leaves - 2 tablespoons
- water - 2 cups (454 g) [https://www.sidechef.com/recipes/8333/no_knead_thyme_dinner_rolls/].

Figure 3.10. Buns with thyme.

Notes!!!

- The addition of aromatic thyme (dry matter as well as essential oil) in the bread recipe resulted in antifungal activity against both *Penicillium* and *Aspergillus*.
- The addition of thyme showed no fungal or bacterial spoilage for four days, indicating its potential as a bread preservative [Skendi et al., 2020].
- By incorporation of the aromatic plant in their dry form *in vitro*, can lead to antifungal effect in bakery products [Skendi et al., 2020].

Rosemary rolls

Step-by-step protocol – Ingredients (Figure 3.11)

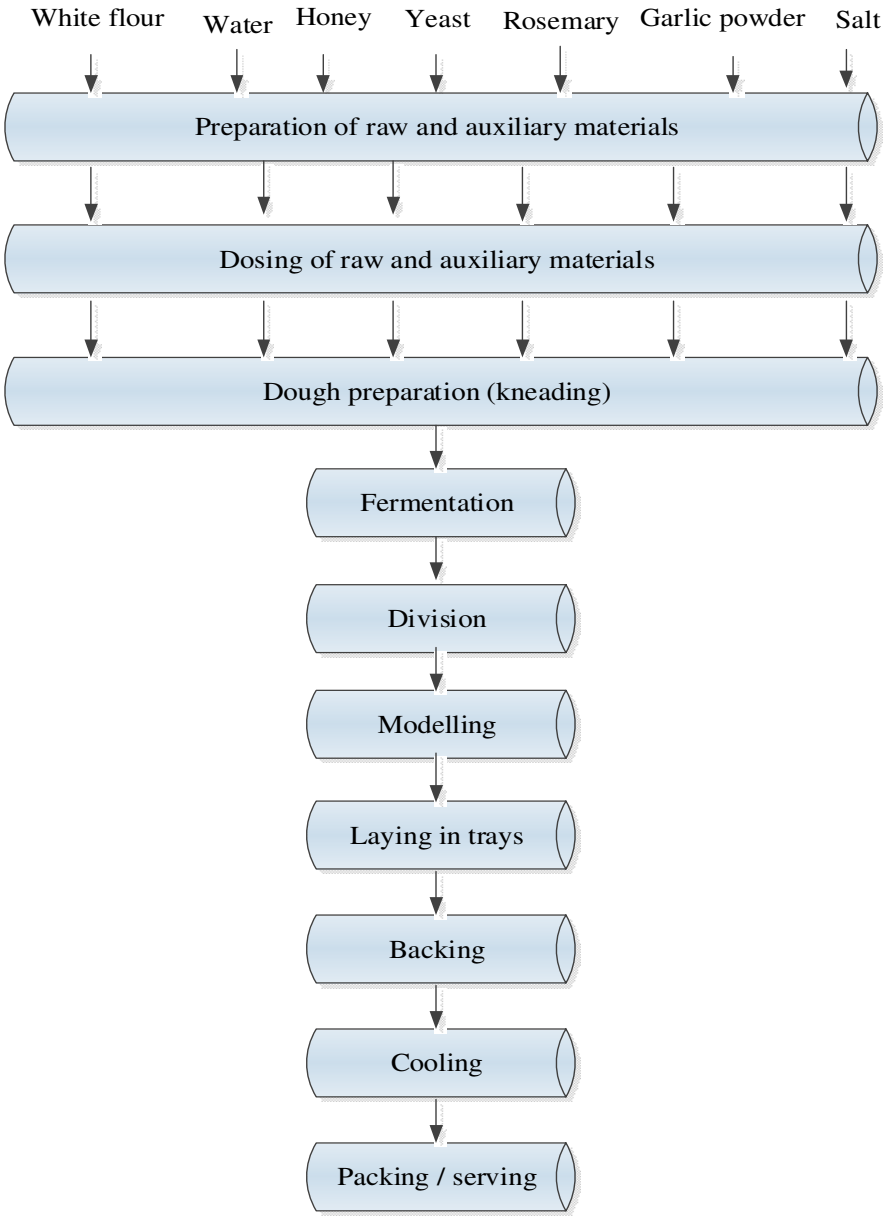

Figure 3.11. Schematic protocol of rosemary rolls preparation [https://www.gimmesomeoven.com/one-hour-rosemary-garlic-dinner-rolls/].

Figure 3.12. Rosemary rolls.

Mint muffins (Figure 3.13)

Step-by-step protocol – Ingredients

- spinach, fresh - 2 cups
- coconut oil, melted - 1/2 cup
- apple sauce - 1 cup
- mint extract - 1 tsp
- eggs (or flax eggs) - 2
- oat flour - 1 3/4 cup
- baking powder - 1 1/2 tsp
- baking soda - 1/2 tsp
- salt - 1/2 tsp
- chocolate chips - 1 cup [https://www.milehighmitts.com/mint-chocolate-chip-oat-muffins-naturally-colored-gluten-free-dairy-free-sugar-free-option-vegan-option/].

Figure 3.13. Mint muffins.

Lavender sweet Bread (Figure 3.14)

Step-by-step protocol – Ingredients

- milk - 3/4 cup
- dried lavender flowers chopped - 2 Tbsp.
- wheat flour - 2 cups
- baking powder - 1 1/2 tsp.
- salt - 1/4 tsp.
- butter softened - 6 Tbsp.
- sugar - 1 cup
- eggs - 2
- Simple Glaze
- Powdered sugar - 1 cup
- Vanilla extract - 1/2 tsp
- lemon juice - 1/2 spoon
- milk - 1 1/2 tbsp [https://www.aliikulalavender.com/lavender-tea-bread/].

Figure 3.14. Lavender sweet Bread.

Biscuits with cloves

Step-by-step protocol – Ingredients (Figure 3.15)

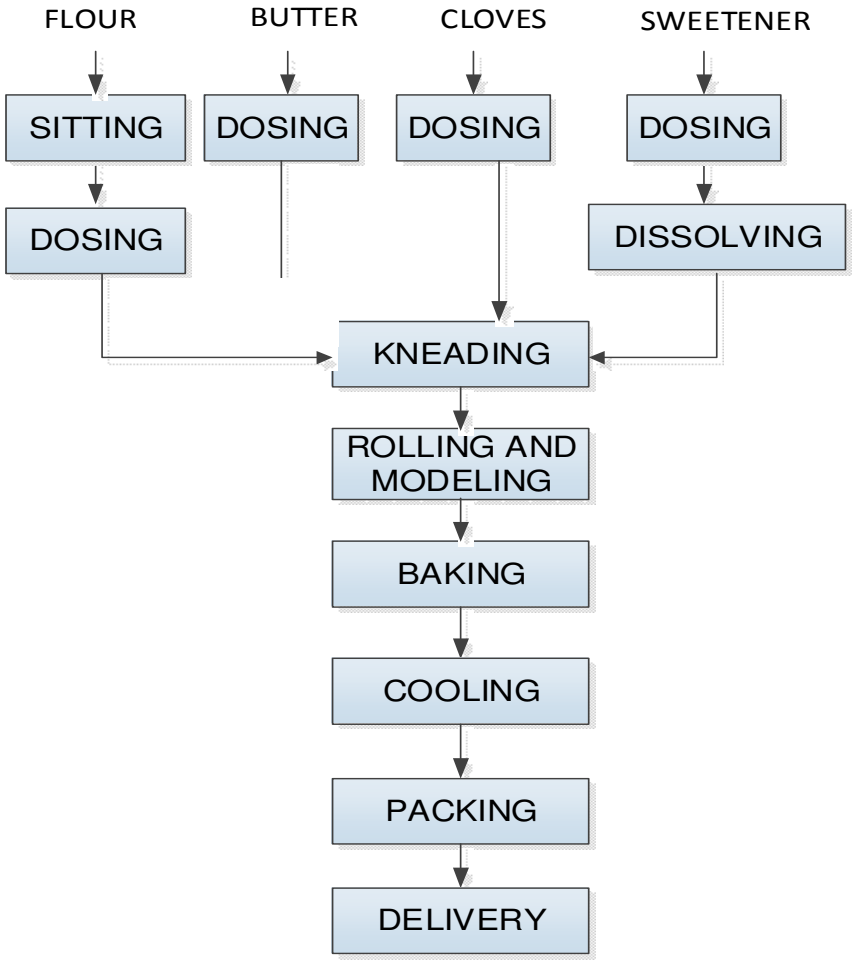

Figure 3.15. Schematic protocol of biscuits with cloves preparation [https://www.bbcgoodfood.com/recipes/clove-sugar-cookies].

Figure 3.16. Biscuits with cloves.

3.4. Medicinal plants as antimicrobial agents in bakery products

Medicinal plants are used in the bakery industry as a spice, to improve the taste and aroma properties of the products, but also as an antimicrobial agent against harmful microorganisms in bakery products. Bread and bakery products are prone to mold spoilage (after a few days of storage) and to avoid this phenomenon food preservatives or modified atmosphere packaging are needed.

The most frequent mold species that occur in bakery products are: *Penicillium, Cladosporium, Aspergillus, Neurospora, and Mucor, Penicillium* being identified as the most common source of bread spoilage.

Several types of essential oils especially those belonging to *Lamiaceae* family and Umbellifere, are mentioned as antimicrobial agents in bakery industry, resulting in a product with extended shelf-life and enhanced safety.

The shelf life of bakery products stored at room temperature is limited to 3-4 days and is influenced by microbial spoilage due to molds, mainly *Penicillium sp*. and other fungi *(Aspergillus, Monilia, Mucor, Endomyces, Cladosporium, Fusarium or Rhizopus*). Due to its chemical composition, *Origanum vulgare* helps to extend the shelf life, nutritional qualities of many products, such as bread and bakery products, cereals.

Two antifungal elements present in essential oils, carvacrol and eugenol, might be considered potent antifungal agents. Essential oils have antifungal properties. Thyme, cinnamon, and clove oils were known to inhibit spoilage fungus, whereas orange, sage, and rosemary oils had only a negligible effect (Figure 3.17).

Figure 3.17. Thyme, cinnamon, and clove used in bakery products.

The risk for human health is given by the fact that contamination with fungi causes the production of mycotoxins with hepatotoxic, carcinogenic effects.

Mycotoxins are fungal secondary metabolites that induce acute and chronic toxic effects in humans and animals. Concurrent contamination of cereal-based products by multiple mycotoxins has been increasingly reported, including in foods commonly consumed by children.

The most important groups of mycotoxins are:

- Aflatoxins (AFLA)
- Ochratoxin A (OTA)
- Trichothecenes (deoxynivalenol DON, nivalenol)
- Zearalenone (ZEA)
- Fumonisins (FUMO)

Mycotoxins can be found in bread, breakfast cereals, pastry products.

- Processing can only reduce the number of mycotoxins, not their total elimination
- Some traditionally used plants have been shown to exhibit fungi-toxic properties

- Medicinal plants as: *Occimum gratissimum, Cymbopogon citratus, Xylopia aethiopica, Monodera myristica, Sizygium aromaticum, Cinnamomum verum* and *Piper nigrum* are effective in inhibiting the formation of non-sorbic acid, a precursor in the aflatoxin synthesis process
- Natural preparations of emulsions based on essential oils were used as antifungal agents in bakery.
- Further studies are necessary for the development of common strategies for the control and prevention of fungal and mycotoxin development in bakery and pastry products.

3.5. The activity of medicinal plants against pathogenic bacteria prevalent in the food industry

The prevention of food spoilage and the emergence of pathogens that cause food poisoning is usually achieved through the use of chemical additives that have a number of negative effects, including: the human health hazards of chemical compounds, the occurrence of chemical residues in the food and feed chains and acquisition of microbial resistance to the chemicals used.

As a result of these worries, it's more important than ever to find a natural, healthy, and safe alternative to preservatives. For some time, plant extracts have been used to prevent food poisoning and preserve food.

Some of the challenges facing bread manufacturers include extending shelf life by reducing rancidity and decreasing microbial spoilage, as these changes lead to spoilage of bread and other bakery products. To overcome these difficulties and increase shelf life, commercially available antioxidants and chemical preservatives such mold inhibitors are used.

A variety of essential oils can stop the development of dangerous germs in bread items, extending their shelf lives and improving their safety, as thyme, cinnamon, oregano, lemongrass, etc.

The majority of edible medicinal plant parts include traces of hydroxybenzoic and hydroxycinnamic acids, two types of phenolic acids that act as plant defences.

Due to their potential as natural food preservatives, flavoring agents, and decontaminating agents, plant essential oils are attracting a lot of interest in the food industry being also Generally Recognized as Safe – GRAS (Figure 3.18).

Figure 3.18. Hydroxybenzoic and hydroxycinnamic acids.

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Chapter 4. Current trends and methods for *in vitro* evaluation of biological activity (CO - UMFVBT)

4.1. Introduction

In vitro cell-based assays represent the first step in preclinical research being applied as a key tool in the screening of novel drug candidates for biological activity. Moreover, the *in vitro* studies offer relevant data concerning their potential mechanism of action (signaling pathways) and their toxicity. Other advantages related to the use of cell lines as *in vitro* models were stated, as follows: (i) involve time- and cost-effective methods, (ii) are "controllable" models (mainly in the case of monolayer cultures), and (iii) multiple standardized techniques are available in the literature [Capula et al., 2019].

Besides the benefits of using *in vitro* models and cell-based assays, there were identified several factors that might impact the results, leading to unreliable data, thus some **basics principles must be considered** when designing and performing *in vitro* experiments [Capula et al., 2019], as:

1) **Selection of an appropriate cell line**

Before designing an *in vitro* experiment, it is mandatory to select the type of cell lines considered eligible models for the intended research aim.

Cell lines can be obtained: (i) *in-house* (require authentication, detailed clinical information and explicit cell designation), (ii) *by acquisition from other laboratories* (require quarantine, authentication and characterization to confirm their specific features) or (iii) by *purchase from approved cell banks* (include authentication data, results of the tests for microorganisms' contamination, and information about cell line storage and subculture method) [Geraghty et al., 2014].

Critical!!! All cell lines used in the experiments must be authenticated and contaminant free!!!

Recognized cell culture collections/cell banks: American Type Culture Collection (ATCC) (www.atcc.org); CellBank Australia (www.cellbankaustralia.com); Coriell Cell Repository (http://ccr.coriell.org); Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (www.dsmz.de); European Collection of Animal Cell Cultures (ECACC) (www.phe-culturecollections.org.uk/); National Institutes of Biomedical Innovation, Health and Nutrition, Japanese Collection of Research Bioresources (JCRB) (https://cellbank.nibiohn.go.jp/english); NIH Stem Cell Unit (https://stemcells.nih.gov/NIH-Stem-Cell-Research); RIKEN Gene Bank

(https://web.brc.riken.jp/en/), UK Stem Cell Bank (UKSCB) (https://nibsc.org/ukstemcellbank); WiCell (www.wicell.org) [Geraghty et al., 2014].

Types of cell cultures and cell lines:

Cell culture is a term used to describe the maintenance or cultivation of cells *in vitro*, including the culture of single cells. Cell cultures can be:

- *primary cultures* – are obtained directly from excised tissue and cultures either as an explant culture or after cleavage into a single cell suspension via enzyme digestion
- *continuous cultures* – are formed of a single cell type that can be sequentially expanded in culture either for a limited number of cell divisions or indefinitely.

A cell line originates from a primary culture and is the result of the first successful subculture. Cell lines can be classified as:

- *primary cells* - are obtained directly from human tissue and are known as "finite" since their proliferation stops after a limited number of cell divisions
- *transformed cells* – can be developed either naturally or by genetic manipulation and are also known as immortalized cell lines
- *self-renewing cells* – have the ability to differentiate into a diversity of other cell types; examples of this kind of cells: embryonic stem cells, neural and intestinal stem cells, induced pluripotent stem cells [Segeritz and Vallier, 2017; Coecke et al., 2005].

2) **Selection of an appropriate solvent for the test compounds:**
   - an improper solvent might affect drug stability leading to incorrect concentration determination
   - the chosen solvent must not be toxic to cells at the final concentration used [Capula et al., 2019]

3) **Drug concentration of the test compounds**
   - a broad range of concentrations should be tested when is performed a screening of novel compounds, followed by a narrow range after it was identified the concentration that triggered a response [Capula et al., 2019].

4) **Drug exposure/treatment duration**
   - the duration of a treatment *in vitro* conditions should be correspondent with the *in vivo* situation
   - it should also be taken into account the metabolism of the tested drug [Capula et al., 2019]

5) **Seeding density and assay timing –** is cell line-dependent [Capula et al., 2019].

Before starting to work with cell lines, it is recommended to know the following fundamental aspects:

- cell lines embody cells that have the capacity to proliferate for extended periods *in vitro* and can be kept by serial subculture (passaging)
- *finite cell lines* can be subcultured multiple times, but after a number of passages (60-70 cell population doublings) they reach senescence state and cease to replicate; can be maintained well-characterized but changes may occur in their morphology as they approach senescence
- *continuous cell lines* can be subcultured indefinitely; are derived from tumors or normal embryonic tissues; show a high stability over long-term passages *in vitro*, still they may undergo considerable and irreversible changes [Coecke et al., 2005].

Note!!!It is recommended for all types of cell lines to have cryopreserved stocks of early passage cells!!!

- the growth phases of normal cells are:
  - ***lag phase*** – is the phase following seeding when the cells do not divide, just adapt to the culture conditions; is dependent on the growth phase of the cell line at the time of passaging and on the seeding density
  - ***logarithmic (log) or exponential growth phase*** – it is characterized by an exponential growth of cells that keeps until the entire plate is occupied or the cell concentration surpasses the growth medium's capacity; the cell population is considered to be the most viable at this time; is the appropriate phase for assessing cellular functions and to determine the population doubling time; it is also recommended to perform the subculturing procedure (performing the passaging too late might lead to cells' overcrowding, apoptosis and senescence)
  - ***plateau or stationary phase*** – the cell population becomes confluent and the proliferation slows down; it is the phase when the cells are most susceptible to injury
  - ***decline phase*** – is characterized by increased cell death and reduction of viable cell number due to the natural progression of the cellular cycle.
- the *in vitro* age of a cell culture can be defined by:
  - **passage number** – the number of times a cell line was subcultured/passaged

- **population doubling time (DT)** – the time needed by a culture to double in number

$$DT=T\ ln2/ln(Xe/Xb),$$

Where T is the incubation time in any units

Xb is the cell number at the beginning of the incubation time

Xe is the cell number at the end of the incubation time [ATCC - Animal Cell Culture Guide].

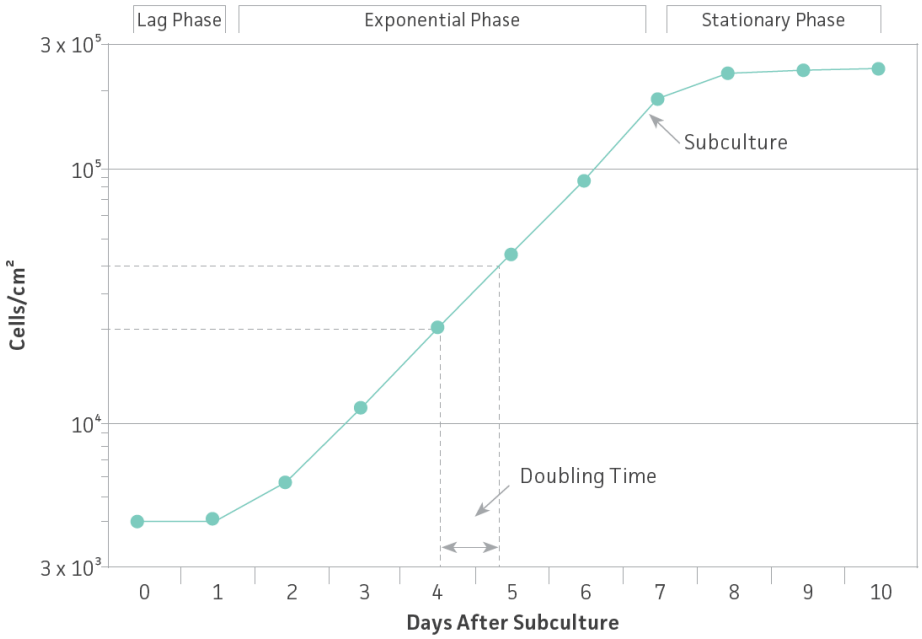

Figure 4.1. Example of a growth curve for cells in culture. It is recommended to do the subculturing procedure when the cells are in the log (exponential) phase of growth [ATCC - Animal Cell Culture Guide].

In the following sections will be provided several guidelines regarding the steps involved in the preparation of a cell line for 2D cell-based assays from the moment of receiving the cryovial from the cell bank until the addition of test compounds. **The protocol refers to mammalian adherent cells cultured in monolayer.**

4.2. Preparation of a cell line for cell based-assays

4.2.1. General rules for safety in cell culture lab

- *The work in the lab should be performed only by trained personnel*
- *The personnel should always wear personal protective equipment (lab coats and gowns, caps, shoe covers, gloves, safety glasses) upon entering into the lab and removing it when leaving*

- *The exposure of skin in open-toed shoes, short trousers, and skirts is not recommended.*
- *Consumption of food, drink, the storage of groceries, smoking, applying cosmetics, or handling contact lenses is forbidden in the cell culture lab.*
- *Cell phones should not be used while working in the cell culture lab.*
- *Loose items of clothing (e.g., scarves, dangling necklaces) should be removed before starting the work and the hair should be tied back.*
- *All work surfaces must be decontaminated before and after experiments, and immediately after any spill or splash of potentially infectious material with an appropriate disinfectant. The laboratory equipment must be cleaned routinely, even if it is not contaminated*
- *All laboratory items that were in contact with potentially infectious or hazardous agents must be decontaminated before and after working with them.*
- *The work surfaces should be thoroughly disinfected with ethanol (EtOH) 70% before and after use.*
- *For sterile handling, the hands and the work areas must be always wiped with EtOH 70%, and all the outside flasks, media bottles, plates, or other containers must be EtOH 70% wiped before introducing them in the cell culture hood.*
- *Cell growth media and other reagents should not be poured directly from bottles or flasks to avoid contamination; sterile serological pipetted should be used.*
- *Hands should be washed before leaving the cell culture lab.*
- *The laboratory's safety officer should be notified upon exposure or spillage of infectious or hazardous agents to advise on a suitable strategy for containment and decontamination* [Segeritz and Vallier, 2017; Gibco Cell Culture Basics; ATCC - Animal Cell Culture Guide; ECACC Handbook].

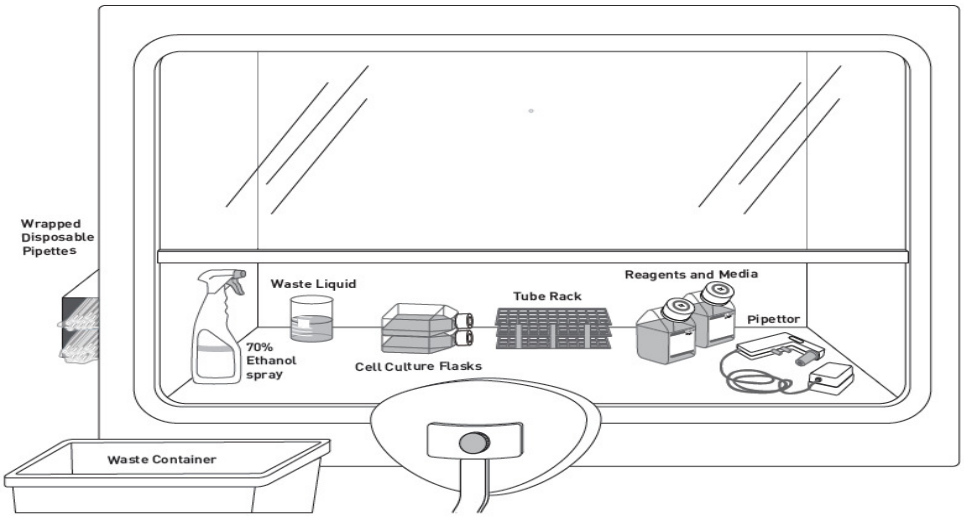

Figure 4.2. Example of a cell culture hood organization layout [Gibco Cell Culture Basics].

4.2.2. Key resources table for thawing/ subculturing/cryopreserving a cell line

Table 1. Materials and equipment required for cell culture thawing/subculturing and cryopreserving steps

Cell line	**Manufacturer**
• Cell line tested	• American Type Culture Collection (ATCC), Manassas, US • The European Collection of Authenticated Cell Culture (ECACC), Salisbury, UK • ThermoFisher Scientific, Waltham, MA, USA
Reagents/Chemicals	**Manufacturer**
• Cell culture growth medium (specific for each cell line)	• American Type Culture Collection (ATCC), Manassas, US • The European Collection of Authenticated Cell Culture (ECACC), Salisbury, UK • ThermoFisher Scientific, Waltham, MA, USA • PAN-Biotech GmbH, Bayern, Germany
• Fetal bovine serum (FBS/FCS)	• ATCC, Manassas, US • ThermoFisher Scientific, Waltham, MA, USA • PAN-Biotech GmbH, Bayern, Germany
• Specific supplements for cells' growth (dependent on the cell line)	• ATCC, Manassas, US • ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• Antibiotic solution for cell culture (dependent on the cell line)	• ATCC, Manassas, US • ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany

• Trypsin/EDTA (TE) solution	• ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• Trypan blue solution 0.4%	• ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• Ethanol 70% (for disinfection)	• Chimopar SA, Bucharest, Romania • ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• Phosphate buffered saline (PBS), pH=7.4 for cell culture	• ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• Dimethyl sulfoxide (DMSO)	• ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• H_2O (pyrogen free)	• Millipore, Merck KGaA, Darmstadt, Germany

*** Note: Equivalent reagents/chemicals of other suppliers can be used as well.**

Consumables	**Manufacturer**
• Cell culture flask (75 cm^2)	• Eppendorf, Hamburg, Germany
• Cell culture flask (25 cm^2)	• Eppendorf, Hamburg, Germany
• 96-well plates (flat bottom) (Eppendorf, Cyto-one)	• Eppendorf, Hamburg, Germany • Starlab Schweiz AG, Switzerland • ThermoFisher Scientific, Waltham, MA, USA
• Centrifuge Falcon tubes (15 mL)	• Eppendorf, Hamburg, Germany • ThermoFisher Scientific, Waltham, MA, USA
• Centrifuge Falcon tubes (50 mL)	• Eppendorf, Hamburg, Germany • ThermoFisher Scientific, Waltham, MA, USA
• Eppendorf tubes (1.5 mL, 2 mL, 5 mL)	• Eppendorf, Hamburg, Germany
• Tube racks	• Eppendorf, Hamburg, Germany
• Cryovials (2 mL)	• Merck KGaA, Darmstadt, Germany
• Pipettes tips	• Eppendorf, Hamburg, Germany • ThermoFisher Scientific, Waltham, MA, USA
• Sterile serological pipettes (10 mL, 25 mL)	• Eppendorf, Hamburg, Germany • ThermoFisher Scientific, Waltham, MA, USA
• Sterile Pasteur pipettes	• ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• Reagent reservoirs for multichannel pipetted	• ThermoFisher Scientific, Waltham, MA, USA • Merck KGaA, Darmstadt, Germany
• Countess™ Cell Counting Chamber Slides	• ThermoFisher Scientific, Waltham, MA, USA
• Glass Neubauer Counting Chamber Slides	• Merck KGaA, Darmstadt, Germany
• Cell counter	• Merck KGaA, Darmstadt, Germany

• Waste containers
• Permanent marker
• Pencil
• Sterile nitrile gloves
• Gloves for liquid nitrogen handling
• Lab personal protective equipment (lab coat and gowns, cap, shoe covers, face shields, safety glasses or goggles)

*** Note: Equivalent consumables of other suppliers can be used as well. All the consumables must be sterile when are used for cell culture.**

Equipment/ Designation	Manufacturer
• Microbiological safety cabinet (Microflow ABS Class II Cabinet ABS 1500 CLS2-MK2)	• Bioquell UK LTD
• Incubator for cell culture (Binder CB-170)	• BINDER GmbH, Germany
• Phase contrast inverted microscope with fluorescence (Olympus IX73 and Cytation 1)	• Olympus, Tokyo, Japan • BioTek Instruments Inc., Winooski, VT, USA
• Water bath (Raypa)	• R. Espinar, S.L., Spain
• Centrifuge (Boeco S-8)	• Boeco, Hamburg, Germany
• Centrifuge with freezing module (Hermle Centrifuge Z 326 K)	• Hermle Labortechnik GmbH, Wehingen, Germany
• Refrigerator (4°C)	• Arctic, Romania
• Freezer (-20°C)	• Arctic, Romania
• Freezer (-80°C) (Ultra Low Temperature Freezer MDF-U3386S)	• Sanyo, Osaka, Japan
• Nitrogen tank (MVE Cryosystem 750)	• MVE Biological Solutions, GA 30107
• Cell counting chamber (Neubauer chamber)	• Neubauer Hemacytometer
• Automatic cell counter (Countess II FL Automated Cell Counter)	• ThermoFisher Scientific, Waltham, MA, USA
• Pipette with adjustable volume	• Eppendorf, Hamburg, Germany
• Multichannel pipette	• Eppendorf, Hamburg, Germany
• Pipetting aid (Easypet® 3)	• Eppendorf, Hamburg, Germany
• Analytical scale (Sartorius CP 324S)	• Sartorius, Göttingen, Germany
• Mr. Frosty freezing container	• ThermoFisher Scientific, Waltham, MA, USA
• Autoclave (Pol-EKO SLW 53)	• POL-EKO®, Wodzisław Śląski, Poland

*** Note: Equivalent equipment of other suppliers can be used as well.**

4.2.3. Step-by-step method details

General notes!!! *For each cell line acquired it should be followed the protocol recommended by the manufacturer!!! All solutions and equipment that come in contact with the cells must be sterile!!! Unless stated differently, all procedures should be conducted under sterile laminar flow conditions, the cells should be kept in humidified incubators at 37°C, 5 % CO_2 and 95 % humidity, and the complete growth media should be pre-warmed in the water bath at 37°C for*

30 min!!! All the items that will be introduced into the laminar flow hood must be wiped with EtOH 70%!!! During all the steps of the protocol, the personnel must wear the personal protective equipment!!!

A. First culturing guidance - Thawing procedure of frozen cells upon receipt

Timing 30 min – 1 hour

1) prepare the complete growth medium specific for the cell line according to the data sheet and pre-warm it in the water bath at 37°C *(!!! The medium is prepared in aseptic conditions in the laminar flow hood!!!*)
2) place a sterile centrifuge tube (15 mL Falcon tube) into the laminar flow hood and add 10 mL of warm complete growth medium into the tube *(!!! Write on the tube with permanent marker the name of the cell line!!!)*
3) prepare an appropriate sterile cell culture flask (25 cm^2) by adding a 10 mL complete cell growth medium (!!! If a different volume of medium is recommended in the data sheet, then it should be followed the instructions of the manufacturer!!!). The type of cell culture flask or the amount of growth medium can be cell-dependent. On the plate it should be written with permanent marker, the following: date of thawing procedure, the name/ code of the cell line, the number of passage, and the name of the person who performed the procedure.
4) transfer the cryovial containing the frozen cells purchased/received from the storage container (dry ice or liquid nitrogen) into a warm water bath set at 37°C (!!! Use safety gloves and a face shield in case of liquid nitrogen handling!!!)

Note!!! *The cryovials with frozen cells should be handled one by one; don't thaw two or more cryovials at once!!!*

5) keep the cryovial in the water bath < 1 minute and thaw the content by gently swirling it until a small bit of ice was left in the vial
6) transfer the cryovial into the laminar flow hood and wiped it with EtOH 70% before opening
7) transfer the thawed cells from the cryovial **drop by drop** into the previously prepared centrifuge tube (step 2). Gently swirl the tube while adding the drops to reduce the osmotic shock to the cells*!!! This is a crucial step and the cells should be treated as gently as possible!!!*
8) verify the cryovial to ensure that all content was removed and if not, rinse with 1 mL of complete growth medium specific for the cell line

9) centrifuge the cells at 1700 rpm for 5-10 minutes (dependent on the cell line – info found in the data sheet)
10) gently discard the supernatant in the waste container placed in the laminar flow hood without disturbing the pellet of cells
11) gently resuspend the pellet in 1-2 mL of complete growth medium and transfer the suspension into the cell culture flask previously prepared (step 3). Rock the plate easily, side to side and back and forth (!!! the medium should not get into the plug!!!) to spread the cells evenly across the plate and incubate at 37°C and 5 % CO_2.

Note!!! *It is recommended to take pictures of the cells immediately post-thaw, at 24 and 48 hours and when 70-80% of confluence is reached* (Figure 4.3)!!!

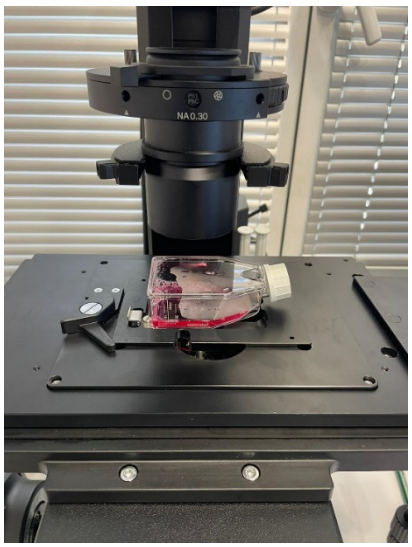

Figure 4.3. Evaluation of cells' aspect using the inverted microscope after the cell thawing procedure.

!!! Troubleshooting tips!!!

Problem	Reason	Solution
No viable cells after thawing stock	Cells were stored incorrectly	Obtain new stock and store in liquid nitrogen. Keep the cells in liquid nitrogen until thawing.
	Home made freezer stock is not viable	Freeze cells at a density recommended by the supplier.
		Use low-passage cells to make your own freezer stocks.
		Follow procedures for freezing cells **exactly** as recommended by the supplier. Note that the freezing procedure recommended in this handbook is a general procedure provided as a guideline only.
		Obtain new stock.
	Cells were thawed incorrectly	Follow procedures for thawing cells **exactly** as recommended by the supplier. Note that the thawing procedure recommended in this handbook is a general procedure provided as a guideline only.
		Make sure that you thaw the frozen cells quickly, but dilute them slowly using pre-warmed growth medium before plating.
	Thawing medium is not correct	Use the medium recommended by the supplier. Make sure the medium is pre-warmed.
	Cells are too dilute	Plate thawed cells at high density as recommended by the supplier to optimize recovery.
	Cells not handled gently	Freezing and thawing procedures are stressful to most cells. Do **not** vortex, bang the flasks to dislodge the cells (except when culturing insect cells), or centrifuge the cells at high speeds.
	Glycerol used in the freezing medium was stored in light (if applicable)	If stored in light, glycerol is gets converted to acrolein, which toxic to cells. Obtain new stock.
Cells grow slowly	Growth medium is not correct	Use pre-warmed growth medium as recommended by the supplier.
	Serum in the growth medium is of poor quality	Use serum from a different lot.
	Cells have been passaged too many times	Use healthy, low passage-number cells.
	Cells were allowed to grow beyond confluency	Passage mammalian cells when they are in the log-phase before they reach confluence.
	Culture is contaminated with mycoplasma	Discard cells, media, and reagents. Obtain new stock of cells, and use them with fresh media and reagents.

Figure 4.4. Potential solutions for troubleshooting issues encountered during the thawing procedure of a cell line [Gibco Cell Culture Basics].

B. Subculturing adherent mammalian cell lines

Timing 30 min – 1 hour

When the cells reach an appropriate confluence (70-80%), it must be applied the subculture protocol, as follows:

12) prepare a sterile Falcon tube – 15 mL and the number of cell culture flasks needed for the seeding. Both the Falcon tube and the cell culture flasks must be labelled (date of subculturing procedure, cell line name/code, number of passages, name of the person that performed the procedure)
13) take the flask with cells from the incubator and check the cells' morphology using an inverted microscope

Note!!! *It is recommended to take pictures of the cells immediately post-thaw, at 24 and 48 hours and when 70-80% of confluence is reached!!!*

14) discard the old growth medium from the cell culture flask using a vacuum pump or a sterile serological pipette attached to the pipetting aid (Easypet ® 3, Eppendorf)
15) wash the cells with pre-warmed phosphate buffer saline (PBS) without Ca^{2+}/Mg^{2+}– 10 mL for cell culture flasks of 75 cm^2 (T75) and 5 mL for cell flasks of 25 cm^2 (T25*)!!! PBS solution should be added gently to the opposite site of the flask were are attached the cells to prevent the detachment of the cells, and do the wash by swaying the flask back and forth several times!!!* ***If the cells are known to adhere strongly, it is recommended to repeat the washing step!!!***

Note!!! *This washing step is mandatory in order to remove the traces of bovine serum and other components of the medium (calcium, magnesium) that inactivate the action of the enzyme (Trypsin/EDTA solution) used to detach the cells' layer!!!*

16) discard the PBS
17) add 3 mL of Trypsin/EDTA (TE) solution (the concentration of TE solution is mentioned in the data sheet received from the manufacturer) for T75 flask and 1.5 mL for T25 flask and rotate the flask to spread uniformly the TE solution
18) return the flask into the incubator for 2-10 minutes (the incubation time is dependent on the cell line)
19) examine the cells under an inverted microscope to verify if all the cells are detached and floating. When less than 90% of cells are detached, the incubation time should by increased by several minutes, with the specification that the examination for cell detachment must be done at every 30 seconds*!!! the lateral side of the flask can be gently tapped to detach the remaining adherent cells!!! (Figure 4.5).*

Note!!! *A prolonged exposure of cells to Trypsin/EDTA solution could damage the cell surface receptors!!! Neutralization of Trypsin /EDTA solution is mandatory prior the seeding of cells, or the cells will not attach!!!*

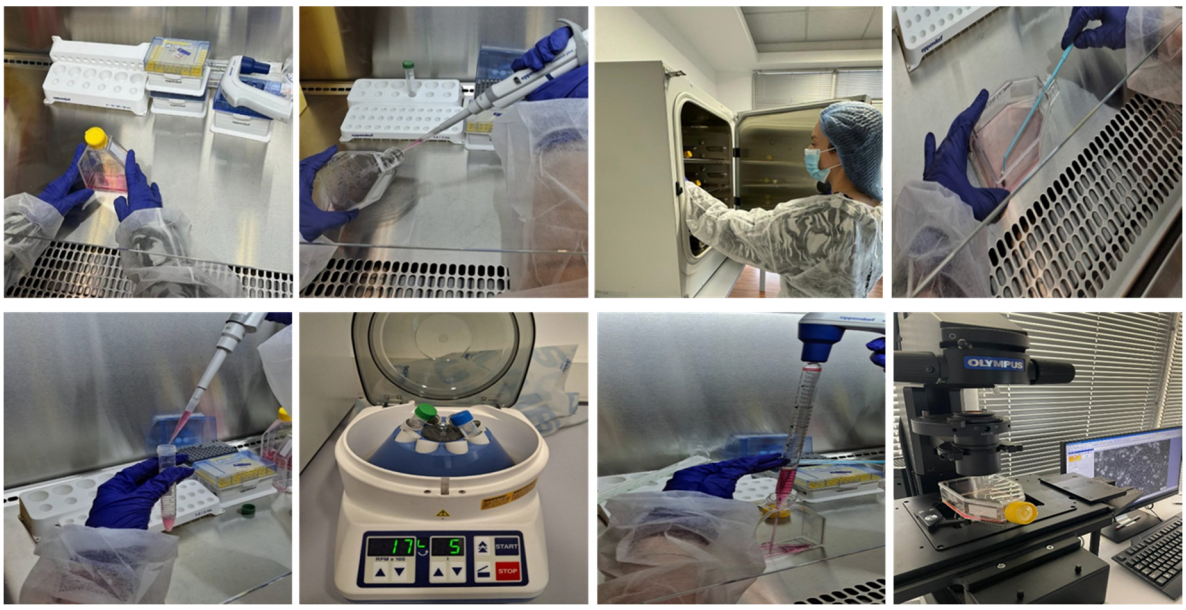

Figure 4.5. Images of the steps specific for the trypsinization procedure.

20) after the detachment of ≥ 90% cells, it should be added 10 mL pre-warmed complete growth medium for T75 flask or 5 mL for T25 flask to inactivate TE solution effect (or twice the volume of TE solution added depending on the recommendation of the manufacturer) and disseminate the medium by pipetting over the cell layer several times.

21) transfer the suspension of cells into the Falcon tube – 15 mL previously prepared (step 12) and centrifuge at 1700 rpm for 5 minutes at room temperature. *!!! The centrifuge speed and time is cell type-dependent!!!*

22) discard the supernatant, resuspend the cells' pellet in a minimal (1 mL) volume of pre-warmed complete growth medium and remove an amount (10 µL) of cells' suspension to perform cell counting

23) determination of the total number of cells and the percentage of viability can be performed using a hemacytometer (Neubauer chamber) in the presence of Trypan blue solution or the Countess II FL Automated Cell Counter.

Cell counting using the hemacytometer – Neubauer chamber

- clean (using EtOH 70%) and dry thoroughly the hemacytometer and the cover slip before use
- prepare an Eppendorf tube (1.5 mL) labelled with the name of the cell line by adding 50 µL Trypan blue and 10 µL cells' suspension and mix it by pipetting

- transfer 10 µL mix into the specific chamber of the hemacytometer and place the cover slip gently to avoid the formation of air bubbles
- place the hemacytometer under an inverted microscope and view the cells at a high magnification (40x or 100x)
- bring into focus the quadrants labelled with 1, 2, 3 and 4, as seen in Figure 4.6.

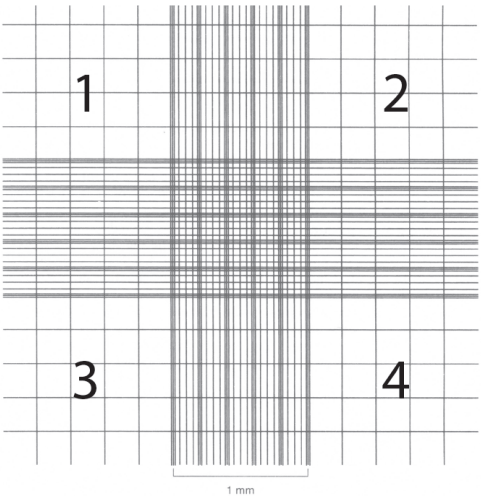

Figure 4.6. Hemacytometer (Neubauer chamber) quadrants.

- count the number of cells from each quadrant using a manual cell counter
- the total number of cells can be calculated using the following formula:

Total number of cells/mL = average of the number of cells obtained from counting the quadrants X 10 000 X dilution factor

Cell counting using the Countess II FL Automated Cell Counter

- prepare an Eppendorf tube (1.5 mL) labelled with the name of the cell line by adding 50 µL Trypan blue and 10 µL cells' suspension and mix it by pipetting
- transfer 10 µL mix into the specific slide for the Countess II FL Automated Cell Counter
- introduce the slide into the Countess II FL Automated Cell Counter and press read button
- in less than 1 minute it will be obtained automatically the total number of cells/mL and it will be further calculated the number of cells correspondent to the whole volume of cells' suspension by multiplication

  For e.g.:

  In 1 mL suspension ……1 400 000 cells

  In 3 mL suspension ……X cells

  X= 4 200 000 cells

24) transfer the required number of cells (the seeding density recommended) into a new labelled flask prepared previously (step 12) containing 10 mL complete growth medium (T25) or 18 mL – 20 mL medium (T75) and incubate the cells according to the recommended conditions
25) repeat the steps 12-24 every time is required by the growth characteristics of the cell line
26) examine the flask the following day to verify if the cells reattached and are actively growing
27) change the culture growth medium as many times as needed; 2-3 times per week is usual for most active growing cells.

Useful tips!!!

- *Before starting the subculturing procedure of a cell line check the product sheet from the manufacturer*
- *Most complete growth media should be kept at 2°C to 8°C for one month and examined periodically*
- *Freezing complete growth medium is not recommended since the supplements added might precipitate*
- *Examination of the cultures should include:*
  - *macroscopic examination of the medium for the existence of microbial contamination: changes of medium color due to pH changes (yellow or purple), turbidity, particles, small fungal colonies that float*
  - *microscopic evaluation of the medium: small, shimmering black dots within the spaces between the cells indicate bacterial contamination; rounded and budding particles – yeast contamination; and thin filamentous mycelia - fungi contamination*
  - *microscopic evaluation of cells morphology: the adherent cells should be firmly attached to the flask surface; some healthy cells round up and detach during mitosis and are refractile, but following mitosis they reattach; dead cells round up and float in the medium and are smaller and darker*
  - *as reference, images of different cell lines can be found on the website of the manufacturer (for e.g. ATCC)*
- *regular use of antibiotics for cell culture is not recommended unless they are specifically required due to their capacity to mask contamination with mycoplasma and resistant*

bacteria; if are used a final concentration of 50 to 100 IU/mL penicillin and 50 to 100 µg/mL streptomycin is recommended.

- *sera from fetal and calf bovine sources are commonly used to support the growth of cells in culture.!!! Use serum that is rigorously tested for infective agents and sourced only from US!!!*
- *don't allow the accumulation of waste into the microbiological safety cabinets or incubators to avoid the risk of contamination*
- *it is not recommended to have too many people in the lab at once*
- *avoid cell cultures from becoming fully confluent; always subculture at 70-80% confluence or as recommended by the manufacturer*
- *avoid keeping cell lines continually in culture without making stocks at early passages and not returning to frozen stocks*
- *keep the water bath clean*
- *keep the equipment calibrated*
- *appoint a regular mycoplasma-testing program* [Geraghty et al., 2014; ATCC - Animal Cell Culture Guide; ECACC Handbook].

!!! Troubleshooting tips!!! [ATCC - Animal Cell Culture Guide]

Table 2. Potential solutions for the issues encountered during the subculturing procedure.

Issues	Potential causes	Solutions
Difficulty to detach the cells following the addition of Trypsin/EDTA (TE) solution	Serum or other supplements present in the medium inactivate TE	Wash the cells twice with PBS solution without Ca^{2+}/Mg^{2+} before adding TE solution Use the TE solution instead of PBS for the first wash
	TE solution was too weak	Use higher enzyme concentrations, higher EDTA concentrations or other enzymes Incubation at 37°C is also recommended to augment the activity of TE solution
	Cells were too confluent for too long and the cell-to-cell junctions are very tight	Subculture the cells before they become confluent
Formation of clumps of cells following dissociation with TE solution	The detachment was too rough and genomic DNA was released from injured cells	Add a sterile DNase solution (1 mg/mL) to the cell suspension to destroy the formed DNA strands
	Pipetting was too vigorous	Perform the pipetting step more gently
	TE solution was too concentrated or too toxic	

	Aggregation of cells before dilution and resuspension into the medium	Use a less concentrated TE solution; reduce the incubation period Keep the cells' suspension on ice if there will be a delay between the moment of
	Long and harsh centrifugation	detachment of cells and seeding into a new flask Gentle centrifugation
Problems with the reattachment of cells to the flask	The detachment procedure was too long and affected the attachment proteins from cells' membrane	Use a protein-coated flask (collagen, poly-L-lysine, fibronectin, gelatin, etc.).
	Insufficient serum or other components with attachment properties in the growth medium	Addition of serum and attachment factor to the medium
	TE solution was not inactivated or discarded by centrifugation	Addition of serum or other enzyme inhibitors, centrifugate and resuspend the cells in fresh complete growth medium
A decreased viability	The detachment procedure was too rough	Act more gently when performing the procedure
	The pH or osmolality of the balanced salt solution containing the TE solution is wrong	Check the opened bottle and/or use a new one
	A long period of leaving the detached cells' suspension at a too high cell concentration before reseeding	Keep the cells on ice
	The growth medium was damaged	Prepare a new bottle of complete fresh growth medium specific for the cell line in use

C. Cryopreservation protocol of a mammalian adherent cell line

Timing - 1 hour

!!!To perform the cryopreservation protocol of a mammalian adherent cell line it must be followed the steps 12-23 described for Subculturing mammalian cell line procedure!!!

28) prepare a labelled cryovial that contains the following information: date of cryopreservation, cell line name/code, number of passages, number of cells, name of the person that performed the procedure (use a pencil to write on the cryovial)

29) after the count of cells number, centrifuge the cells' suspension at 1700 rpm for 5 minutes. *!!! The centrifuge speed and time is cell type-dependent!!!*

30) resuspend the cells into an appropriate volume of freezing medium (according to manufacturer recommendation) and add 1710 µL of cells' suspension into the cryovial

previously prepared (step 28) + 90 µL DMSO (dimethyl sulfoxide – a cryoprotective agent)

31) place the cryovial into Nalgene Mr. Frosty box that was filled with isopropanol and transfer Mr. Frosty into the -80°C freezer overnight
32) Transfer the frozen cells to liquid nitrogen

Note!!! *It is mandatory that the cells are healthy, contaminant free and in the log phase of growth!!!*

Useful tips!!!

- *Use as cryoprotective agent DMSO – is it the most common used, if the manufacturer recommends otherwise, follow their instructions*
- *To get healthy and in log growth phase cells, it is recommended to use pre-confluent cultures (cells that are below their maximum cell density) and by changing the culture medium 24h before cryopreservation procedure*
- *An alternative of freezing medium is: 70% basal medium+20% FBS+10% DMSO, still the freezing medium is cell type-dependent*
- *Frozen cells kept in above -65°C will be rapidly damaged* [ATCC - Animal Cell Culture Guide; ECACC Handbook].

4.3. CELL BASED-ASSAYS USING 2D CELLS

4.3.1. Cell viability tests

The screening of different collections of compounds, mainly drugs, in terms of assessing their impact on cell proliferation or the cytotoxic potential is conducted using cell-based assays. A variety of methods can be applied to evaluate the number of viable cells, such as: tetrazolium reduction, resazurin reduction, ATP detection, and also flow cytometry and high content imaging [Riss et al., 2016].

Cell viability is a parameter that refers to the number of viable/healthy cells in a test sample and its quantification is often determined in toxicity assays, for screening the response of cells to different drugs or chemical, or to correlate cell behavior to the number of cells [Kamiloglu et al., 2020]. Cell viability assays monitor different cellular functions, as: cell membrane permeability, metabolism or enzyme activity, ATP production, cell adherence, or nucleotide uptake activity [Adan et al., 2016; Kamiloglu et al., 2020]. Dependent on the mechanism of action, cell viability assays are classified as: (i) dye exclusion assays (trypan blue, eosin, congo red, and erythrosine B); (ii) colorimetric assays (MTT, MTS, WST-1, XTT, WST-8, LDH, sulforhodamine B (SRB), neutral red uptake (NRU)); (iii) luminometric assays (ATP assay and

real-time viability assay), and fluorometric assays (Alamar blue and CFDA-AM assay) [Aslanturk, 2017; Adan et al., 2016; Kamiloglu et al., 2020]

In the subsequent sections of the present guide will be presented in detail several cell viability assays as Trypan blue, 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and Alamar blue assays, methods that are frequently used in our cell culture lab.

Trypan blue method

Is a dye exclusion technique developed since 1975 to determine the count of viable cells and is still applied to confirm the changes in viable cell number following exposure to a drug or a toxic compound. This method presents multiple advantages as simplicity, reduced costs and data regarding the integrity of cell membranes [Aslanturk, 2017; Kamiloglu et al., 2020].

Principle of the method: Trypan blue is a large negatively charged molecule that does not penetrate the intact cell membrane of viable cells, whereas the dead cells are permeable and are stained in blue. The dead cells with the cytoplasm stained in blue are observed using light microscopy and can be counted using a manual or an automatic cell counter [Aslanturk, 2017; Kamiloglu et al., 2020].

Step-by-step protocol

Timing 2-4 days (depending on the treatment period – 24/48/72 h)

!!!To determine the viability of a mammalian adherent cell line using the Trypan blue assay the steps 12-22 described for Subculturing mammalian cell line procedure! must be followed!!! The key resources table described for thawing/subculturing/cryopreserving of a cell line is required for this assay!!!

In brief, the following steps should be performed:

(i) clean (using EtOH 70%) and dry thoroughly the hemacytometer and the cover slip before use

(ii) prepare an Eppendorf tube (1.5 mL) labelled with the name of the cell line by adding 50 µL Trypan blue and 10 µL cells' suspension and mix it by pipetting

(iii) transfer 10 µL mix into the specific chamber of the hemacytometer and place the cover slip gently to avoid the formation of air bubbles

(iv) place the hemacytometer under an inverted microscope and view the cells at a high magnification (40x or 100x)

(v) bring into focus the quadrants labelled with 1, 2, 3 and 4, as seen in Figure 4.7

(vi) count all the cells from each quadrant using a manual cell counter

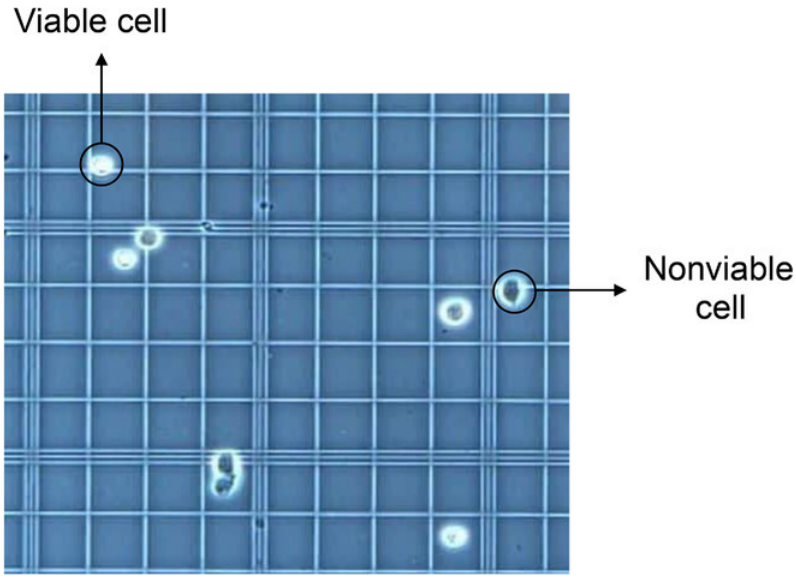

Figure 4.7. Trypan blue assay principle [Kamiloglu et al., 2020].

(vii) count the blue stained cells from each quadrant using a manual cell counter

(viii) cell viability (%) should be at least 95% in the case of healthy cells in the log phase growth.

The percentage of viable cells can be calculated using the following formula [Kamiloglu et al., 2020]:

$$\%\ \text{Viable cells} = \frac{\text{Total number of viable cells per milliliter of aliquot}}{\text{Total number of cells per milliliter of aliquot}} \times 100.$$

Total number of cells/mL = average of the number of cells obtained from counting the quadrants x 10 000 x dilution factor.

MTT - 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay

The MTT assay represents the first cell viability technique developed for the 96-well format, being highly applied in research studies as a test for cell viability/proliferation assessment [Kamiloglu et al., 2020].

Principle of the method: this test is based on measuring the activity of succinate dehydrogenase (a unique enzyme known as Complex II that is part of the Krebs cycle): a yellow tetrazolium salt suffers a chemical reaction, resulting crystals colored in purple. These

crystals are then dissolved and the solution that results is analyzed in order to measure the absorbance [Aslantürk, 2017].

The MTT reagent or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a mono-tetrazolium salt that can pass through the cell membrane and mitochondrial inner membrane of viable cells and is subsequently reduced to formazan by metabolically active cells. Formazan product of MTT tetrazolium accumulates as an insoluble precipitate, and the formazan must be solubilized before the absorbance reading. The redox chemical reaction produced in this assay provides a colorimetric measurement of intracellular formazan production and is now used with extensive utility [Ghasemi et al., 2021].

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- Cell proliferation Kit I (MTT) Roche (Merck KGaA, Darmstadt, Germany)
- Positive control to induce cell death - SDS (0.05% in pyrogen free water, final concentration – 0.005%) or Triton-X 100
- Cytation 5 Multimode Reader (BioTek Instruments Inc., Winooski, VT, USA)
- Negative control – untreated cells
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent reagents, consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol

Timing 2-4 days (depending on the treatment period – 24/48/72 h)

(i) preparation of the specific complete growth media for each cell line according to the manufacturer protocol

(ii) subculture of the cell line in cell culture flasks (**steps 12-22 from subculturing protocol**)

(iii) count of cell number using hemacytometer or the Countess II FL Automated Cell Counter (**step 23 from subculturing protocol**)

(iv) seeding of cells at an appropriate seeding density (specific for each cell line) - $1x10^4$ cells/well/ 200 µL complete growth medium into 96-well plates

(v) microscopic inspection of the cells confluence in order to start the stimulation/treatment phase

(vi) preparation of test compounds dilutions in sterile 1.5 mL Eppendorf tubes labelled with the compound name/code and concentration

(vii) preparation of the positive control solution (SDS or Triton-X 100 – depending on the manufacturer recommendation)

(viii) patterning a template for the plate corresponding to the experiment (the evaluation of different concentrations of test compounds) that include: negative control (untreated cells), positive control (SDS or Triton-X 100 solutions), blank (well only growth medium), dilution series for test compounds and dilution series for the solvent

(ix) when the cells reach the appropriate confluence (70-80%), the old medium is discarded and replaced with fresh growth containing the test compounds (triplicates), and the solvent dilutions, respectively

(x) incubation of the plate for 24/48/72 h (depending on the aim of the research) at 37°C and 5 % CO_2.

(xi) after the incubation period, the medium is removed and 100 µL of complete growth medium is added in all the wells

(xii) the cells' morphology is verified using the inverted microscope and pictures are taken to detect the potential changes following the treatment applied

(xiii) a volume of 10 µl of MTT solution (kit 1 MTT) is added to each well and the cells are incubated for 3 hours at 37°C and 5 % CO_2 (Figure 4.8)

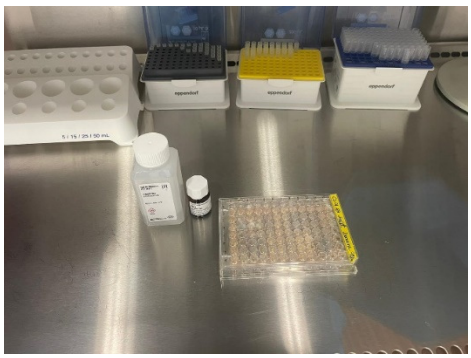

Figure 4.8. Addition of kit-1 MTT in each well (10 µl/well)

(xiv) after the 3h incubation period, 100 µl of solubilization buffer (kit 2) is added to each well to dissolve the formazan crystals and the plate is incubated for another 30 minutes protected from light at room temperature (Figure 4.9)

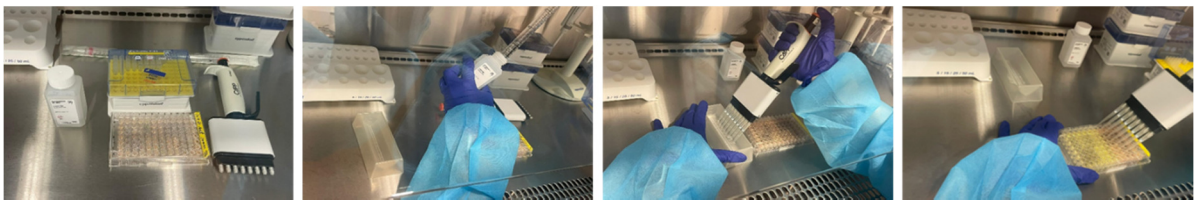

Figure 4.9. Addition of kit-2 MTT in each well (100 µl/well)

(xv) the absorbance is read at 570 nm using the Cytation 5 Multimode Reader (BioTek Instruments Inc., Winooski, VT, USA) (Figure 4.10).

Figure 4.10. Absorbance reading at 570 nm.

(xvi) cell viability percentage is calculated using the following formula [Kamiloglu et al., 2020], where OD is the absorbance value measured:

$$\% \text{ Viability} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \times 100.$$

(xvii) data interpretation: absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation; conversely a higher absorbance rate indicates an increase in cell proliferation [MTT Cell Proliferation Assay ATCC® 30-1010K]

!!! Troubleshooting tips!!! [MTT Cell Proliferation Assay ATCC® 30-1010K]

Problem: MTT Reagent is blue-green.

Cause	Remedy
Contamination with a reducing agent or cell/ bacterial contamination.	Discard. Remove aliquots of new MTT Reagent using sterile technique.
Excessive exposure to light.	Store solution in the dark at 4°C.

Problem: Blanks (medium only) give high absorbance readings.

Cause	Remedy
The medium is contaminated with cells/ bacteria/yeast (visible under microscope).	Discard. Check medium before plating. Use sterile technique for cell plating in biological hood. Use sterile 96-well plate.
The medium contains ascorbic acid.	Incubate plate in the dark. Find alternative medium if possible.

Problem: Absorbance readings too high.

Cause	Remedy
Cell number per well too high.	Decrease cell density at plating.
Contamination of culture with bacteria or yeast.	Discard. View wells prior to addition of MTT Reagent to check for contamination.

Problem: Absorbance readings are too low.

Cause	Remedy
Cell number per well is too low.	Increase cell density at plating.
Incubation time for reduction of MTT is too short. No purple color visible in cells when viewed under microscope.	Increase incubation time with MTT Reagent until purple color is evident inside cells when viewed under microscope. Longer incubation of up to 24 hours may be required for some cell types.
Incubation time for solubilization of formazan dye too short (intact cells with intracellular dye visible when viewed under the microscope).	Increase incubation time with Detergent Reagent or incubate at 37°C. View under microscope to ensure no crystals remain out of solution.
Cells not proliferating due to improper culture conditions or inadequate time of recovery after plating.	Check that culture conditions (medium, temperature, humidity, CO_2, etc.) are appropriate. View cells periodically to check condition. Increase time in culture after plating for cell recovery.

Problem: Replicates have different values.

Cause	Remedy
Inaccurate plating or pipetting.	Increase accuracy of cell plating, check accuracy of pipette.

Figure 4.11. Troubleshooting tips recommended for MTT assay.

Alamar blue method

This technique is also known as resazurin reduction assay. Resazurin is a phenoxazin-3-one dye and a nontoxic cell permeable redox indicator. This compound can be used to determine viable cell number applying protocols similar to those utilizing the tetrazolium compounds [Aslanturk, 2017].

Principle of the method: after entering cells, resazurin is reduced to resorufin. Resorufin is red in color and highly fluorescent compound. Viable cells convert continuously resazurin to resorufin, increasing overall fluorescence and color of the cell culture medium. The quantity of produced resorufin is related to the number of viable cells [Aslanturk, 2017].

Step-by-step protocol

Timing 2-4 days (depending on the treatment period – 24/48/72 h)

(i) preparation of the specific complete growth media for each cell line according to the manufacturer protocol

(ii) subculture of the cell line in cell culture flasks (**steps 12-22 from subculturing protocol**)

(iii) count of cell number using hemacytometer or the Countess II FL Automated Cell Counter (**step 23 from subculturing protocol**)

(iv) seeding of cells at an appropriate seeding density (specific for each cell line) - $1x10^4$ cells/well/ 200 µL complete growth medium into 96-well plates

(v) microscopic inspection of the cells confluence in order to start the stimulation/treatment phase

(vi) preparation of test compounds dilutions in sterile 1.5 mL Eppendorf tubes labelled with the compound name/code and concentration

(vii) preparation of the positive control solution (SDS or Triton-X 100 – depending on the manufacturer recommendation)

(viii) patterning a template for the plate corresponding to the experiment (the evaluation of different concentrations of test compounds) that include: negative control (untreated cells), positive control (SDS or Triton-X 100 solutions), blank (well only growth medium), dilution series for test compounds and dilution series for the solvent

(ix) when the cells reach the appropriate confluence (70-80%), the old medium is discarded and replaced with fresh growth containing the test compounds (triplicates), and the solvent dilutions, respectively

(x) incubation of the plate for 24/48/72 h (depending on the aim of the research) at 37°C and 5 % CO_2.

(xi) after the incubation period, the medium is removed and 200 µL of complete growth medium is added in all the wells

(xii) the cells' morphology is verified using the inverted microscope and pictures are taken to detect the potential changes following the treatment applied

(xiii) a volume of 10 µl of Alamar blue reagent is added to each well and the cells are incubated for 3 hours at 37°C and 5 % CO_2

(xiv) the absorbance is then read at 570 and 600 nm. [https://assets.thermofisher.com/TFS-Assets/LSG/manuals/AlamarBluePIS.pdf]

(xv) the percentage of viable cells is calculated using the following formula:

Percentage of viable cells (%) = {[$(\varepsilon_{OX})\lambda_2$ $A\lambda_1$-$(\varepsilon_{OX})\lambda_1$ $A\lambda_2$ of test agent dilution]/[$(\varepsilon_{OX})\lambda_2$ $A°\lambda_1$-$(\varepsilon_{OX})\lambda_1$ $A°\lambda_2$ of untreated positive growth control]} x100,

Where: ε_{OX}=molar extinction coefficient of Alamar blue oxidized form (BLUE)

A=absorbance of test wells

A°=absorbance of positive growth control well (cells without tested compounds)

λ_1=570 nm and λ_2=600 nm [Iftode et al., 2021].

4.3.2. Immunofluorescence staining methods for the assessment of cell morphology

Immunofluorescence is a staining method known since 1950s, considered an excellent tool in preclinical experiments, modern biology and related areas of medical-pharmaceutical research. It can also be described as a combination of morphological technology and immunofluorescence techniques for the development of fluorescent cells. This method allows the visualization of multiple components of a tissue or of a cell (cell nuclei, proteins, nuclei acid or other cellular components). Among the advantages of immunofluorescence technique are stated the high signal amplification, targeted specificity, high resolution and analytical capabilities [Im et al., 2019].

In general, the principle of the procedure is based on the use of specific fluorescent antibodies to observe the impact of the test samples on the expression of cellular markers.

In the subsequent sections will be presented several tests applied for the visualization by immunofluorescence staining of the nucleus of and cytoskeletal features of adherent cultured cells in the presence or absence of a specific compound/treatment.

Nuclear Staining

Hoechst 33342 Staining

Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell-permeable stain that binds to the adenine-thymine (AT) sequences of double-stranded DNA, extensively applied as a nuclear dye with blue fluorescence at 460-490 nm. It is especially well known for distinguishing condensed nuclei in

apoptotic cells. Hoechst 33342 stain is used for living cells and is cell permeable [Thermo Fisher Scientific Hoechst 33342 protocol].

Principle of the method: after the addition to the culture cells, Hoechst 33342 dye enters the cell and concentrates into the nucleus, allowing its visualization in blue; is considered an indicator of the nucleus status and can also be used to assess the cell cycle status or in apoptosis assays.

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- Hoechst 33342 (ThermoFisher Scientific, Waltham, MA, USA)
- Positive control to induce apoptosis - Staurosporine solution (5 µM; Merck KGaA, Darmstadt, Germany) or necrosis Triton-X 100 (0.5%; Merck KGaA, Darmstadt, Germany)
- Lionheart FX automated microscope (BioTek Instruments Inc., Winooski, VT, USA)
- Negative control – untreated cells
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent reagents, consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol

(i) preparation of the specific complete growth media for each cell line according to the manufacturer protocol

(ii) subculture of the cell line in cell culture flasks (**steps 12-22 from subculturing protocol**)

(iii) count of cell number using hemacytometer or the Countess II FL Automated Cell Counter (**step 23 from subculturing protocol**)

(iv) seeding of cells at an appropriate seeding density (specific for each cell line) - $1x10^5$ cells/well/ 1500 µL complete growth medium into 12-well plates

(v) microscopic inspection of the cells confluence in order to start the stimulation/treatment phase

(vi) preparation of test compounds dilutions in sterile 1.5 mL Eppendorf tubes labelled with the compound name/code and concentration

(vii) preparation of the positive control solution (5 µM Staurosporine and 0.5% Triton-X 100 – depending on the manufacturer recommendation)

(viii) patterning a template for the plate corresponding to the experiment (the evaluation of different concentrations of test compounds) that include: negative control (untreated cells), positive control (5 µM Staurosporine and 0.5% Triton-X 100 solutions), dilution series for test compounds and dilution series for the solvent

(ix) when the cells reach the appropriate confluence (70-80%), the old medium is discarded and replaced with fresh growth containing the test compounds, and the solvent dilutions, respectively

(x) incubation of the plate for 24/48/72 h (depending on the aim of the research) at 37°C and 5 % CO_2.

(xi) after the desired time has elapsed, the culture medium is discarded from each well

(xii) the prepared staining solution (20 mM Hoechst stock solution diluted in PBS 1:2000) is added to each well in an adequate volume (300 – 500 µL) to cover the cells

(xiii) the well plate is incubated for 5-10 minutes in a place protected from direct light at room temperature

(xiv) after the incubation time, the staining solution is removed

(xv) each well is then washed 2-3 times with 1500 µL PBS solution

(xvi) the final step is to photograph the cells using inverted microscope or special devices for this purpose [https://www.thermofisher.com/ro/en/home/references/protocols/cell-and-tissue-analysis/protocols/hoechst-33342-imaging-protocol.html]

Note!!! *Hoechst 33342 staining protocol can be also performed in 96-well or 6-well plate formats with the mention that the number or seeded cells and the volumes of reagents must be adjusted accordingly!!!*

DAPI (4',6-diamidino-2-phenylindole)

DAPI is used in modern biology, in *in vitro* techniques, as a blue fluorescent dye for DNA. It can be used for analysis of cell nuclei, quantification of apoptosis or DNA-based analysis. It is known to stain fixed cells and is impermeable to cells.

Principle of the method: DAPI stain passes through the intact cells membrane and binds to the DNA strands located into the nucleus and emits a blue fluorescence. It can be used to stain both live cells and fixed cells (require fixation and permeabilization).

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- DAPI Staining solution (ThermoFisher Scientific, Waltham, MA, USA)

Note!!! *DAPI stock solution (5mg/mL) can be obtained using deionized water (diH2O) or dimethylformamide (DMF) as solvent. DAPI has poor solubility in water, so sonicate as necessary to dissolve. The 5 mg/mL DAPI stock solution may be stored at 2–6°C for up to 6 months or at ≤–20°C for longer periods!!!*

- Positive control to induce apoptosis - Staurosporine solution (5 µM; Merck KGaA, Darmstadt, Germany) or necrosis Triton-X 100 (0.5%; Merck KGaA, Darmstadt, Germany)
- Lionheart FX automated microscope (BioTek Instruments Inc., Winooski, VT, USA)
- Negative control – untreated cells
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

* **Note: Equivalent reagents, consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol

(i) preparation of the specific complete growth media for each cell line according to the manufacturer protocol

(ii) subculture of the cell line in cell culture flasks (**steps 12-22 from subculturing protocol**)

(iii) count of cell number using hemacytometer or the Countess II FL Automated Cell Counter (**step 23 from subculturing protocol**)

(iv) seeding of cells at an appropriate seeding density (specific for each cell line) - $1x10^5$ cells/well/ 1500 µL complete growth medium into 12-well plates

(v) microscopic inspection of the cells confluence in order to start the stimulation/treatment phase

(vi) preparation of test compounds dilutions in sterile 1.5 mL Eppendorf tubes labelled with the compound name/code and concentration

(vii) preparation of the positive control solution (5 µM Staurosporine and 0.5% Triton-X 100 – depending on the manufacturer recommendation)

(viii) patterning a template for the plate corresponding to the experiment (the evaluation of different concentrations of test compounds) that include: negative control (untreated cells), positive control (5 µM Staurosporine and 0.5% Triton-X 100 solutions), dilution series for test compounds and dilution series for the solvent

(ix) when the cells reach the appropriate confluence (70-80%), the old medium is discarded and replaced with fresh growth containing the test compounds, and the solvent dilutions, respectively

(x) incubation of the plate for 24/48/72 h (depending on the aim of the research) at 37°C and 5 % CO_2.

(xi) after the desired time has elapsed, the culture medium is discarded from each well and the cells are washed 2-3 times with 1.5 mL PBS

(xii) addition of 300 nM DAPI staining solution/well in the amount needed to cover all the cells

(xiii) the plate is incubated for up to 5 minutes in a place protected from direct light

(xiv) staining solution is aspirated

(xv) cells are washed with PBS 2-3 times

(xvi) cells are photographed using an inverted fluorescence microscope or Cytation 1 [https://www.thermofisher.com/ro/en/home/references/protocols/cell-and-tissue-analysis/protocols/dapi-imaging-protocol.html]

Note!!! *DAPI staining protocol can be also performed in 96-well or 6-well plate formats with the mention that the number or seeded cells and the volumes of reagents must be adjusted accordingly!!!*

Cytoskeletal Staining

The cytoskeleton is a matrix that supports the form and function of cells performing essential roles in organelle transport, division, motility and signaling, as well as in cellular health and disease processes.

TexasRed™-X Phalloidin staining on fixed cells

Texas Red-X phalloidin is used for the quantification of F-actin, the stain is compatible with other fluorescent dyes used *in vitro*. This method is used for high contrast discrimination of actin staining. In general, Texas red phalloidin can also be investigated in combination with other stains for broader observations and analysis with greater specificity.

Principle of the method: Phalloidin is a bicyclic peptide and binds to F-actin with high selectivity allowing the visualization of actin fibers in red fluorescence. Phalloidin is not a cell-permeable reagent, so it is recommended the fixation and permeabilization of the cells prior the addition of Texas Red-X phalloidin dye [https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0001777_Phalloidins_UG.pdf].

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- Texas Red™-X Phalloidin (ThermoFisher Scientific, Waltham, MA, USA)
- Lionheart FX automated microscope (BioTek Instruments Inc., Winooski, VT, USA)
- Negative control – untreated cells
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent reagents, consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol

(i) preparation of the specific complete growth media for each cell line according to the manufacturer protocol

(ii) subculture of the cell line in cell culture flasks (**steps 12-22 from subculturing protocol**)

(iii) count of cell number using hemacytometer or the Countess II FL Automated Cell Counter (**step 23 from subculturing protocol**)

(iv) seeding of the cells ($1x10^4$ cells/well/ 200 µL) in black 96-well plates with flat glass bottom clear wells

(v) microscopic inspection of the cells' confluence in order to start the stimulation/treatment phase

(vi) preparation of test compounds dilutions in sterile 1.5 mL Eppendorf tubes labelled with the compound name/code and concentration

(vii) patterning a template for the plate corresponding to the experiment (the evaluation of different concentrations of test compounds) that include: negative control (untreated cells), dilution series for test compounds and dilution series for the solvent

(viii) when the cells reach the appropriate confluence (70-80%), the old medium is discarded and replaced with fresh growth containing the test compounds, and the solvent dilutions, respectively

(ix) incubation of the plate for 24/48/72h (depending on the aim of the research) at 37°C and 5 % CO_2.

(x) after the incubation period, culture medium is removed and the cells are washed with PBS twice

(xi) the sample is fixed in 3.7% methanol-free formaldehyde solution in PBS for 15 minutes at room temperature - 100 µL/well

(xii) wash 2x with PBS

(xiii) incubate the sample in blocking solution containing 1% BSA in PBS for approximately 45 minutes at room temperature in a volume of 100 µL/well

(xiv) the samples are incubated in the staining solution for 30-60 minutes at room temperature;

(xv) wash 2x with PBS;

(xvi) if necessary, add a DNA counterstain (for nuclei analysis). For example, DAPI staining can be added (staining performed according to the steps described above in the section - **DAPI staining**);

(xvii) Image the cells (adequate storage of the plates for about one month for conclusive images)

Note!!! *The plates can be stored at 4°C Celsius and a preservative can be added; the plates stored are sealed with a specific adhesive and then wrapped with aluminum foil!!!*

CellLight™ GFP Tubulin staining protocol on live cells

Principle of the method: CellLight reagents contain fluorescent protein-signal peptide fusions targeting specific cellular structures, in the present case, tubulin fibers, for live-cell imaging applications. It can also be applied on fixed cells [https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp10582.pdf].

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- CellLight™ Tubulin-GFP, BacMam 2.0 reagent (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA)
- Lionheart FX automated microscope (BioTek Instruments Inc., Winooski, VT, USA)
- Negative control – untreated cells
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent reagents, consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol

(i) follow the steps (i) – (ix) described previously for TexasRed™-X Phalloidin staining

(ii) calculate the volume of CellLight® for the number of cells with the formula given in the manufacturers protocol [https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp10582.pdf]

$$\text{Volume of CellLight® Reagent (mL)} = \frac{\text{number of cells} \times \text{desired PPC}}{1 \times 10^8 \text{ CellLight® particles/mL}}$$

Where the number of cells is the estimated total number of cells at the time of labeling, PPC is the number of particles per cell, and 1×10^8 is the number of particles per mL of the reagent.

For example, to label 40,000 cells with a PPC of 30:

$$\text{Volume of CellLight® Reagent (mL)} = \frac{40{,}000 \times 30}{1 \times 10^8} = 0.012 \text{ mL } (12 \mu\text{L})$$

(iii) the CellLight® reagent is mixed by inversion to form a homogeneous solution;

(iv) the previously calculated volume of reagent is mixed gently with the complete growth cell medium and added into the wells with cells

(v) the cells are incubated overnight

(vi) image the cells using the appropriate equipment (Cytation 1/ Lionheart FX automated microscope)

Note!!! *If is required, the cells' nuclei can also be stained with Hoechst 33342 (staining performed according to the steps described above in the section –* ***Hoechst 33342 staining).***

4.3.3. Methods to assess cell migration capacity

Scratch Assay

Cell migration plays a central role in different fundamental biological processes, as embryonic development, immune defense, tissue formation, wound repair, inflammation, and also, in cancer progression. The capacity of cells to migrate highlights their ability to adapt to a given environment in terms of regaining a proper position and accomplishing their functions; however, an impaired cell migration is frequently encountered in multiple pathologies (inflammation, cancer progression, invasion and metastasis), or following exposure to different compounds [Pijuan et al., 2019].

In vitro 2D wound healing assays, as scratch assay, represent excellent and reliable approaches to study/to monitor the cell migratory behavior in response to different stimuli (substances of interest) or in different pathologies [Pijuan et al., 2019]. For healthy cells, wound

repair is often used to observe cell migratory behavior after treating cells with the compounds of interest. For diseased cells, especially cancer cells, the method targets the evaluation of treatment impact on cells capacity to invade and to develop metastases.

Principle of the method: this method is used to measure the capacity of cells to migrate in a given time frame (24 h) following exposure to different stimuli (compounds analyzed for biological or for noxious effects).

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- Lionheart FX automated microscope (BioTek Instruments Inc., Winooski, VT, USA)
- BioTek AutoScratch wound making tool (BioTek Instruments Inc., Winooski, VT, USA)
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent reagents, consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol

(i) seeding the cells depending on the cell line according to the manufacturer protocols (**see steps 12-22 from subculturing protocol**)

(ii) an appropriate number of cells (depending on the cell line) are grown in 96-well or 24-well plates until the desired confluence (generally 90-95%)

(iii) patterning a template for the plate corresponding to the experiment (the evaluation of different concentrations of test compounds) that include: negative control (untreated cells), dilution series for test compounds and dilution series for the solvent

(iv) when the desired confluence is reached, the old medium is discarded and the scratch line is drawn manually (using a sterile pipette tip) or automatically using the BioTek AutoScratch wound making tool in the middle of each well

(v) remove the debris and the non-attached cells by washing 2x with pre-warmed PBS

(vi) replace the PBS with fresh complete growth medium containing the compounds of interest and the solvent dilutions, respectively

(vii) incubation of the plate for 24 h at 37°C and 5 % CO_2.

(viii) during the whole procedure the evolution of the cells is followed and pictures are taken so that at the end of the time allocated for the experiment the results can be analyzed

(ix) data analysis using specific bioinformatics tools.

Notes!!!

- ❖ *The method aims to evaluate the effect of different compounds of interest or substances on cell lines, this effect can be stimulatory or inhibitory on the migration/invasion process of cultured cells.*
- ❖ *For a better analysis of the data, the wells are photographed at different time points (0h, 3h, 8h and 24h) until the end of the treatment period.*
- ❖ *Scratching can be carried out manually or automatically using special devices for this purpose (auto-scratchers) (Figure 4.12).*

Figure 4.12. BioTek AutoScratch wound making tool

4.3.4. Molecular biology tests for the study of mechanisms of action

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a laboratory technique that involves making several copies of a genetic sequence in order to analyze it. It is also known as reverse transcription-polymerase chain reaction [https://www.cancer.gov/publications/dictionaries/cancer-terms/def/rt-pcr].

Principle of the method: the procedure determines the effect induced by the samples to be analyzed in the gene expressions, at the messenger RNA level, of specific markers depending on the proposed experiment. This technique evaluates the changes that can occur in a gene, a chromosome or for the activation of genes, which often leads to the diagnosis of a pathology.

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA)
- Tadvanced Biometra Product line (Analytik Jena AG, Göttingen, Germany)
- DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA)
- ThermoMixer C (Eppendorf, Hamburg, Germany)
- peqGold RNAPureTM Package (Peqlab Biotechnology GmbH, Erlangen, Germany)

- Maxima® First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA)
- Power SYBR-Green PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA)
- Primers pairs of interest (Thermo Fisher Scientific, Inc., Waltham, MA, USA)
- PCR specific 96-well plates or tubes (Eppendorf, Hamburg, Germany, Thermo Fisher Scientific, Inc., Waltham, MA, USA)
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent reagents, consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol

(i) seeding the cells depending on the cell line according to the manufacturer protocols (**see steps 12-22 from subculturing protocol**)

(ii) an appropriate number of cells (depending on the cell line) are grown in 6-well plates until the desired confluence

(iii) patterning a template for the plate corresponding to the experiment (the evaluation of different concentrations of test compounds) that include: negative control (untreated cells), dilution series for test compounds and dilution series for the solvent

(iv) cells are stimulated with the compounds of interest and then incubated under optimal conditions for the desired time - 24, 48 or 72 hours

(v) RNA isolation step, this step is carried out on ice as follows:

a. the old culture medium is removed and the cells are washed with 2 mL of PBS
b. the cells are detached from the plate using the cell scraper in 1 mL PBS (Figure 4.13)

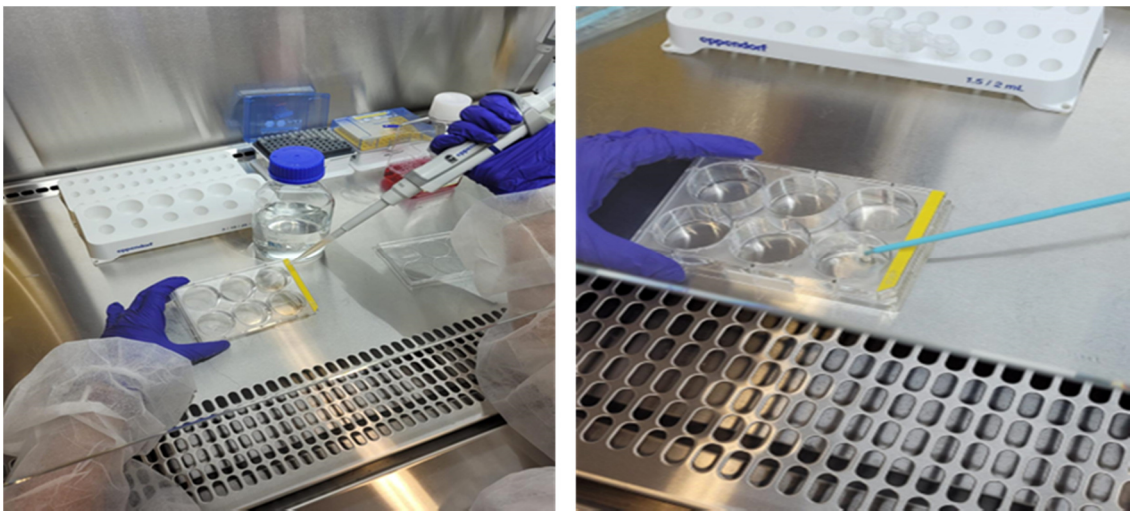

Figure 4.13. Preparation of cells for RNA isolation step

c. the suspension formed in each well is transferred to 1.5 mL Eppendorf tubes previously prepared (labelled with the name/code of the sample and the date of the procedure and wrapped with Scotch tape) and kept on ice until the suspension is added (Figure 4.14)

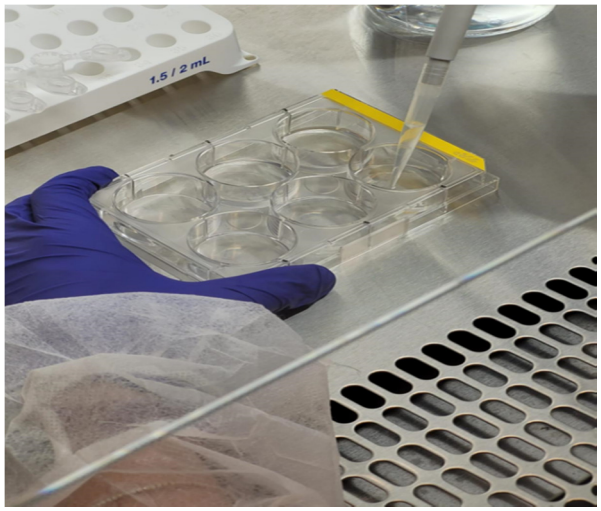

Figure 4.14. Cell suspension aspiration

d. the suspension is centrifuged at 12,000xg for 5 minutes at 4°C
e. the supernatant is removed and the sediment is kept at -20°C in case RNA isolation does not occur at that time
f. the RNA isolation is performed using the peqGold RNAPureTM Package (or other equivalent kit) and the protocols follows the instructions of the manufacturer

g. the sediment obtained after RNA isolation is resuspended in 50 µL DEPC-Water, is heated for 30 minutes at 55°C (to increase ARN solubility) and then it is measured the RNA concentration using the DS-11 Spectrophotometer

(vi) the following step is the synthesis of the complementary DNA using a specific kit (Maxima® First Strand cDNA Synthesis Kit)

(vii) the RT-PCR step involves the following steps:

a. Selection of the pairs of primers of interest
b. Preparation of the mix containing the primers, the cDNA resulted and the SYBR-Green PCR Master Mix
c. Pipetting the mix into the plate
d. PCR reaction using the QuantStudio 5 Real-Time PCR System

(viii) Data analysis

4.4. Cell-based assay using 3D (three-dimensional) models

The 2D *in vitro* models, which represent monolayer adherent cell cultures, are the most used *in vitro* models. However, due to the limitation of planar geometry, this type of model poorly predicts *in vivo* behavior. Therefore, the newly formulated 3D *in vitro* models are a bridge between *in vitro* and *in vivo* models and reproduce the physiological and pathophysiological characteristics of the original tissues, simulating the *in vivo* 3D structure. The 3D structures, due to the spatial configuration, possess a better representation of the cell-cell and cell-matrix contact in the native microenvironment *in vivo*. These models produce a realistic impression of the transport of gases, nutrients, or signaling molecules. Furthermore, 3D *in vitro* models can recreate the complex structure of the tumor and maintain genotypic and phenotypic features along with tumor heterogeneity [Leonard and Godin, 2016; Jubelin et al., 2022; Mu et al., 2023].

The 3D models used in our laboratory as tools to perform pharmaco-toxicological studies were the 3D Reconstructed Human Epidermis (RHE), EpiDerm tissues from MatTek Corporation (Slovakia). These 3D reconstructed EpiDerm tissues are ready-to-use systems consisting of normal, human-derived epidermal keratinocytes (NHEK) cultured on specially prepared tissue culture inserts [https://www.mattek.com/mattekproduct/epiderm/]. The 3D EpiDerm tissues present the human epidermal structure, including the proliferative basal layer, the spinous and granular layers, and also, the cornified epidermal layers [https://www.mattek.com/mattekproduct/epiderm/]. This 3D *in vitro* model was validated by ECVAM (European Centre for the Validation of Alternative Methods) and OECD (The

Organisation for Economic Co-operation and Development) guidelines as an *in vitro* model system for chemical, pharmaceutical and skin care product testing.

4.4.1. Determination of the irritant potential (EpiDerm Skin Irritation test – OECD TG 439)

Principle of the method: the test consists of a topical exposure of the neat test compounds to a reconstructed human epidermis (RhE) model followed by a cell viability test. The reduction of the viability of tissues exposed to chemicals in comparison to negative controls (treated with water) is used to predict the skin irritation potential [https://www.mattek.com/wp-content/uploads/EPI-200-SIT-Skin-Irritation-MK-24-007-0023.pdf].

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- EPI-200-SIT Kit Components (MatTek Corporation)
- MTT-100 kit components (MatTek Corporation)
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol (according to manufacturer's recommendations)

Timing 4 days

(i) upon receipt, the inserts containing the tissues are removed from the agarose and transferred to a 6-well plate in 900 µL /well of test medium, for 4 hours, under specific conditions (37 °C and 5% CO_2)

(ii) after the 4h period, the initially added medium is replaced with 900 µL /well of fresh pre-warmed medium, and incubated until the next day

(iii) the next day, the medium is changed and the test samples are applied to the top of the inserts containing the tissues; initially, the tissue surface is moistened (25 µL of Dulbecco's phosphate-buffered saline - DPBS), after which 100 mg of sample/insert is added and incubated for 18 hours (Figure 4.15)

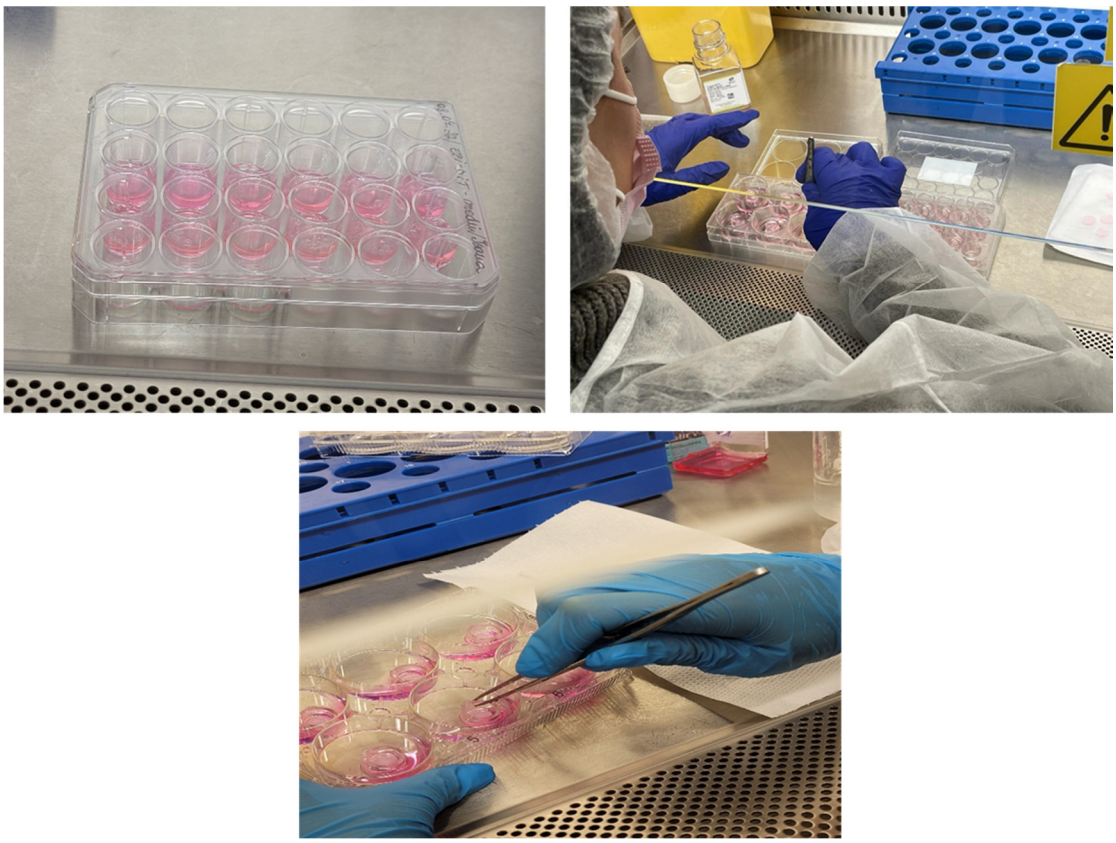

Figure 4.15. Manipulation of 3D reconstructed EpiDerm tissues upon receipt.

(iv) the positive control (PC) - Triton X-100 1% and the negative control (NC) - ultrapure water, are also added to the top of the inserts with tissues and incubated for 18 hours;

(v) the MTT test is performed as follows:

a. after the 18h stimulation period, the inserts are washed with DPBS and transferred to a 24-well plate containing 300 µL/well of MTT (1 mg/mL) and incubated for 3 h

b. following the 3h of incubation, the inserts are removed and placed in 2 mL of extractant solution in a new 24-well plate for 2 hours at room temperature, protected from light

c. then, 200 µL/well of the extraction solution is added to a new 96-well plate, and the absorbances are measured spectrophotometrically at the corresponding wavelengths (570 nm and 650 nm).

d. The viability of the samples is calculated using the formula [Pinzaru et al., 2021]:

Viability % = OD $_{sample}$ / OD $_{NC}$ × 100

Relative viability TS % = OD $_{TS}$ / Mean of OD $_{NC}$ × 100

Relative viability NC % = OD $_{NC}$ / Mean of OD $_{NC}$ × 100

Relative viability PC % = OD $_{PC}$ / Mean of OD $_{NC}$ × 100

where: TS = test substance, NC = negative control, PC = positive control, OD = optical density.

4.4.2. Determination of the phototoxicity (EpiDerm Phototoxicity Assay – OECD TG 498)

Principle of the method: measures acute toxic response after exposure of skin to certain chemicals and subsequent exposure to light or that is induced similarly by skin irradiation after systemic administration of a chemical substance. [https://www.mattek.com/application/phototoxicity/].

Materials needed for conducting the assay in addition to **the key resources table described for thawing/subculturing/cryopreserving of a cell line**:

- EPI-200 Kit Components (MatTek Corporation)
- MTT-100 kit components (MatTek Corporation)
- UVA-vis Irradiation equipment - Biospectra
- Test compounds
- Selection of the appropriate solvent for the solubilization of test compounds

*** Note: Equivalent consumables and equipment of other suppliers can be used as well.**

Step-by-step protocol (according to manufacturer's recommendations)

Timing 4 days

(i) upon receipt, the inserts containing the tissues are removed from the agarose and transferred to a 6-well plate in 900 µL /well of test medium, for 1 hours, under specific conditions (37 °C and 5% CO_2)

(ii) after the 1h period, the initially added medium is replaced with 900 µL /well of fresh pre-warmed medium, and incubated until the next day

(iii) the next day, the medium is changed and the test samples are applied to the top of the inserts containing the tissues; initially, the tissue surface is moistened (25 µL of Dulbecco's phosphate-buffered saline - DPBS), after which 100 mg of sample/insert is added and incubated for 18 hours

(iv) the coated plates are exposed to UVA at 6 J/cm^2, and in parallel the plates without exposure are kept at room temperature in the dark. Label the lids of the plates to identify the UVA exposed tissues.

(v) after this step, each insert is washed with DPBS and transferred to new plates containing 900 µL medium/well and is incubated for 18-24 hours at 37 °C and 5% CO_2.

(vi) On the third day, the inserts are washed with DPBS and transferred to a 24-well plate with 300 µL MTT/well, after which they are analyzed according to the above protocol.

$$\textbf{RTV \%} = \textbf{OD}_{\textbf{sample}} / \textbf{OD}_{\textbf{vehicle control}} \times \textbf{100}$$

$$\textbf{RTV control \%} = \textbf{OD}_{\textbf{vehicle control}} / \textbf{OD}_{\textbf{mean vehicle control}} \times \textbf{100},$$

where: RTV = relative tissue viability, OD = optical density.

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Chapter 5. The use of *in ovo* methods for the evaluation of biological activity (CO - UMFVBT)

5.1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, plays a crucial role in various physiological and pathological processes, including tumor growth and metastasis. Evaluating the antiangiogenic potential of compounds is essential in developing novel therapeutic strategies. In this context, the *in ovo* chorioallantoic membrane (CAM) assay has emerged as a valuable tool for assessing the biological activity of substances on angiogenesis [Moreno-Jiménez et al., 2016].

The CAM assay involves the use of the chorioallantoic membrane of developing chicken embryos, which is highly vascularized and sensitive to angiogenic stimuli. This model allows for the direct observation of blood vessel formation and provides a cost-effective and ethically acceptable alternative to traditional *in vivo* assays. The CAM assay has been widely utilized in studying angiogenesis due to its simplicity, reproducibility, and relevance to human physiology [Moreno-Jiménez et al., 2016].

Researchers have employed the CAM assay to evaluate the antiangiogenic potential of various compounds. For instance, rhodamine B□oleanolic acid derivative bioconjugate has been shown to exhibit antiangiogenic abilities, demonstrating significant inhibitory effects on blood vessel formation [Mangır et al., 2019]. Similarly, natural compounds like the ones isolated from the aerial parts of *Salvia officinalis* have been investigated for their antiangiogenic activity using the CAM assay, showing promising results in inhibiting blood vessel formation necessary for embryonic growth [Reichling et al., 2006].

Moreover, the CAM assay has been instrumental in assessing the antiangiogenic properties of diverse compounds, including organometallic complexes [Fathalla et al., 2017], dendrimers [Kluge et al., 2016], and venom fractions [Coelho et al., 2019]. These studies highlight the versatility of the CAM assay in evaluating the effects of different substances on angiogenesis, providing valuable insights into potential therapeutic interventions targeting aberrant blood vessel formation.

Furthermore, the CAM assay has been utilized not only in the field of oncology, but also in tissue engineering, demonstrating its applicability in studying bone regeneration and the biocompatibility of biomaterials [Butt et al., 2018]. The assay's ability to assess both the biocompatibility and angiogenic response to biomaterials underscores its utility in various research areas beyond angiogenesis studies. The *in ovo* CAM assay serves as a valuable and

versatile tool for evaluating the antiangiogenic potential of compounds. Its simplicity, cost-effectiveness, and relevance to human physiology make it a preferred choice for researchers investigating angiogenesis and developing novel therapeutic agents targeting blood vessel formation.

5.2. Determination of the antiangiogenic potential using CAM assay

Step-by-step protocol

Note !!! This is a slightly modified version of the ICCVAM-Recommended Test Method Protocol!!!

1) Eggs preparation:
   - the incubator is prepared a few hours before the eggs are incubated. The entire surface of the incubator is disinfected with wipes and 70% ethanol. The temperature is set at 37.5 ^{0}C and the humidity is at 60 %.
   - fresh, fertile eggs weighing between 50-60 g from hens (Figure 5.1.) reared under optimum conditions are used for hatching. Before being placed in the incubator, the eggs are washed with lukewarm water and a sponge, or they may be wiped with tissues and 70% ethanol without shaking.

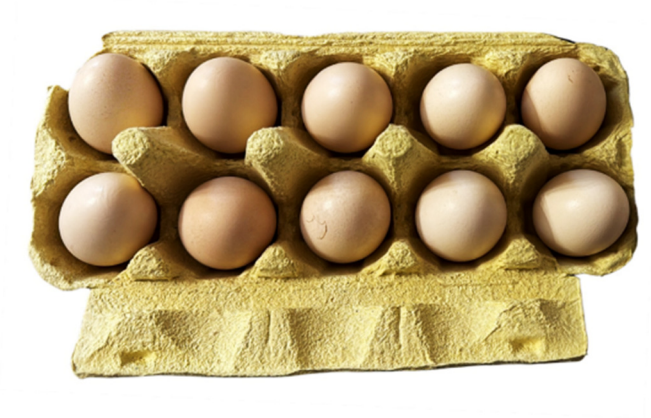

Figure 5.1. Fertile hen eggs weighing between 50-60 g.

   - A pencil is used to mark on the eggs the date they are placed in the incubator (Figure 5.2).

Figure 5.2. The eggs are dated and disinfected.

- The eggs are placed in the incubator tray in a horizontal position (Figure 5.3).

Figure 5.3. Eggs placed in the incubator (1st day).

- On the 4th day of incubation, two holes are made at the two ends of the egg using forceps, and 5-8 mL of egg white (albumen) is extracted from the top of the egg using a syringe and decanted into a collecting container. The two holes are then covered with medical adhesive tape and placed back in the incubator (Figures 5.4 and 5.5).

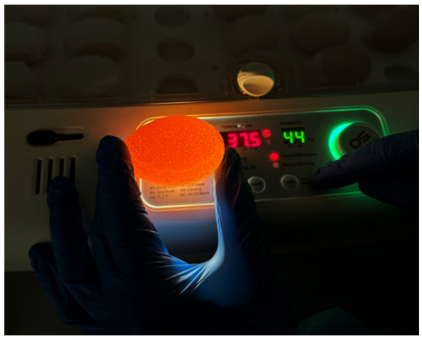

Figure 5.4. Checking with a light source whether embryos are developing or not (4th day).

Figure 5.5. Eggs placed in the incubator aftera albumen extraction (4th day).

- On the 5th day of incubation, a large piece of adhesive tape is glued to the horizontally held egg to cut out a large window with forceps to expose the chorioallantoic membrane and blood vessels. The window of the eggs showing viable embryos is then covered with a large piece of adhesive tape to avoid dehydration, and they are reintroduced into the incubator until experimental tests are performed (Figures 5.6 - 5.8).

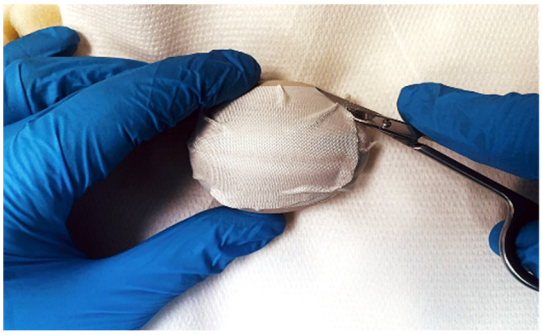

Figure 5.6. Cutting a large window with forceps to expose the chorioallantoic membrane and blood vessels (5th day).

Figure 5.7. Eggs covered with adhesive tape to avoid dehydration (5th day).

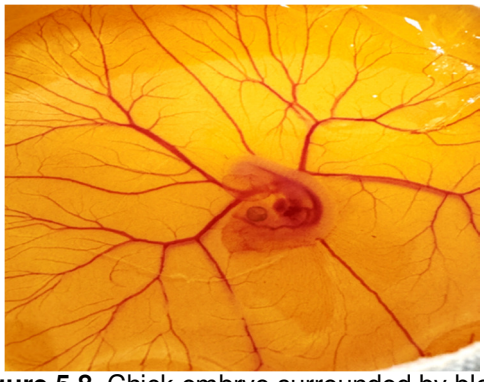

Figure 5.8. Chick embryo surrounded by blood vessels (5th day).

- Every day, it is checked that the temperature and humidity parameters are maintained at the set values for optimal embryo development [Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), ICCVAM Recommended Test Method Protocol: Hen's Egg Test–Chorioallantoic Membrane. 2010. Available online: http://iccvam.niehs.nih.gov/].

2) application of a ring to a vascularized area on day 8 of egg incubation, within which 10 µL of the test sample is applied (Figure 5.9)

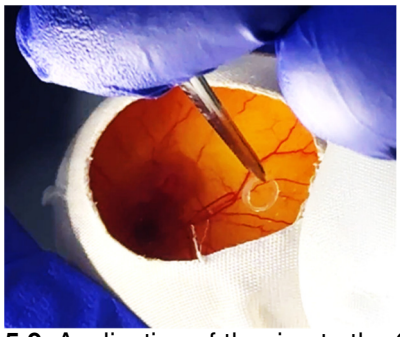

Figure 5.9. Application of the ring to the CAM in order to apply samples for angiogenesis testing (8^{th} day).

3) samples are applied for 5 days in the ring, taking pictures before each application and after the application of the samples
4) throughout the experiment, the eggs are kept in the incubator at the optimal set parameters.

5.3. Determination of the irritant and anti-irritant potential

The HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) assay is a widely used method for evaluating the irritant and anti-irritant potential of various substances. This assay involves observing the chorioallantoic membrane of fertilized chicken eggs to assess the effects of test substances. The CAM assay has been recognized as a reliable model for studying tissue responses, angiogenesis, and biocompatibility [Moreno-Jiménez et al., 2016; Mangır et al., 2019].

Researchers have adapted the HET-CAM assay to determine the irritation potential of substances like essential oils and their components, providing a valuable tool for assessing non-severe eye and mucous membrane irritants [Reichling et al., 2006]. Additionally, studies have utilized the HET-CAM assay to confirm the non-irritancy of formulations such as *in situ* gels, demonstrating their ability to protect against strongly irritant substances [Fathalla et al., 2017].

The HET-CAM assay has also been employed in various fields beyond biocompatibility assessments. For instance, it has been used to evaluate the mutagenicity of plasma jet treatments and to assess the cytotoxicity of nano-hydroxyapatite in oral care cosmetics [Kluge et al., 2016; Coelho et al., 2019]. Furthermore, the assay has been instrumental in studying the conjunctival irritation potential of microemulsions designed to combat ophthalmia neonatorum caused by pathogens like *Neisseria gonorrhoeae* and *Staphylococcus aureus* [Butt et al., 2018].

Moreover, the HET-CAM assay has been recognized as a valuable tool for predicting ophthalmic irritation potential, showing a good correlation with *in vivo* situations [McKenzie et al., 2015]. This highlights its significance in the pharmaceutical industry for assessing the irritancy of various formulations intended for ocular use.

Step-by-step protocol

Note!!! *The preparation of the eggs is done as described in step 1 of the protocol for the determination of the antiangiogenic potential!!!*

1) on the eighth day of incubation, 600 µL of the test sample is applied to the chorioallantoic membrane of the embryos, and the changes in the vascular plexus are observed stereomicroscopically for 300 seconds (Figure 5.10).

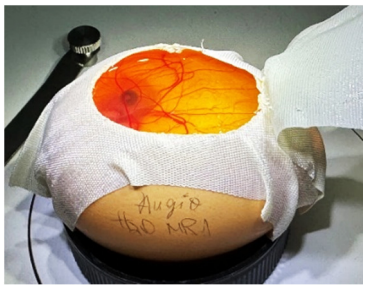

Figure 5.10. An open window revealing the CAM and blood vessels of the chick embryo (8^{th} day).

2) signs of lysis, hemorrhage, or clotting are observed and the time of occurrence of these processes is noted in a table
3) based on the values obtained, an irritability score is calculated, from which the irritancy potential of the test samples is estimated.

In conclusion, the HET-CAM assay is a versatile and reliable method for evaluating irritant and anti-irritant potential in various applications. Its ability to provide valuable insights into tissue responses, biocompatibility, and irritancy makes it a valuable tool for researchers and industries involved in developing products ranging from cosmetics to pharmaceuticals.

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Chapter 6. Methods to obtain preparations from plants (Extract and Essential oils)

6.1. Introduction

Plant-based industries are dependent on the preparation of products from plants. Extraction is the first step of any medicinal plant study, plays a significant and crucial role on the final result and outcome (Figure 6.1).

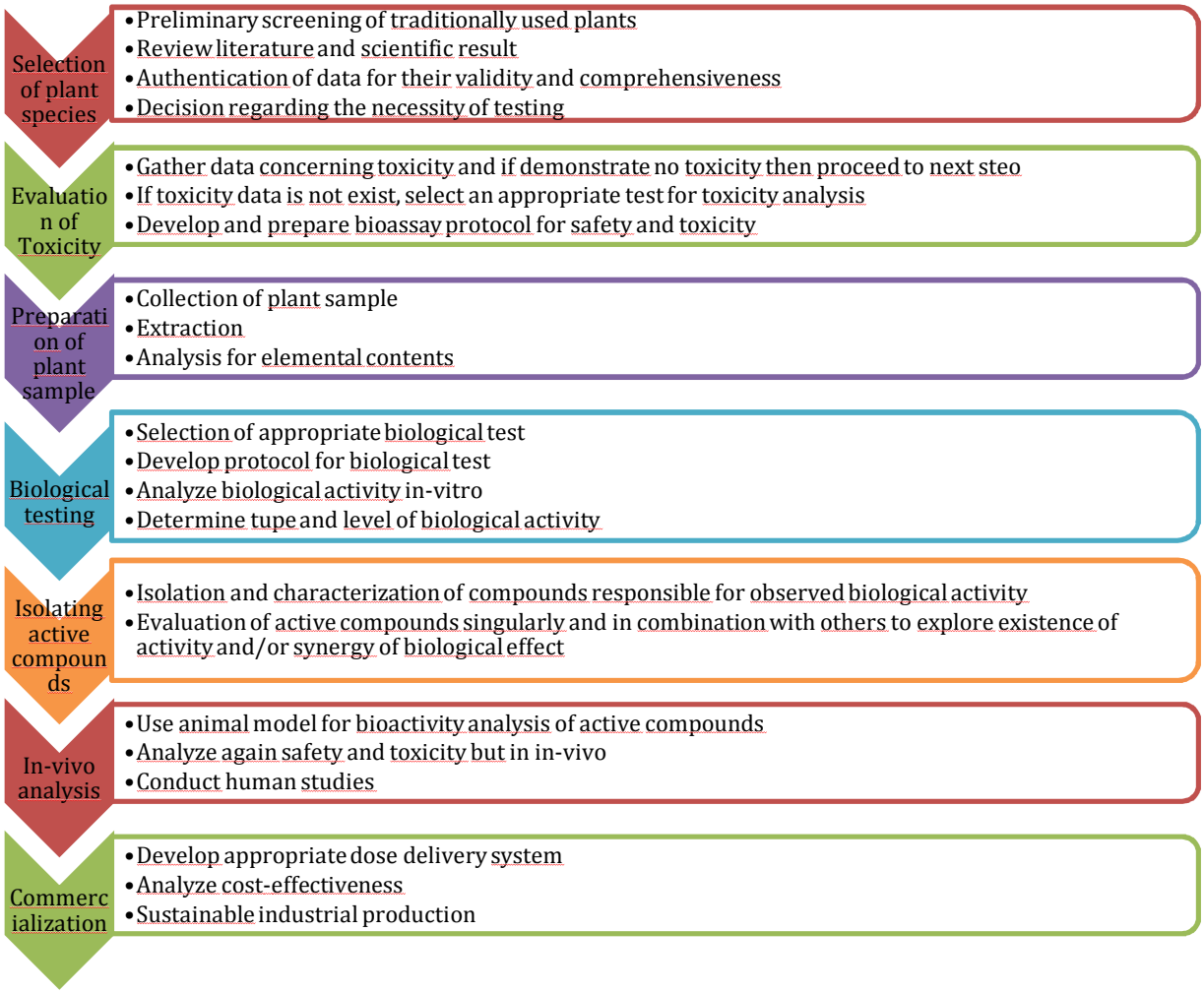


Figure 6.1. The flow chart of medicinal plant study and position of extraction techniques [Azmir et al., 2013].

Bioactive compounds from plant materials can be extracted by various extraction procedures (Figure 6.2).

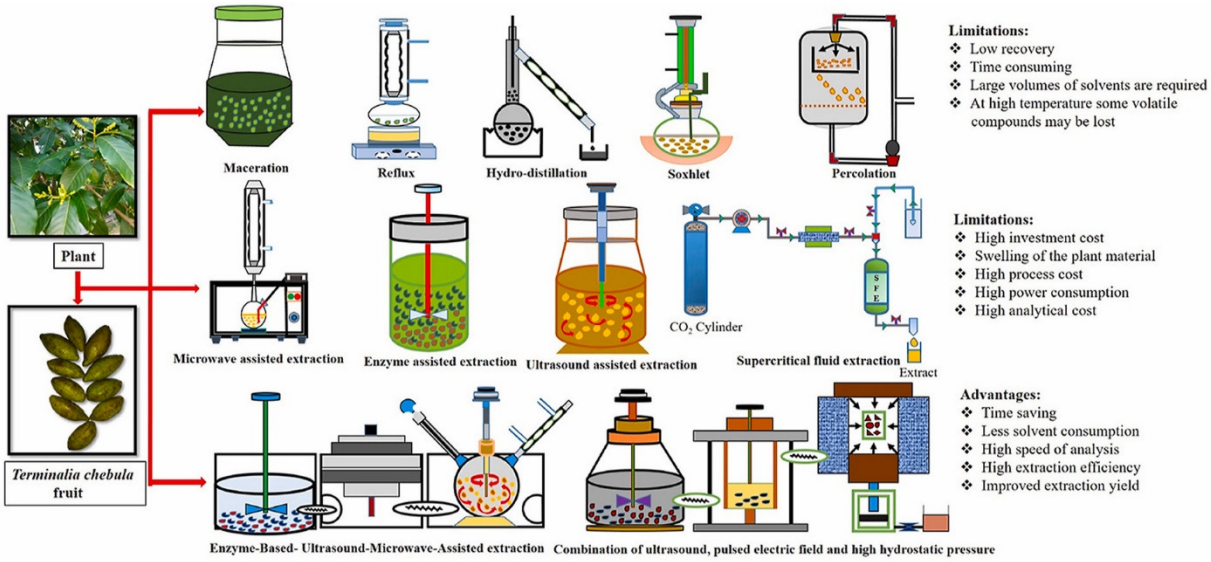


Figure 6.2. Techniques for extraction of bioactive compounds [Jha and Sit, 2022].

6.2. Maceration

Most of these techniques are based on the extracting power of different solvents in use, like the maceration (Figure 6.3-6.4).

Figure 6.3. Maceration of *Pinus nigra* subsp. *laricio* Poiret (Original pictures)

Figure 6.4. Maceration of *Capsicum baccatum.* (Original pictures)

Extraction means the separation of one or more substances from a matrix, through treatment with mechanical, chemical and thermal means.

The maceration takes place in steel containers which they can have both small and large capacities (1,000–10,000 liters) or other inert material towards both the solid matrix and the extracting solvent. The solid to be extracted is introduced into the container completely covered by the solvent, in order to obtain the most complete extraction possible. The extraction process is usually quite long and demanding days or even weeks to reach completion. Both diffusion and osmosis phenomena intervene in this extraction process, which are strongly dependent on temperature.

Usually, the use of heat alters the thermolabile component of the drug; therefore, in the majority of cases the use of chemical reagents is required, generally organic solvents which make the chemical structure of the cell membrane permeable.

The maceration, conducted at room temperature, reduces the risk of thermal degradation of the sample.

Step-by-step protocol

1) The plant is initially separated from foreign material such as soil and parts unsuitable for extraction.

In order to increase the contact between the sample to be extracted and the solvent, the plant was appropriately chopped.The particles must not be too large otherwise the solvent would not be able to penetrate the innermost cells, but they must not be reduced to a powder state either; this would lead to the loss of the volatile active ingredients contained within the plant and the difficult separation by filtration of the plant material from the liquid used, once the maceration time has ended.

2) Subsequently, each sample is immersed in organic solvent for 48 hours, inside an airtight container, repeating the procedure 3 times.

The solvents indicated by the Pharmacopoeia for the extraction of the active ingredients from herbal drugs are: water, ethyl alcohol and ethyl ether. Industrially, others can be used such as isopropyl alcohol and toluene, which may have advantageous characteristics from a commercial point of view or for their extractive power. The solvent must be chosen based on the chemical nature of the compounds contained within the sample. Alcohol is generally the solvent that is most used since it is able to extract most of the molecules contained within the plant (active ingredients), whether they are hydrophilic, i.e. soluble in water, or lipophilic and therefore soluble in oil or other organic solvents.

3) Subsequently the sample is filtered and dried using the rotary evaporator with a water bath at 37 °C, whose peculiarity is low pressure evaporation with a vacuum pump which allows boiling points much lower than those at ambient pressure in order to lower the boiling temperature of the solvent and favor its evaporation.

The combination of bath temperature and vacuum pressure allows precise distillation of solvent fractions (Figure 6.5).

Figure 6.5. Drying extract (Original pictures)

The maceration technique regarding extraction in aqueous phase presents some variations: infusion, which can be represented as a maceration for very short times in boiling water. In this case the extraction is certainly faster but the degradation phenomenon affecting the thermally labile substances also becomes faster. Another variant of the classic maceration is decoction. It is done placing the matrix in contact with the solvent, operating at boiling temperature, for a variable time of up to 30 minutes or more.

This technique is therefore reserved for compact materials that have heat-resistant active ingredients, the extraction of which requires the intervention of heat. Decoctions, like infusions, are easily altered and have a limited validity.

6.3. Soxhlet extraction

The Soxhlet extractor (Figure 6.6) is a device capable of continuously separating i.e. from a solid mixture poorly soluble components from insoluble ones using a volatile solvent.

The device takes its name from Franz von Soxhlet (1848-1926), who invented it in 1879 to the extraction of lipids from a solid material, using ethyl ether as a solvent; the complex of the separated chemical components took the name of ethereal extract.

Figure 6.6. Soxhlet extractor (Original pictures)

The Soxhlet extractor essentially consists of 3 parts: a) flask containing the solvent; b) the actual extractor; c) reflux condenser, water cooled. Also, in the extractor a lateral arm (1) allows the passage of solvent vapors to the condenser, while the siphon (2) allows periodic emptying of the extractor. The solid material containing the compound from extract, is placed in a thimble, often made of porous cellulose (3), which is inserted into the chamber extractor main. The key advantages of Soxhlet extraction are: i) its simplicity, ii) applicability at high temperatures, which boosts process kinetics, iii) low start-up costs, iv) no filtering is required, v) the constant presence of solvent and sample throughout the extraction.

One of the major problems associated with this extraction method is that they are limited due to their poor extraction efficiency, time taking procedure, and involvement of lot of solvents.

The preparations that can be obtained from the plants are numerous. Extracts are preparations that can be liquid, semisolid, or solid in consistency, in function of the extraction technique [Azwanida, 2015]. Using maceration or percolation processes, the extracts obtained can be classified as follows: (i) fluid extracts: these extracts contain the same amount of active ingredient found in the starting plant drug; (ii) soft extracts: during the concentration process, a honey-like consistency is achieved and they are 2 to 6 times more concentrated than fluid extracts; (iii) dry extracts: these are solid powders obtained by complete evaporation of the solvent used for extraction [Abubakar and Haque, 2020].

Other preparation obtained through maceration or percolation are the tinctures. These are liquid solutions obtained by processing plant drugs with an appropriate solvent. Commonly, a hydroalcoholic solution (a mixture of water and alcohol) is used, the alcohol content of which is chosen according to the solubility of the active ingredients to be extracted. The main distinction between extracts and tinctures lies in the fact that, in the former, an evaporation process is carried out to increase the concentration of the active ingredients in the preparation. In contrast, tinctures can also be obtained by simply diluting the corresponding fluid extract. Another substance extracted plant materials is oleoresin, which contains a mixture of essential oils (volatile aromatic components) and resins (nonvolatile components). This extraction is usually done by solvent or high-pressure extraction processes [Hudz et al., 2020].

6.4. Innovative extraction techniques

The application of innovative extraction methods in the food industries has been extensively investigated, due to increased consumer expectations for greener options. These methods, which are more environmentally friendly due to decreased use of synthetic and organic chemicals, reduced operational time, and better yield and quality of extract.

Some of the most promising techniques are ultrasound assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, pulsed electric field assisted extraction, supercritical fluid extraction and pressurized liquid extraction. The use of novel and combined novel technologies increases extractability, resulting in yields with higher extraction rates.

6.4.1. Ultrasound-assisted extraction

Ultrasound-assisted extraction uses ultrasound energy and solvents to extract target compounds from various plant matrices [Kumar et al., 2021]. It is a process that allows the extraction of the bioactive compounds contained in the plant by exploiting the mechanical action of ultrasound on the plant walls.

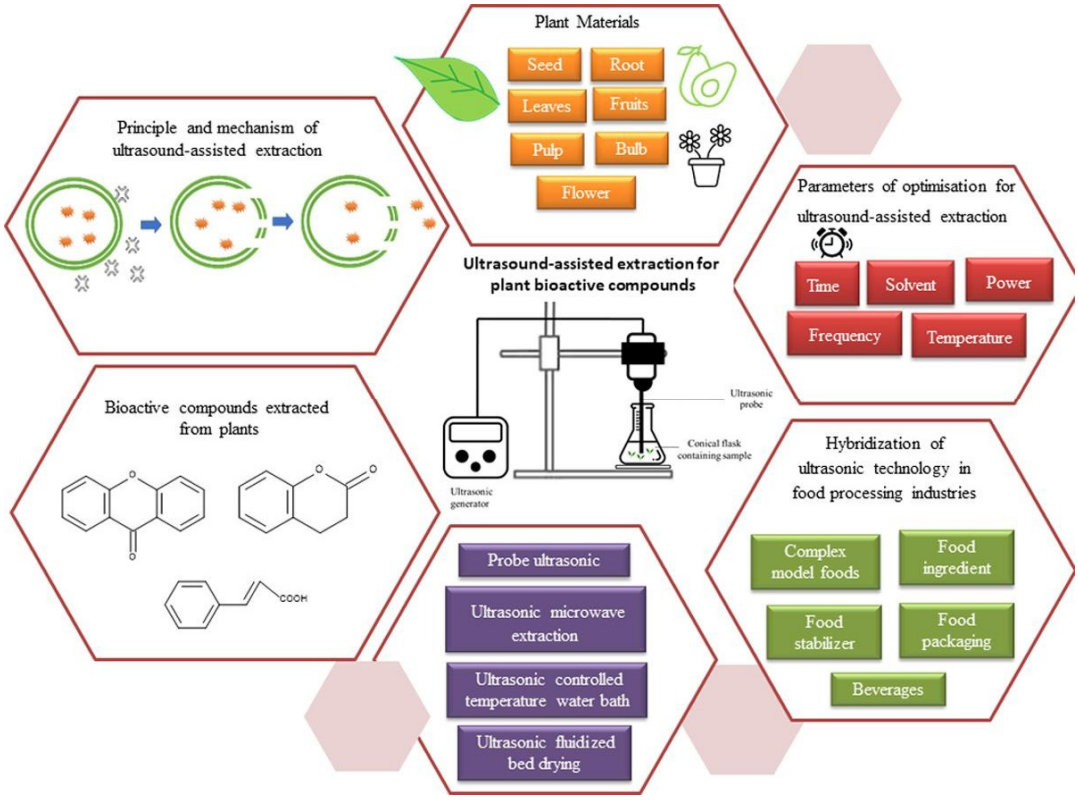


Figure 6.7. Ultrasound-assisted extraction [Yusoff et al., 2022].

Ultrasonic extraction generally uses a smaller frequency range (16 KHz – 100 KHz) as higher frequencies would be too energetic and could degrade the active ingredients of the plant matrices. These waves consist of a series of compression and rarefaction cycles that can be propagated through solid, liquid or gas medium inducing displacement and dislodgement of the molecules from their original positions. Ultrasound technology allows the complete extraction of the plant material, preserving the integrity of all the molecules contained in the plant, whether they are thermos-labile (proteins, amino acids, vitamins, enzymes, etc.), thermostable, water-soluble or fat-soluble.

This is made possible thanks to the shock wave produced by the ultrasound which causes the mechanical rupture of the cell walls.

Table 1. Advantages and disadvantages of ultrasound extraction compared to conventional maceration.

Main advantages of ultrasound extraction compared to conventional maceration	Disadvantages of ultrasound extraction
Significant reduction in production times since the ultrasounds break the cell walls and reduce the transfer times of the active ingredients from the plant material to the solvent.	With ultrasound extraction it is not possible to carry out a selective extraction since there is a total escape of all the molecules contained in the plant material, regardless of the affinity with the solvent used.
It is possible to obtain a liter of macerate in just 15 minutes.	If it is desired to obtain the separation of the active ingredients contained in the plant mass, subsequent methods must be carried out.
It is an extraction technique which has been awarded Bio accreditation as this extraction is a purely physical process in which no chemical products are used or added. In fact, natural solvents (oil, water and alcohol) are used, regardless of the solubility of the active ingredients.	
Very satisfactory yields are obtained in terms of active ingredients since there is an almost total exhaustion of the vegetable.	

Step-by-step protocol

1) the plant material is ground or chopped into small pieces to increase the extraction surface area
2) the plant material is then mixed with a solvent (such as ethanol or water)
3) ultrasound is then used to aid the extraction process by applying high-intensity, low-frequency ultrasound waves at approximately 20 kHz to the mixture. This causes acoustic cavitation and rapid vibration of the solvent, which promotes the disintegration and rupture of plant cells and the release of bioactive substances such as polyphenols, flavonoids and vitamins
4) the mixture is then filtered to separate the solid plant material from the liquid containing the extracted bioactive compounds
5) The liquid is then evaporated or subjected to further treatment to remove the solvent and concentrate the bioactive molecules
6) The final product is a bioactive-rich extract that can be used in various applications, such as dietary supplements, functional foods and cosmetics.

6.4.2. Enzyme-assisted extraction

Phytochemicals, that are present in specific matrices which contain polysaccharide lignin stabilized by hydrogen bonding and hydrophobic interactions, remain dispersed in the cell cytoplasm and are inaccessible with the solvent extraction process. The bound

phytochemicals in such samples are effectively released at high yields by pre-treating the plant material with specific enzymes (cellulose, pectinase and amylase) are added during extraction to enhance phytochemical yield by breaking down cellular walls. Further, these enzymes hydrolyze carbohydrates such as cellulose and lipid bodies [Bitwell et al., 2023].

Step-by-step protocol

1) pulverization of the herbal drug
2) suspension in water
3) use of specific hydrolytic enzymes which have the task of releasing all the molecules present in the phytocomplex
4) addition of glycerine
5) filtration
6) addition of citric acid to stabilize and make the product stable over time
7) packaging.

The advantages of using hydro-enzymatic extracts compared to classic phytotherapeutic remedies such as mother tinctures or glycerine macerates are first of all the effectiveness, the high bioavailability thanks to the use of hydrolytic enzymes, the prompt action and the safety of the product in as this contains neither alcohol nor sugar.

6.4.3. Microwave-assisted extraction

Microwaves are non-ionizing waves of electromagnetic nature with frequency between 300 MHz and 300 GHz. These waves are positioned in the spectrum electromagnetic wave between infrared rays and X-rays. The direct action of these waves on the substance is to transform electromagnetic energy into thermal energy. The microwaves consist of two oscillating perpendicular fields: the magnetic and the electric one, responsible for heating. The extraction with microwave (MAE) (Figure 6.8) depends on the heating process of the solvent and the sample. Furthermore, it is governed by two phenomena: dipole rotation and ionic conduction. Dipole rotation means the realignment of the dipoles of the molecule with the rapid electric field change; this causes both dielectric material and solvents with persistent dipoles are heated by the action of microwaves. By ionic conduction instead we mean the transfer of ions caused by the change in the field electric. As a result, migration generates friction (due to the resistance offered from the solution) responsible for heating the solution.

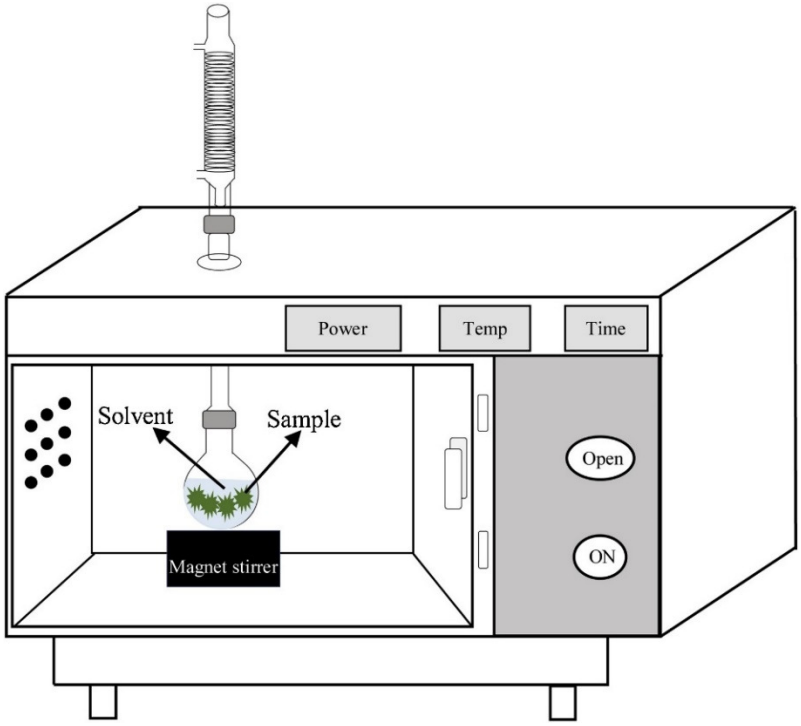


Figure 6.8. A schematic representation of the Microwave-assisted extraction system [Mirzadeh et al., 2020].

The extraction can be carried out on both wet and dry matrices, exploiting the traces of humidity present in the sample that are heated (e.g. water contained in the biomass plant cells). The resulting evaporation creates extremely high pressures within the cells which ultimately cause the cell wall to rupture, increasing recovery yields of phytocondensates in the medium.
Alvarez *et al.* (2017) demonstrated how using microwaves as a pretreatment for pomace increase both the extraction yields and the total polyphenols extracted. Caldas *et al.* (2018) instead observes how the polyphenol yields are greater using methods that involve the use of microwaves as an extraction medium compared to methods of conventional extraction.

6.4.4. Supercritical fluid extraction

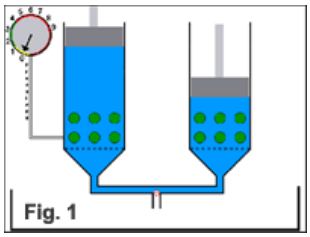
Supercritical fluid extraction is an important separation technique used in the food, nutraceutical and pharmaceutical sectors through which some active ingredients are isolated by subjecting the material to high pressure in the presence of a gas, mainly CO_2.
Extraction with supercritical fluids is a Green Technology that guarantees environmental sustainability to the process and a high degree of quality and purity of the extracted product. It is a clean, selective technology, does not require high temperatures and represents an alternative to the common extraction with organic solvents.

This technique allows the extraction of compounds sensitive to heat and oxidation such as polyunsaturated fatty acids (ω3, ω6), vitamins, cannabinoids, flavonoids, sterols, tocopherols and other compounds with high added value with the certainty of not damaging them. The result is extracts of extreme purity and very refined organoleptic characteristics.

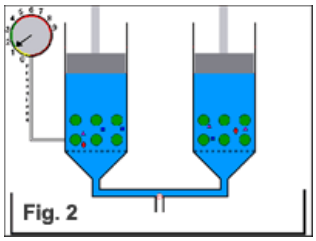
6.4.5. Naviglio extractor ®

The rapid dynamic solid-liquid extractor (**Naviglio extractor ®**) (Figure 6.9) represents an innovative solid-liquid extraction technology that allows the solid matrices containing substances extractable in an organic or inorganic solvent and in their mixtures to be exhausted in a short time, compared to other currently existing extraction techniques. The novelty of the extractor lies in the fact that the extraction philosophy changes as the trend of current methods which aim to heat the extraction system to increase yield and speed up extraction times is reversed; the Naviglio Estrattore® carries out the extraction at room or sub-room temperature and exploits an increase in pressure of the extracting liquid on the solid matrix to be extracted. The importance of extracting at low temperatures lies in the fact that in this way thermal stress on thermolabile substances is avoided.

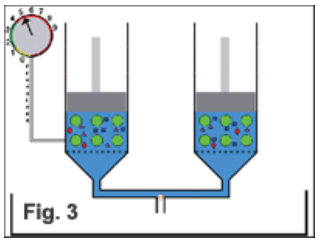
Schematic representation of the functioning of the Naviglio extractor ®



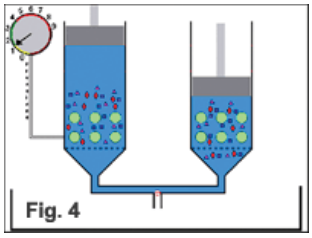
Step 1. The extraction chambers are filled with the solid matrix to be extracted and the extracting solvent, at atmospheric pressure. At the bottom of the two cylindrical extraction chambers there are two porous partitions which allow the passage of the liquid and block the solid matrix.



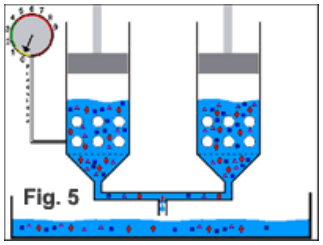
Step 2. After filling the system is closed and put under pressure (between 7 and 9 bar, generally). The pressure exerted by the pistons is transferred to the liquid as the two extraction chambers are connected via a duct.



Step 3. Once the set pressure has been reached, the system remains still for the time necessary to establish a balance between the inside and outside of the solid matrix. This is the static phase.



Step 4. After this period, the pistons are set in motion, generating an immediate lowering of the system pressure. In this way the dynamic phase begins in which the extractive principle is realized; the extractable substances are transferred into the extracting solvent due to a suction effect due to the negative pressure gradient created between the inside and outside of the solid matrix.



Step 5. The dynamic phase has among its functions that of mixing the extracting liquid, avoiding the formation of concentration gradients in the immediate vicinity of the exposed surface of the solid. The alternation of a static phase with a dynamic one constitutes an extraction cycle; repeating several cycles leads to the complete exhaustion of the solid matrix. At the end of the extraction the extracting solvent is expelled through a solenoid valve and collected in a special container.

Figure 6.9. Naviglio extractor ®

Gallo *et al.* (2020) evaluated the kinetics of the extraction process from female inflorescences of *Canapa sativa* subsp. *sativa* var. *sativa*, on the basis of determination of the content of cannabinoids: cannabidiolic acid (CBDA), Δ9-tetrahydrocannabinolic acid (THCA), cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC), before and after decarboxylation in the oven, in

order to evaluate the possible use of the hemp extract obtained in the food sector. The extraction yield was the same in the two cases examined and the final qualities were almost identical. However, the extraction times were significantly different: the maceration was completed in no less than 24 h, while with Naviglio extractor the extraction was completed in only 4 h (Figure 6.10).

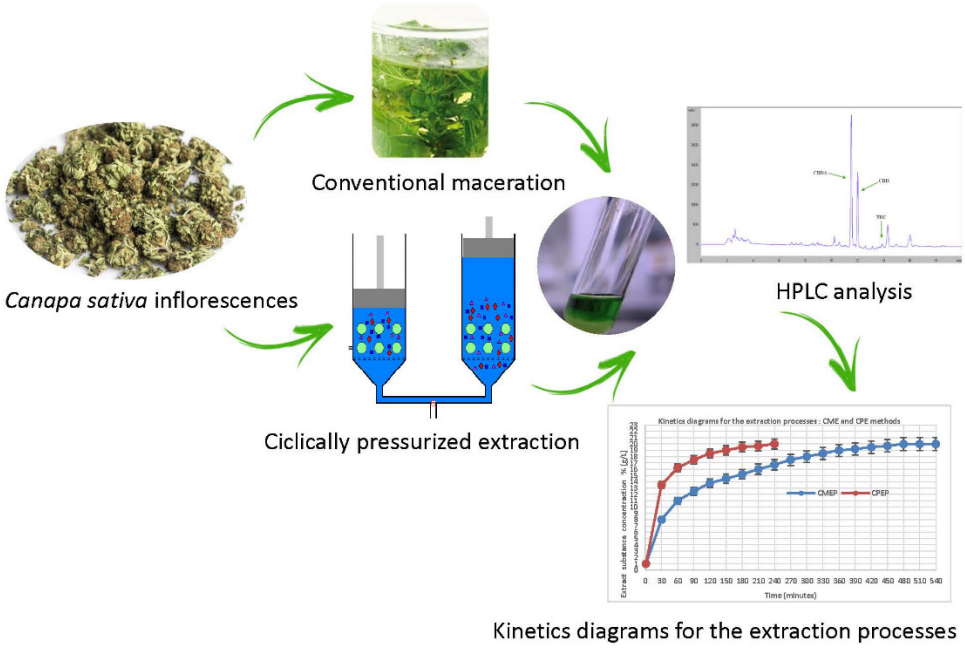


Figure 6.10. Extraction Process for The Production of Hemp Inflorescences Extracts by Means of Conventional Maceration and Rapid Solid-Liquid Dynamic Extraction (Naviglio extractor) [Gallo et al., 2020].

6.5. Essential oil extraction

Essential oils, which can be obtained from plant material that undergoes steam distillation, hydrodistillation or cold pressing, depending on the plant and the type of oil to be obtained. Content of essential oil in plant material is low, usually 1 to 3% of the plant weight. They consist of a complex mixture of chemical compounds, including terpenes, aldehydes, ketones, alcohols and others, which give them both their distinctive aroma and potential therapeutic properties [Aziz et al., 2018].

The essential oils are a complex mixture of volatile and fragrant organic substances of various chemical nature.

Some components of essential oils:

- MONOTERPENES antifungal, antimicrobial, antiphlogistic and healing properties (e.g. lavender, thyme, cypress, sage, lemon…);
- SESQUITERPENES calming, antifungal and disinfectant properties (e.g. lemon balm, ylang-ylang, cedar, juniper…);
- PHENOLS antifungal and antimicrobial properties (e.g. thyme, oregano, savory, nails carnation…);
- ALDEHYDES antibacterial, anti-inflammatory properties and mainly responsible for perfume (e.g. lemon, lemon balm, cinnamon, eucalyptus…);
- KETONES with balsamic and cholagogue-choleretic properties (they can also be toxic constituents) (e.g. mugwort, wormwood, rosemary, thuja, peppermint, sage officinal…);
- ESTERS with balancing, decongestant, antispasmodic and calming properties (e.g. laurel, lavender, chamomile, pink geranium…).

Characteristics of essential oils

1. composed of a complex mixture of hydrocarbons mono- and sesquiterpene and oxygenated materials, phenyl derived propanoids, compounds of acid metabolism fats and amino acids, nitrogenous and sulfur compounds;
2. they can be mono-, bi- or trimolecular, but for the most are polymolecular (>250);
3. strong, pleasant, mostly colorless or slightly odorless colorful;
4. made up of extremely volatile molecules;
5. they are insoluble or slightly soluble in water;
6. very soluble in alcohol, fat, acetic acid, ether, chloroform…;
7. lower specific gravity of water (EXCEPTIONS: essential oil of Cinnamon, Carnation, Mustard...).

The most-used method for essential oil extraction is steam distillation (Figure 6.11). It is used by different sectors as supplying raw materials for cosmetics, pharmaceuticals, aromatherapy, food flavorings, preservation, and homecare businesses [Machado et al., 2022].

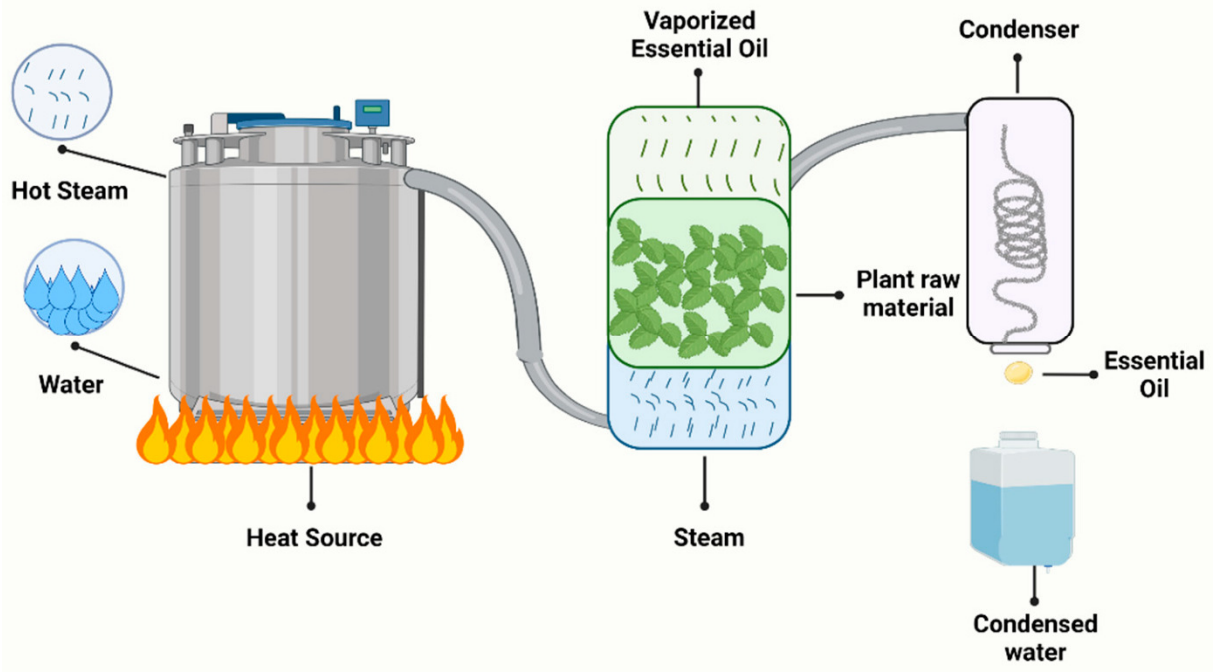


Figure 6.11. Overview of the steps involved in the steam distillation process [Machado et al., 2022].

This method takes advantage of some of the physical properties of essential oils previously reported. More precisely those concerning: volatility, solubility in water and density. Distillation is, in fact, a process through which the Essential Oils are separated from the tissues of the plant that contains them, through their "transport" by water vapour. A physical property of Essential Oils is volatility, that is, the ability to be easily transported (vaporized) by water vapor, despite their high boiling point. Once vaporized, Essential Oils are easily carried by the flow of water vapor.

The vegetable, suitably prepared, is placed in a "distillation chamber", into which water vapor is then introduced. This drags the essential oils with it, forming a mixture of Essential Oil + Water Vapor which, passing through a refrigerating column, condenses and quickly separates the water from the Essential Oil. This separation occurs because water and Essential Oils are immiscible with each other and are also of different densities. They separate by simple decantation. This type of extraction is the most used method for extracting Essential Oils as it offers a series of advantages:

A. The boiling temperature of the steam/Essential Oil mixture is close to 100 °C, still far from the boiling temperature of Essential Oils. It does not increase for the entire duration of the processing and this avoids the risks of deterioration of the Essential Oils and ensures good volatility of the same.

B. The water vapor swells the plant tissues, dilates the pores, breaks the essential cells, facilitating the escape of the Essential Oils.
C. The Essential Oil thus obtained is easily separated from the condensed water by decantation thanks to the fact that the two compounds are immiscible with each other and of different densities.
D. There is minimal loss of Essential Oil due to its very low solubility in water. This loss can be further contained by using the aromatic water, which has just separated from the Essential Oils, to regenerate steam.
E. The process has a very low cost (drinkable tap water is used) and does not create particular problems for safety at work.
F. The water vapor flow does not contain oxygen; therefore, Essential Oils are not subject to oxidation. The only disadvantage is the risk of experiencing possible thermal degradation and hydrolytic processes.

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