

***Tragopogon porrifolius:*  
phytochemical characterization and  
evaluation of the potential antitumor  
effect of the extract on the prostate cells**

Experiência profissional em Investigação  
e em Farmácia Comunitária

“Versão Final Após A Defesa”

Pedro Gabriel Plácido de Mendonça Rocha

Relatório de Estágio para a obtenção do Grau de Mestre em

Ciências Farmacêuticas



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**Orientadora:** Professora Doutora Ana Paula Coelho Duarte

**Co-orientadoras:** Professora Doutora Silvia Cristina Socorro  
Mestre Lara Raquel dos Santos Fonseca

**fevereiro 2024**

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## **Declaração de Integridade**

Eu, Pedro Gabriel Plácido de Mendonça Rocha, abaixo assinado, aluno com matrícula 38952 do Mestrado Integrado em Ciências Farmacêuticas da Faculdade de Ciências da Saúde, declaro ter desenvolvido o presente trabalho e elaborado o presente texto em plena conformidade com o Código de Integridade da Universidade da Beira Interior.

Mais especificamente, afirmo que não incorri em nenhuma das formas de Fraude Académica, de que declaro ter conhecimento e que, nomeadamente, cumpro a obrigatoriedade de referência de frases, excertos, imagens e outras formas de trabalho intelectual, assumindo assim plenamente as responsabilidades de autoria.

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Universidade da Beira Interior, Covilhã, 28 de fevereiro de 2024

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***“Give me a lifetime of promises and a world of dreams  
Speak a language of love like you know what it means...”***

- Tina Turner.

Excerpt from the song *"The Best"*,

from the album *"Foreign Affair"*, released in 1989.

***“Cos we've shared the laughter and the pain and even shared the tears  
You're the only one who really knew me at all...”***

- Phil Collins.

Excerpt from the song *"Against All Odds"*,

from the album *"Against All Odds"*, released in 1984.

***“I heard somebody whisper "Please adore me"  
And when I looked, the moon had turned to gold...”***

- The Marceles.

Excerpt from the song *"Blue Moon"*,

from the album *"Blue Moon"*, released in 1961.

***“The band was jumpin' and the joint began to swing  
You should've heard those knocked out jailbirds sing...”***

- Elvis Presley.

Excerpt from the song *"Jailhouse Rock"*,

from the movie *"Jailhouse Rock"*, released in 1957.

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## **Dedicatória**

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## Abstract

The present document is divided into 2 chapters being part of the unit "Internship in Pharmaceutical Sciences" incorporated in the curricular plan of the Integrated Master's in Pharmaceutical Sciences, culminating in its defense in public evidence.

The first chapter refers to a detailed subjective testimony based of the internship made in Farmácia Modelar from october 2021 to january 2022, under the supervision of technical director João Paiva. During this time, I explored a variety of areas, from management to filling patients prescriptions, gaining practical experience.

In the second part, an experimental study was conducted to assess the potential antitumor effect of an extract on prostate cells, under the supervision of teachers Ana Duarte and Silvia Socorro and also master Lara Fonseca.

In the previous mentioned study, *Tragopogon porrifolius*, commonly named salsify, is an edible herb used in traditional medicine with proven anti-tumorigenic activity in human breast and colon cancer cells, controlling oxidative stress, proliferation, and apoptosis. However, the effect of this plant in prostate cancer remains largely unknown.

This study aims to investigate the effect of *T. porrifolius* extract in modulating cell proliferation in human prostate cells.

*T. porrifolius* extract was obtained by an ultrasound-assisted solid-liquid extraction with ethanol/water (70/30) followed by a solvent evaporation in a rotovapor.

The chemical characterization was performed by determination of the total phenolic compounds using the *Folin-Ciocalteu* colorimetric method and the total flavonoid content by the Aluminum Chloride colorimetric method. The antioxidant activity was determined by DPPH and  $\beta$ -carotene methods.

Non-neoplastic (PNT1A) and neoplastic (PC3) cells were treated with 10 – 2000  $\mu\text{g/mL}$  of extract for 24, 48 and 72 hours. The results showed that this plant extract takes time to exercise his effects. Cell viability and proliferation were evaluated by the MTT essay.

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The *T. porrifolius* extract exhibited significant contents of total phenolics and total flavonoids, as well as a strong antioxidant activity using the DPPH method in reducing the radical to hydrazine.

Although preliminary, the results obtained indicate that *T. porrifolius* can inhibit prostate cancer cell growth with more pronounced effects in neoplastic cells. These findings support the further investigation on the possible use of this good cost-accessibility plant as a new potential therapeutic tool.

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## **Keywords**

“Antioxidant activity”; “Community pharmacy”; “Phenolics”; “Proliferation”; “Prostate cancer”; “Salsify” and “*Tragopogon porrifolius*”.

## Resumo

O presente documento está dividido em 2 capítulos integrantes da unidade curricular “Estágio em Ciências Farmacêuticas” incorporada no plano de estudos do curso de Mestrado Integrado em Ciências Farmacêuticas, lecionado na UBI, culminando na sua defesa em prova pública.

O primeiro capítulo refere-se a um detalhado testemunho subjetivo baseado no estágio realizado na Farmácia Modelar de outubro de 2021 a janeiro de 2022, sob a supervisão do farmacêutico diretor técnico João Paiva. Durante esse período, explorei diversas valências, desde a gestão de armazém até ao atendimento ao balcão, adquirindo experiência prática.

Na segunda parte, realizou-se um estudo experimental para avaliar o potencial efeito anti-tumoral de um extrato nas células da próstata, sob a supervisão das professoras Ana Duarte e Silvia Socorro e ainda da mestre Lara Fonseca.

A *Tragopogon porrifolius*, comumente denominada de “Salsifi”, é uma raiz comestível utilizada na medicina tradicional. Estudos demonstraram que a *T. porrifolius* exibe uma atividade antitumoral em linhas celulares de cancro da mama e do cólon, através de um mecanismo de controlo do stress oxidativo, a proliferação e a apoptose. Um possível efeito antitumoral desta planta no cancro da próstata permanece em grande parte desconhecido.

Este estudo teve como objetivo investigar o efeito do extrato de *T. porrifolius* na modulação da proliferação celular em células da próstata.

Obteve-se o extrato de *T. porrifolius* através de uma extração sólido-líquido auxiliada por ultrassom em etanol-água (70/30) seguida de evaporação do solvente em rotovapor.

A caracterização química realizou-se pela determinação dos compostos fenólicos totais pelo método colorimétrico de *Folin-Ciocalteu* e do teor de flavonóides totais pelo método colorimétrico do Cloreto de Alumínio. A atividade antioxidante determinou-se pelos métodos de DPPH e  $\beta$ -caroteno.

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As células não neoplásicas (PNT1A) e as células neoplásicas (PC3) trataram-se com 10 – 2000 µg/mL de extrato durante 24, 48 e 72 horas. Os resultados demonstram que este extrato demora algum tempo até produzir um efeito quantificável. A viabilidade e a proliferação celular foram avaliadas pelo ensaio de MTT.

Em suma, o extrato de *T. porrifolius* exibiu teores significativos de compostos fenólicos e de flavonóides, além de uma relativa forte atividade antioxidante. Embora preliminares, os resultados obtidos indicam que o extrato de *T. porrifolius* pode inibir o crescimento de células do cancro da próstata, com efeitos mais pronunciados em células neoplásicas. Estas descobertas apoiam a investigação adicional sobre o possível uso desta planta de boa acessibilidade como uma nova ferramenta terapêutica.

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## **Palavras-Chave**

“Atividade antioxidante”; “Farmácia Comunitária”; “Fenólicos”; “Proliferação”; “Cancro da Próstata”; “Salsifi” e “*Tragopogon porrifolius*”.

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## List of Acronyms

ADIFA	Association of Pharmaceutical Distributors.
ANF	National Association of Pharmacies.
APA	Portuguese Environment Agency.
APIFARMA	Portuguese Pharmaceutical Industry Association.
ASP	Average Sales Price.
BMI	Body Mass Index.
BPH	Benign prostatic Hyperplasia.
CGD	General Cash Deposits.
CNPEM	National Code for the Electronic Prescription of Medicines.
COVID-19	Disease caused by the <i>SARS-CoV-2</i> virus beginning in 2019.
DGAV	Directorate-General for Food and Veterinary.
DGAE	Directorate-General for Economic Activities.
EtOH	Ethanol.
E.G.	<i>Exempli gratia</i> .
FEFO	First-Expire, First-Out.
FIFO	First in, first out (FIFO).
FGP	Portuguese Galenic Form.
FM	Modelar Pharmacy.
H <sub>2</sub> O	Water.
INN	International Non-Proprietary Name.
INFARMED	National Authority for Medicines and Health Products, I.P..
MNSRM	Non-Prescription Drugs.
MNSRM-EF	Non-Prescription Drugs Exclusively for Pharmacy Use.
MSRM	Prescription Drugs
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide.
NHS	National Health System.
NPS	Portuguese National Pharmacovigilance System.
NDC	National Drug Code.
PVA	Sale Price to Stockist.
PVF	Sale Price to Pharmacy.
QR code	Quick-response code, a two-dimensional matrix barcode.
RAM	Adverse Drug Reactions.
SAMS	Union of Bank Employees of the South and Islands.

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SARS-Cov	Severe acute respiratory syndrome caused by the Corona Virus.
SNF	National Pharmacovigilance System.
SDS	Sodium Dodecyl Sulfate.
URF	Regional Pharmacovigilance Units.
UBI	University of Beira Interior.
VD	Veterinary Drugs.
VAT	Value Added Tax.
WHO	World Health Organization

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# **Chapter 1 – Internship Report in Community Pharmacy – Farmácia Modelar**

## **Introduction**

The present curricular unit "Internship" is part of the 10<sup>th</sup> – and last – semester of the Integrated Master in Pharmaceutical Sciences, taught at the Faculty of Health Sciences, part of the University of Beira Interior (UBI), based in Covilhã, district of Castelo-Branco. This document consists of 2 components, the "Research Strand" and the "Community Pharmacy Strand", which together form the "Internship Report".

This report as a ponderation of 30 ECTS (*European Credit Transfer and Accumulation System*) – in a total of 300 ECTS in the course - and is mandatory for the completion of the course of "Integrated Master's in Pharmaceutical Sciences" and subsequent obtaining the degree of "Master in Pharmaceutical Sciences" by UBI if the final grade is higher than 10 values - on a scale of 1 to 20 values.

This measuring instrument is based on the European Credit Transfer and Accumulation System, created to standardize academic qualifications in European territory.

The internship took place at Farmácia Modelar (FM), located in Teixoso, municipality of Covilhã, under the tutelage of the pharmacist technical-director João Paiva, leader of the team that welcomed me from October the 4<sup>th</sup> of October of 2021 to the 28<sup>th</sup> of January of 2022 lasting for about 116 days.

In the beginning, I was presented by the team with an action program divided into several objectives and outlined steps to make my learning process as succinct and productive as possible, with the primary focus on my training as a health professional through weekly summaries (written or oral).

Currently, the community pharmacy is one of the main contacts of the patient with the National Health System (NHS). It is one of the places closest to users and offers several services to the population, including pharmacotherapeutic counseling and monitoring, thus distinguishing itself from other NHS institutions [1,2]. The community pharmacy is a space of special importance. It not only has the simple role of dispensing medicines to the user, it also has a very relevant role with regard to minimizing the risks associated with medicines and raising awareness and educating the population, thus contributing to the reduction of the overload in hospital emergencies [3].

Increasingly, the pharmacist's activity and concerns are focused on the well-being of the patient and the quality of the services provided. This health promoter must go beyond the act of dispensation, but must also assume a role in promoting adherence to therapy and the rational use of medication [1].

## Organization and characterization of the Pharmacy

### Geographic Location

The FM is located at Rua João de Deus, number 38 in the União de Freguesias Teixoso-Sarzedo, in the municipality of Covilhã, district of Castelo-Branco as shown in the satellite view of **figure 1** about the geographic location.



**Figure 1:** Geographical view (satellite) of FM.

### Public Service Hours

It's opening hours are from 9:00 a.m. to 7:30 p.m., closing on Saturday and Sunday afternoons complying with the weekly obligation of a minimum of 44 hours, according

to the published in the Portaria n<sup>o</sup> 277/2012 [4], presenting the following temporal distinctions:

- Monday to Friday: Service from 9:00 a.m. to 19:30 p.m;
- Saturday: Service from 9:00 a.m. to 1:00 p.m;
- Sunday and Holidays (covering national and religious): Attendance from 10:00 a.m. to 1:00 p.m.

The FM has two times of greater affluence, the morning time of 10:00 to 12:00 hours and the afternoon time of 17:00 to 19:00 hours, despite the changes due to external factors (eg: presence of the doctor in the office, weather conditions, the user's desire to move ...).

## **Population**

A pharmacy must assume the characteristics (for example, medication, extra services available and decoration, except for mandatory exceptions) of the population in which it operates, which in this specific case, translates into an older population, with a tendency to illiteracy.

## **Human Resources**

The technical team is the first line of contact of the user with the establishment, in this case the FM. It becomes crucial to recruit a serious, qualified and available team for a correct management of the various areas that make up the pharmacy.

This team of 6 elements, represented in Table 1, currently consists of 3 Pharmacists, 2 Pharmacy Technicians and 1 Accountant, people with distinct functions but symbiotic with each other. In correct nomenclature, “Pharmacists” are the professional directly involved in patient care, working with existing drugs, while the “Pharmaceutical” are scientists who create new drugs, therapies, and approaches to maximize benefit established therapies.

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**Table 1:** Human resources actively present on FM.

<b>Name</b>	<b>Function</b>
Álvaro Míngote	Chartered Accountant
Cristina Paiva	Pharmacy Technician
Diana Lopes	Pharmacist
João Paiva	Pharmacist and Technical Director
José Paiva	Pharmacy Technician
Tânia Correia	Pharmacist

According to the Republic Diary, article nº 21 of Decree-Law nº 75/2016 of 8 November, as amended by Decree-Law nº 171/2012, of 1 August, the responsibilities are subdivided into the following topics.

Pharmacist Technical Director: Assumes full responsibility for the pharmacist acts practiced in this pharmacy, ensuring compliance with the rules and informing and ensuring the due clarifications like the manipulation of medicines, promoting their rational use and ensuring a constant evolution of thought in order to accompany both the pharmaceutical market and its innovations.

He should also insure the team follows the rules of dispensing Prescription Drugs and justify if required.

Also ensure compliance with the requirements of hygiene, security and supply of space [5].

Pharmacy Technician: Ensures the correct service and advice to the user, with pharmaceutical supervision, also ensuring the maintenance of the internal requirements of the medicines, from their receipt, computer notes, stock management, storage of orders with parallel verification of the expiration dates (a way to facilitate future work), verification of biochemical parameters, control of the conditions of the pharmacy [5].

### **Computer Resources**

Currently, the pharmacy has the software Sifarma® (and SifarmgEST) created by Glintt© as an indispensable tool to the daily life of the pharmacy, a program with information of each user, making the whole process of care safer, faster and not conducive to human errors [6].

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It is subdivided into 3 modules "Sifarma Atendimento", "Sifarma Clínica" and "Sifarma Encomendas".

Being a software, it is amenable to updates and internal improvements, so the pharmacy has both versions "Sifarma 2000" (the past one) and the "Sifarma.MA" (the current one) integrating functions that the current one does not yet have.

The "Sifarma.MA" presents an increased efficiency in the management of the time of the professional in attendance, allowing the organization of essential information about the user.

In the duration of the internship, I worked primarily with the current version, using the old one in specific situations such as getting the profile of older users to confirm medication marks.

The layout of the page, the simplicity of the color palette and the assignment of tabs allow a faster and more concise service.

Among some facilities:

- Simultaneous attendance of one or more users;
- Receipt and consequent processing of orders;
- Management of orders and pharmaceutical inventory;
- Control of validity limits;
- Price assignment to prescription drugs.

In the upper right corner there is a button that allows you to view information about the product in question:

- Quantitative and qualitative composition;
- Secondary adverse reactions;
- Contraindications;
- Therapeutic group;
- Therapeutic indications;
- Dosage;
- Drug interaction (Light, Mild and Severe).

In the "new user" tab, there is the possibility of creating a user form, an essential record of the most prevalent information about it.

It is essential to have an organized user file updated so that the professional who is dispensing the drug can consult the information without ever having seen the user, being

an added value when the user cannot describe the packaging or the designation of the drug. Overall, it improves pharmacotherapeutic follow-up.

The software allows the pharmacist to have full control over the existing pharmaceutical inventory, allowing the order of a product - whose inventory is necessary to reinforce - through daily orders or if the product has exhausted or does not have a defined inventory (instant orders).

The system is divided in different modules and offers notifications of daily orders for a particular product depending on the established limits of replacement.

The patient care module is the most used, allowing access to the clinical characterization of the user, as well as notes on the same (e.g. if you usually comply with the doses, some extra warning) and previous medication.

There is also the distinction between “prescription drugs”, compliant or not, and “free dispensing products”, that is, those that do not require a prescription.

The old program allows the pharmacist to make the "Suspended Sale", where the professional can give the drugs at the moment without the presentation of a medical prescription, committing the user to bring a prescription later, a condition that is not present in the Sifarma.MA, and all products subject to medical prescription are subject to reimbursement.

## **Equipment**

The team has properly differentiated rooms with enough material to perform the most varied tasks [7]:

- Communication Technology (e.g. landline, mobile, computers, makes, printer of booklets and information labels and citizen card readers).
- Security Equipment (e.g. internal video surveillance system in closed circuit, alarm, fire extinguishers, exit plates);
- Thermo hygrometers;
- Standard blood pressure measurement equipment;
- Instruments for measuring biochemical parameters.

According to the “Manual of Good Practices for Community Pharmacy”, the pharmacist technical director ensures the levels of quality and safety, as well as the plans for hygiene, calibration, maintenance and correct handling [1].

## **Physical Space**

### **Outdoor space**

The FM is located on the ground floor of a residential building and is duly identified with a sign inscribing the name of the pharmacy and with a green cross illuminated during opening hours, as stipulated by article 28 of Decree-Law n<sup>o</sup> 75/2016 of 8 November [5]. Along the front of the pharmacy are also displayed various information, such as the name of the technical director, the opening hours, the shift schedules of the pharmacies in Covilhã, the services provided by the pharmacy, the license number and the existence of a complaints book [5].

With regards to access to the pharmacy, accessibility is guaranteed to all users, since the entrance to the pharmacy is at the level of the sidewalk and there are no stairs or unevenness that hinder access for users with reduced mobility and/or disabilities [3].

The front of the pharmacy, composed of two glazed windows, also features billboards and advertising for some products that inform about public health campaigns and that are changed periodically according to the campaigns in force.

### **Interior Space**

It complies with resolution n<sup>o</sup> 1502/2014 of July 3, with the following mandatory spaces:

- Warehouse;
- Space of general service to the community;
- Private and personalized service space;
- Sanitary Installation;
- Biochemical laboratory (adapted).

The pharmacy also has an office with exclusive access to the pharmacist technical director [8].

### **Warehouse**

Contains the majority of the pharmaceutical inventory at the level of Prescription Drugs and natural products, dressings, compresses, compression stockings, blood glucose measurement. VD are on the shelf next to COVID-19 (surgical masks).

There is also a shelf containing products in terms of validity, considering less than 4 months.

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The organization of the inventory follows a logic of steps, starting with the separation by pharmaceutical form and/or place of applicability and, after this division, sub-divide alphabetically still paying attention to the increasing dosage. It is in my opinion a viable option, easy to implement and correct execution, facilitating access with training.

The Medicines considered "DANGEROUS" by the legislation are confined to a closet of restricted access, including Narcotic and Psychotropic Drugs.

### **Biochemical laboratory (adapted)**

Of reduced activities, the main utility is the storage of raw materials and material to perform pharmaceutically manipulated products, which includes extemporaneous preparations, by joining a powder with ultra-purified sterilized water.

### **Private and personalized service space**

It is the space where the user can expose the problem without compromising his security, being in a closed space with a safe perception.

At the same time, it is used to administer injectables (e.g. insulin), measure blood pressure and some biochemical parameters using its own machinery (e.g. glycaemia, total cholesterol, triglycerides and or uric acid), as well as evaluation consultations.

### **Office of the Pharmacist Technical Director**

It is the administrative space properly equipped with a desk, computerized, multifunction printer and a cabinet with vital documentation for the legal and financial papers, containing files properly organized with material such as invoices for sales of narcotics and psychotropics, prescriptions, accounting aspects, temperature and humidity records and return notes. They also contain relevant pharmaceutical bibliography for consultation in doubts, including the *Portuguese pharmacopoeia*, the *Portuguese galenic form* and the *Pharmaceutics medical record* [3].

Behind the warehouse, there's a common area where the team can make the reception of orders, containing a Sifarma computer system and a refrigerator for thermolabile medicines (e.g. Vaccine, Colirium or Insulins).

## **Scientific Valencia**

The community pharmacy is one of the points of interaction between the user and his health, which is why the pharmacist, as an advocate of adequate dispensing, must have the means to access updated and constantly relevant information, accompanied by the demands of the current market.

As discussed earlier, the Sifarma software presents a cluttered database of scientific information, as well as fast internet access to Infomed (drug database) and the search for scientific articles.

In their visits, the medical delegate brings information leaflets and magazines that allow a consultation by health professionals and additional copies for delivery to the user.

## **Pharmacy Management**

### **Suppliers**

One of the initial phases of a pharmaceutical company is the right choice of suppliers of drugs. Since the market is competitive, it is essential to analyze the offer and try to understand the most advantageous for the pharmacy, whether in the short or long term. Some of the reasons are related to the periodicity of deliveries, the general quality of the service, availability of products, policy of returns, prices charged and discounts.

In the FM, *Plural* dominates the majority of the delivery of inventory, followed in reserve by Alliance Healthcare and OCP, safeguarding the possibility of inventory breakage of a given product in one supplier and its existence in another. There is also another way of acquiring products, through contact with the laboratories and/or through delegates, who regularly visit the pharmacy presenting the appropriate promotions. This factor is of some importance, allowing the pharmacy to keep up to date in the market.

### **Inventory**

One of the objectives of the pharmacy at a financial level is to maintain a diversified and at the same time cost-accessible inventory, capable of satisfying the needs of users, taking into account the discounts, the existing publicity in the media, the monthly rotation (taking into account its availability) the selling price to the public (P.V.P.) and the seasonality, but the type of users (age and economic condition) and their individual needs are the important factors.

## **Orders**

There are 4 types of orders that can be placed:

- Daily;
- Direct;
- Instant;
- Green Way.

### **Daily Order**

The pharmacist enters the data in Sifarma, analyzes the minimum and maximum inventory of the products and then creates a list of products, taking into account the reservations.

The person in charge confers, approves (or amends if necessary) and sends.

There´s 2 being held: 1 in the late morning and 1 in the late afternoon.

### **Direct Order**

It applies if the benefit of the pharmacy is visible, acquiring at the most competitive price.

### **Instant Order**

During the service to the user, it can happen that the pharmacy has not a determined product in stock. To solve the situation, Sifarma is used to order and book with the "User Name" and "Date", delivering an estimated arrival to the user, usually in the same day.

### **Order using Green Way**

If the user requests a drug in market shortage, this function can be used, guaranteeing the supply within a maximum period of 48 hours after the order.

Alternatively, telephone is also feasible, with the intention of needing a correct and consistent record so as not to conflict with the Sifarma inventory and inventory duplication to occur [9].

### **Order Reception**

In this situation, we chose to receive each other at different times:

*Tragopogon porrifolius*: phytochemical characterization and evaluation of the potential antitumor effect of the extract on the prostate cells

Professional Experience in Research and in Community Pharmacy

- Morning – Reception of orders from the previous afternoon.
- Afternoon – Reception of morning orders.

In the case of sensitive products, they are packed in plastic boxes duly identified with the supplier and the pharmacy are intended not to be confused with other pharmacies, always having within the respective invoice or packing slip the following information:

- Invoice Number;
- National Drug Code (NDC);
- Date of delivery;
- Quantity supplied;
- Quantity ordered;
- Retail Resale Price (RRP);
- Sale Price to the Storekeeper;
- Sale Price to the Pharmacy.

The cycle of receipt of the order begins with the arrival of the order to the warehouse and subsequent opening of the boxes, verifying their content and integrity of the boxes, checking that the order is correct, being the recipient, products and quantities correct.

In the second instance, the operator must enter the new products in the Sifarma system in order to allow the updating of the inventory. To do this, the "Order Reception" tab opens where all orders to be received are located.

Each order from each supplier has associated a NDC and many times in the same invoice in several orders from the same supplier, so it becomes necessary to group the various NDC and generate a single order by entering the following details:

- Barcode (alternatively, the invoice number);
- Total Order Value (if applicable, account for the transport fee);
- Number of units ordered (one unit is a pharmaceutical product)

After this, the insertion of the products in the system begins, using the Quick-Response code (QR code) on each package (the new legislation has approved this) in favor of the barcode for becoming a technological advance, however in MNSRM it is not yet mandatory.

After entering the QR code of the product, the shelf life (the length of time for which an item remains usable) must be checked, changing in the system whenever the validity of the product received is lower than that of the same product in inventory.

The thermolabile medicines come in bags suitable for this purpose with 2 strips of ice to prevent degradation by temperature, which, if present in the order, should be a priority upon reception and stored immediately in the refrigerator.

It is important to find discrepancies between the RRP, that is updated whenever there are differences and “not existing product” in inventory or if there is such a pipeline should be grouped in the same group with new price and sell last.

Finishing the insertion in the system, the registered quantity equivalent to the quantity received is checked one last time, the PVF and the respective discounts (information present in the invoice) are manually corrected, checking in the last instance if the total invoiced and the number of packages correspond to that indicated on the invoice.

The pharmacy stipulates (ethically, following a market evaluation) the price of the class of OTC drugs, such as dermo-cosmetic products, cough lozenges, oral elixirs and dietary supplements taking into account profit margins and PVF.

We must also check the necessary times to minimize the error inherent in the human aspect to improve the pharmaceutical resources available and the final check-up, the receipt of the orders is finalized and the list is verified where all the products including those that did not come can be reordered from another supplier or, if there are any outstanding products, wait for the next delivery.

If necessary, label the products and identify the reservations with the name of the customer and in order to better organize the resources, the chosen drug can be near the end of validity meaning that has arrived in the first instance to the pharmacy.

Opting for the telephone route, the only difference is the necessity to create a complete order in the system.

As previously seen, one of the first and most important organizational tasks in any pharmacy is to understand where all the products are stored and the correct way the pharmacy operates.

## **Pricing**

It is subdivided into two types [10,11]:

- Prescription Drugs – The attribution of RRP is the responsibility of Infarmed, these being pre-defined by decree-law and duly printed on the packaging of the drug next to the QR code.
- Non Prescription Drugs - The allocation of RRP is the responsibility of the Pharmacy, and these are defined based on the most concentrated profit margin

of the pharmacy, considering the Value Added Tax (VAT) at 6% or 23%, respectively.

The decree-law n<sup>o</sup>4699/2020 establishes the limit of the percentage of profit of pharmacies, a fact verified in time of COVID Pandemic where the profit in disinfectant gel, ethyl alcohol and personal protective equipment was limited to 15% [12].

## **Returns**

A return note must be made if either the product has reached its end of the shelf-life, damaged packaging, wrong product, recall of the product from the market or suspension of its marketing.

The previous version of Sifarma (version 2000) contains a module called "Returns" that creates a return note that should contain:

- Distributor;
- Product name;
- Quantity to be returned;
- Reason for return;
- Invoice number.

Then, print in triplicate to file a copy at the pharmacy as proof of the return and stamp the other two along with the product you want to return.

Upon receipt of the returned product, the supplier analyzes the situation and decides whether to approve (or not) a credit note, returning all or part of the value or even the replacement of the product with a new or other different product of equal value.

If the return is not accepted, the product is forwarded to the pharmacy and is ultimately destroyed.

## **Inventory Management**

The core of the pharmaceutical profession, it presents in the form of an inventory, a set of medicines inherent in a pharmacy is essential a rigorous and controlled management so that there are no unnecessary expenses or additional ones due to lack of attention.

The inventory of a product should be controlled so as not to be excessively high (in order to monopolize this product and force the resale in higher quantities) because it causes the pharmacy monetary losses caused by 2 inherent situations, the non-disposal of the product and the approach of the end of validity.

On the other hand, the inventory should not be equally low to combat user demand and should not make up sales.

A dynamic balance between the products ordered (inventory) and the products dispensed (sale) is crucial, having as a criteria the turnover and seasonality, with periodic updates and special attention to the associated media marketing, because a product that uses the media is liable to higher sales.

The programs “Sifarma 2000” and “Sifarma Gest” allow an analysis of the turnover of a product through the registration of entry or exit of the same.

### **Shelf Life**

A factor that if uncontrolled can amount to significant expenses leaving the pharmacy without the respective profit.

In the FM, a complete inventory is constantly carried out upon receipt of new orders (to make sure that the new products are well registered), at the end of each month (ensuring the incorporation of the new inventory with the existing one) and quarterly on the day designated for inventory collection.

The person responsible for receiving orders must check and enter the date if there is no inventory of the product or if the validity of the same is lower than the validity recorded in the program.

At the end of each month, the survey and control of the validity periods is carried out through a list of products whose shelf life is equal to or less than 4 months and whenever the product has a shelf life greater than that indicated in the listing, this period is updated in Sifarma. Also, if the product is less than or equal to 4 months, it's placed on a proper shelf with indication for future return to the supplier.

### **Atmospheric Parameter Logging**

Temperature and humidity are 2 factors that are indispensable to correctly store and preserve the products, being controlled by 4 thermohydrometers located in the front office, back office/warehouse, laboratory and refrigerator.

This control is done every Friday to verify the minimum legal requirements:

- Warehouse and Service: 15 – 25 °C;
- Cold: 2 – 8 °C.

The humidity should be equal or less than 60%.

In an exceptional regime, it should be pointed out why it has not been possible to mark these precise values and their solution.

## **Drug Preparation**

### **Raw materials**

They are the basis of the products handled, with the criteria being specified in the monography where they are registered, accompanied by the respective analysis report that ensures compliance with the text and are mainly purchased from suppliers authorized by INFARMED.

### **Manipulated products**

Once a pharmaceutical perk, it ends up falling into disuse, becoming a practice replaced by the technological development of the industry.

It's function doesn't change, filling the gap of not having a product that meets the needs of the user, that does not have the desired pharmaceutical form or without the necessary dosage.

Its classification is divided into [13]:

- Magistral Preparation – Preparation following the guidelines inscribed in the medical prescription, which the doctor must follow the guidelines present in the Portuguese Pharmacopoeia, in the Pharmacopoeias of other Member States of the European Union and in published scientific documentation.
- Officinal Preparation – Preparation following the guidelines of the *Portuguese pharmacopoeia*, the *Portuguese galenic form*. This practice can also be designated by "Make ByArt" (F.S.A.) giving the pharmacist freedom of choice.

Before beginning, the manipulated sheet must be filled with all the crucial information, and at the end it is mandatory to perform a quality control check-up, with pharmaceutical supervision through 3 conditions [14]:

- Organoleptic characteristics (e.g. color, smell, opacity,...);
- Hydrogen potential (pH);
- Amount dispensed.

A validity interval shall be assigned according to the *portuguese galenic form*, taking into account:

- Aqueous liquid preparation – Store at refrigerator temperature (4°C) for a maximum of 14 days;
- Solid and liquid non-aqueous preparations – Considering the presence of an industrialized product as an active substance, it has a shelf life equal to one quarter (25%) of the value that remains until the expiration of the shelf life of the same, up to a maximum limit of 6 months.
- Other preparations – Follow indication in the treatment up to a maximum of 30 days.

### **Retail Resale Price (RRP)**

The equation listed below (1) sums the value of raw materials with the value of packaging material and the value of handling fees, the latter of which does not apply to bulk substances (raw goods with huge quantities) [15]:

$$\text{RRP (€)} = [(\text{Raw material}_{\text{value}} + \text{Packaging material}_{\text{value}} + \text{Handling fees}_{\text{value}}) \cdot 1.3] + \text{Added Tax}_{\text{value}} \quad (1)$$

### **Labelling**

It presents has the final step, with printed paper that will include important details, namely [14]:

- Identification of the pharmacy (e.g. name, contact, location,...);
- Name of the pharmacist technical director;
- Retail Resale Price (RRP);
- Lot number;
- Formula of the manipulated;
- Validity Interval;
- Recommended dosage;
  - Officinal Preparation - Name of the costumer for which it is intended;
- Extra instructions (e.g. "EXTERNAL USE", "SHAKE BEFORE USE", ...).

## **Extemporaneous Preparation**

In this type of preparation, all formulations need reconstitution at the time of giving medication to ensure their viability.

Being a reconstitution process, it presents a solid side (previously lyophilized granules or powders) and a liquid side (purified water) that combined give rise to solutions (water/soluble solvent) or suspensions (immiscible solvent with water).

The technician should start by shaking the closed bottle (releasing the powder present on the walls due to the time on the shelf) and the bottom (sedimentation by time) added then 2/3 of the total volume in purified water, close and shake vigorously to loosen the powder as much as possible. After this step, complete to the reference meniscus, close and shake vigorously until obtain a mixture as homogeneous as possible.

At the time of dispensing, the costumer must be warn that “before using should shake well” and also pay attention to the conservation information (e.g. sun exposure and humidity).

## **Patient-Pharmaceutical-Drug Cycle**

### **Community Service**

A cycle is characterized by a set of actions that end up repeating themselves, which happens a lot in our profession, where the patient presents a situation to the pharmacist that will solve with professional advices and, if applicable, with the use of medication.

Thus, the pharmacy team presents an extremely active role in promoting the health and well-being of the patient, promoting the rational and optimized use of the drug and alerting to the undesirable effects if the user does not adhere to the prescribed therapy.

The patient, as a an individual for whom a practitioner has prescribed a drug, is the focus of our entire profession and should always be our number 1 priority.

We also act as teachers, helping solving the doubts that the user has in relation to the current disease, its changes and the respective practical solutions, always remembering that each patient is an isolated case and, even in similar circumstances of having happened, should be considered unique focusing on the therapeutic adherence.

In a first contact, the pharmacist must find techniques to be able to understand the general life of the patient, namely his degree of literacy (in order to try to frame the explanation as simplistic as possible) and his financial capacity.

In a first approach, in order to comply with the principles of our profession, we must opt for non-pharmacological measures (in case of counseling) and also visualize other problems (e.g. obesity, sedentary lifestyle,...) and offer advice to the problems exposed. Having communication as an essential role, the pharmacist must be empathetic, positive, with eloquent and careful language, an elegant posture and prompt care, passing the information as clear and concise as possible promoting a correct understanding by the patient.

### **Conflict Management**

Like most professions, conflicts are a recurring fear that, after some training, becomes spontaneous situations of simple resolution.

These conflicts stem primarily from disbelief in pharmacists, much of it sparked by the human inherence that we try to deceive each other and mainly motivated by the information presented by the media.

In order to solve this situation, the pharmacist must understand that inside the pharmacy it represents the institution and the health industry. Any complaint (as long as it is not on personal offense) should be understood by the professional as “tired state” by the patient in relation to stress, personal life or actions of the day. In that instance, we must remain calm, listen to complaints to understand the reasons for the conflict and argue as best as we can according to the situation, always applying a calm and honest approach, starting with simple statement like *"I Understand Your Situation"*, trying to put ourselves in the position of the same and uncomplicating the situation as much as possible. In sum, the conflict should not be seen as a problem but as a need to be resolved with the techniques that have the professional has learned.

### **Pharmacovigilance**

As defined by the World Health Organization (WHO), “Pharmacovigilance” is the act of detecting, recording and evaluating Adverse Drug Reactions (ADRs) in order to determine the incidence, severity and causal link with the medication [16,17].

The Portuguese National Pharmacovigilance System (NPS) consists of 10 Regional Pharmacovigilance Units with the purpose of ensuring the safest possible use of a drug, providing adjacent information present on the Infarmed website in the section "Medicines for Human Use", sector "Pharmacovigilance" in the sub-sector "Pharmacovigilance System":

**Pharmacovigilance Unit of Azores:**

- Divino Espírito Santo Hospital of Ponta Delgada, EPER.
- Address: Avenida D. Manuel I, 9500-370 Ponta Delgada.
- Phone: 296 203 030
- Email: [ufa@azores.gov.pt](mailto:ufa@azores.gov.pt)

**Pharmacovigilance Unit of the Algarve and Baixo Alentejo (UFALBA):**

- University of Algarve – Department of Biomedical Sciences and Medicine (DCBM).
- Address: Campus de Gambelas, North wing of building 2, room 2.52, 8005-139 Faro.
- Phone: 289 800 065
- Email: [ufalba@abcmedicalg.pt](mailto:ufalba@abcmedicalg.pt)

**Pharmacovigilance Unit of Beira Interior:**

- Faculty of Health Sciences (FCS) - University of Beira Interior.
- Address: Avenida Infante D. Henrique, 6200-506 Covilhã.
- Phone: 275 329 070
- Email: [ufarmabi@fcsaude.ubi.pt](mailto:ufarmabi@fcsaude.ubi.pt)

**Pharmacovigilance Unit of Braga:**

- Braga Hospital.
- Address: Sete Fontes – São Victor, 4710-243 Braga.
- Phone: 253 027 611.
- Email: [farmacovigilancia.braga@hb.min-saude.pt](mailto:farmacovigilancia.braga@hb.min-saude.pt)

**Pharmacovigilance Unit of the Center and North Alentejo - UFV-CNA:**

- University of Évora.
- School of Nursing of São João de Deus, Largo do Senhor da Pobreza, 7000-811 Évora.
- Phone: 266 730 318 (319)
- Email: [ufv\\_cna@uevora.pt](mailto:ufv_cna@uevora.pt)

**Pharmacovigilance Unit of Coimbra:**

- AIBILI.
- Address: Azinhaga de Santa Comba, Celas, 3000-548 Coimbra.
- Phone: 239 480 138 (111)
- Email: [ufc@aibili.pt](mailto:ufc@aibili.pt)
- Website: [www.ufc.aibili.pt](http://www.ufc.aibili.pt)

**Pharmacovigilance Unit of Guimarães:**

- Guimarães Hospital.
- Address: Rua dos Cutileiros, Creixomil, 4835-044 Guimarães.
- Phone: 253 421 011
- Email: [farmacovigilancia@hospitaldeguimaraes.min-saude.pt](mailto:farmacovigilancia@hospitaldeguimaraes.min-saude.pt)

**Pharmacovigilance Unit of Lisbon and Setúbal and Santarém:**

- Faculty of Pharmacy of the University of Lisbon.
- Address: Avenida das Forças Armadas, 1649-019 Lisboa.
- Phone: 217 971 340
- Email: [ufs@ff.ulisboa.pt](mailto:ufs@ff.ulisboa.pt)

**Pharmacovigilance Unit of Madeira:**

- Institute of Health Administration, IP-RAM.
- Rua das Pretas, number 1, 9004-515 Funchal.
- Phone: 291 212 352
- Email: [farmacovigilancia.drs@madeira.gov.pt](mailto:farmacovigilancia.drs@madeira.gov.pt)

**Pharmacovigilance Unit of Porto:**

- Faculty of Medicine of the University of Porto.
- Address: Rua Doutor Plácido da Costa, 4200-450 Porto.
- Phone: 220 426 952 /220 426 943
- Email: [ufporto@med.up.pt](mailto:ufporto@med.up.pt)
- Website: [www.ufporto.med.up.pt](http://www.ufporto.med.up.pt)

The pharmacist and the patient can report any suspected unwanted reactions resulting from the use of a given drug, regardless of its severity, in order to expand the safety profile.

The general population can also notify any unwanted ADR, through the portal of Infarmed, providing an update essential to a continuous monitoring of the safety of the drug.

The notification process can be made in 3 ways:

- Call the respective pharmacovigilance unit;
- Form (on paper);
- Form (online) in the reporting portal for suspected adverse drug reactions (PORTAL RAM).

There is also some essential information to take into account:

- Concomitant preparation;
- Date of initiation and discontinuation of the therapy;
- Dose;
- Duration and evolution of ADR;
- Lot;
- Route of administration.
- Trade name and brand of the drug;
- User information (diagnosed health conditions);

## **ValorMed**

Created in 1999, this non-profit company ascended from the collaboration between the pharmaceutical industry, community pharmacies and drug distributors with the responsibility of collecting and ensuring the treatment of empty packaging waste and discarded or expired medicines, using the acronym SIGREM, meaning Integrated Management System for Packaging and Medicines Waste and is supervised by the Portuguese Environment Agency (APA) and the Directorate-General for Economic Activities (DGAE) [18].

Currently, it has a share capital with members such as the APIFARMA (The Portuguese Pharmaceutical Industry Association), the ANF (The National Association of Pharmacies), the GROQUIFAR (The Association of Chemical and Pharmaceutical Products Wholesalers) and the ADIFA (The Association of Pharmaceutical Distributors) [18].

In this pharmacy, there are 2 containers: 1 in the service area (patients) and 1 in the warehouse area (pharmacy team).

When filled, they must be properly closed and labeled with the receipt printed on Sifarma that contains:

- Signature of the warehouseman;
- Pharmacist's signature;
- Pharmacy identification;
- Serial number of the collection container.

## **Drug Dispensing**

It is the most recurrent situation in the community pharmacy, being the core of the pharmaceutical act, with due responsibility as the well care of the patient is at risk [19]. Faced with a medical prescription or in responsible self-medication, the patient must possess the majority of information for the *day-on-day* care, like:

- Adverse side effects;
- Dosage;
- Duration of the therapy;
- Method of administration;
- Optimal storage conditions.

As in any dialogue, the spoken word must be self-explanatory, solving the doubts the patient presented .

An information sticker can be placed on the package (alternatively, written on the box) and in the case of illiteracy, some visual means such as drawings can be made.

From an early age, this practice is instilled as of high responsibility, to promote the correct and effective use of drugs and health products, especially in situations of self-medication. To ensure this practices, the pharmacist must internalize, reflect and advance the possible knowledge having some tricks, following the motto "*Work Smart Not Hard*".

## **Prescription**

### **Types of medical prescriptions**

In order to make their work accessible, the pharmacist must be aware of the main existing prescribing models.

The traditional paper model was a companion of our profession from the beginning, presenting a problem: the letter of the prescribing physician could be considered a language barrier.

More recently, from the guideline of the NHS, this model was replaced by the electronic model, following the technological trend and presenting universal characters to eliminate the reading comprehension and giving a correct understanding of both the drug and its indications, materialized or dematerialized.

The following situations are subject to manual prescription opportunity:

- Computer failure of the system;
- Maximum of 40 recipes in a month;
- Prescription at home.
- The prescriber is conditioned;

### **Manual Prescriptions**

Once the dispensing of the medicine is carried out, the pharmacist must print on the back of the prescription the respective medicines and quantities dispensed and their reimbursements.

Before delivering, he must stamp and put the date of dispense and also ask the patient for the signature (if the person cannot perform it, put only the initials of the name).

### **E-Recipes**

They are paperless electronic prescriptions (dematerialized) that consists of revenue transmitted electronically ("Email" or "Mobile") in the form of a treatment guide that can be accessed by users through the application "MySNS Wallet – The Electronic Health Wallet (CeS)".

In this situation, if the patient requests or the pharmacist finds it more appropriate, paper printing can be used to facilitate the control of the medication by the patient.

In sum, the main advantage of the electronic aspect in favor of the paper one is the interpretation by the pharmacist of the medical assumption, either in terms of medication or in terms of notes.

## **Analysis and Validation**

### **Dematerialized Electronic Recipe**

Faced with a dematerialized electronic prescription, the pharmacist must validate it by entering the access and dispensing codes in Sifarma, with a limit of 6 units per medication if it is a drug used for prolonged treatment.

### **Materialized Electronic Recipe (*e-recipe*)**

In the case of a materialized *e*-prescription, there are parameters that must be checked:

- Health system;
- Identification of the drug;
- Identification of the prescribing physician;
- Place of prescription;
- Name of the patient;
- Reimbursement regime (if applicable, orders and ordinances);
- Revenue number;
- Signature of the prescribing physician;
- Patient contact number;
- Validity of the prescription (30 days or 6 months).

In this case, the number of medicines is limited to a maximum of 4 types, and no more than 2 packs of the same medicine or more than 4 packages in the total prescription may be prescribed, except for medicines dispensed in an unit dose where the limit is reduced to 4 packages of the same drug.

### **Manual recipe (on paper)**

In this situation, in addition to the above parameters, other elements are required:

- Green sticker – Particular situation where the patient is a pensioner covered by the special co-participation scheme;

- Stamp or Manual Registration – If the patient had this prescription in private practice or private doctor.
- Vignette to identify the pharmacy;
- Vignette to identify the prescribing physician;

It is important to note that all drugs that have a generic aspect are mandatorily designated by the International Nonproprietary Name (INN) of the active substance, a proposal adopted or proposed by the WHO for active substances of medicines obeying to certain rules that cannot be the subject of trademark or name registration.

There are some exceptions that allow prescription by brand name, namely:

- Drug with a short therapeutic margin;
- Previous ADR with the same active substance but different brand;
- Treatment lasting more than 28 days.

After the validation, the prescribed products should be analyzed, ensuring that there are no contraindications or concomitant drug interactions that endanger the life of the patient, so it is vital to keep a record of the patient and the state of their diseases.

Currently, it is possible to dispense 2 units per drug in a month, with some exceptions that can be justified.

## **Prescription Drugs**

Considering the Decree-Law n<sup>o</sup> 176/2006 of 30 August, drugs that meet any of the following criteria can only be dispensed with a prescription:

- If constitutes a risk to human health when used for its intended purpose;
- It constitutes a risk to human health when used for purposes other than those intended;
- Parenteral administration;
- Substance in need of adverse profile study.

In the presence of a prescription, the pharmacist has the legal (and ethical) duty to inform the patient about the existence of the generics on the market and in the pharmacy (at least 3 generics that correspond to the lowest prices of each homogeneous group) and

present to the patient the lowest price (ethically, eliminating the pharmacy profit margin needs that may not correspond to the lowest generic).

You should also dispense the cheapest drug, among all the generics present, except for:

- The medical prescription features the brand name;
- The patient wants to select another drug with the same CNPEM (National Code for Electronic Prescription of Medicines), medicines that have the same INN, pharmaceutical form, dosage and package size similar to the one prescribed.

The geriatric population (above 65 years old) presents some problems when remembering the products names, some visual factors (e.g. color of the tablets, shape,...) or packaging (e.g. color, size,...), which presents a curious change because, if the brand changes the aesthetics of the drug (interior and/or exterior) or if we have to present another brand to the user the distrust of the same increases, leading to a constant explanation that it is the same drug.

## **Narcotic and Psychotropic Drugs**

All medicines that have in their constitution active substances classified as "Narcotic" or "Psychotropic" are scrutinized under a strict legislation, where they require medical prescriptions with unique special characteristics, presenting as a special prescription where it can only contain this type of medicines because of its site of action, the Central Nervous System, as it presents the possibility of causing habituation or dependence (even in regular use and can increase in uncontrolled or improper use). It can also be used for illicit purposes, so to prevent that, it is subject to a highly judicious control.

It is not possible to carry out suspended sale, the presentation of medical prescription is always mandatory.

Finishing all the electronic aspect, 2 receipts are printed – 1 receipt for the user and 1 receipt for the pharmacy as proof of dispensing, the latter being filed in a logistic file for a minimum period of 3 years.

## **Non-prescription drugs**

This type of drugs present a function of relief or health treatment of transient problems and without severity justification for going to the doctor, being passive as self-medication.

The "Not Subject to Medical Prescription" allows the commercialization in external circuits to the pharmacy, facilitating the acquisition and consequent self-medication.

The Infarmed created a document which includes medicines for which no prescription is required but can only be dispensed in Pharmacy, presenting the designation "Drugs Not Subject to Medical Prescription of Exclusive Dispensing in Pharmacy" – MNSRM-EF, where the dispensing is carried out in pharmacy by a pharmacist with special attention to its safety profile.

If the patient desires an MNSRM it is important that the pharmacist asks questions to determine the adjacent health problem and if it will not be likely to evolve into a situation that requires medical intervention of its own.

The pharmacist should understand if the patient is the first time to feel the symptoms or if he has felt them before, asking for the beginning of them, the concomitant diseases and the usual medication (if taking) gathering a wide range of information before accessing self-medication.

The main problem of self-medication is the consequences that can originate from misuse by the user:

- Adverse reactions;
- Appearance of drug interactions;
- Masking symptoms of more serious diseases;
- Aggravate existing diseases.

The pharmacist must produce an intensive study of knowledge about this category of drugs and know the various options on the market.

### ***Reimbursement Schemes***

These schemes include certain medicines which, when dispensed to the patient, pay only the difference between the total RRP value and the reimbursement value established for the medicinal product in question.

A "contribution value" is considered as the percentage of RRP that is deducted from the total price of the drug and that is paid by the Portuguese state (NHS) and/or by complementarity schemes (e.g. Caixa Geral de Depósitos (CGD) or the Institute of Social Action of the Armed Forces (IASFA)).

The NHS patients may have a general or an exceptional reimbursement scheme, where the latter covers certain pathologies and certain groups of patients.

## **General Contribution Scheme**

The participation of the portuguese state is defined according to the therapeutic classification of these drugs, subdivided in 4 tiers [21]:

- Tier A – the State pays 90% of the value of the drug;
- Tier B – the State pays 69% of the value of the drug;
- Tier C – the State pays 37% of the value of the drug;
- Tier D – the State pays 69% of the value of the drug.

## **Exceptional Contribution Scheme**

The participation of the portuguese state is defined according to the therapeutic classification of these drugs, subdivided by 4 tiers [21]:

- Tier A – the State pays 95% of the value of the medicine;
- Tier B – the State pays 84% of the value of the drug;
- Tier C – the State pays 52% of the value of the drug;
- Tier D – the State pays 30% of the value of the drug.

In order to be able to access these schemes, the prescribing doctor must refer to the order or diploma in the medical prescription, since in the prescription of medicines used for diseases are covered by special contributions, the prescribing physician must refer to the order relating to the situation in question.

## **Complementarity Regime**

They can be associated with the main regimen and thus cover a larger percentage of the total value of the medicinal product.

The NHS pays a percentage of the medicine and the subsystem pays a percentage of the remaining amount.

The patients must present the beneficiary card so that the number on it must be recorded on the complementarity invoice so that it is possible to enjoy the contribution of the card entity.

Covilhã, eternal land of great industrialists and textiles, presents a complementary regime of pensioners of the Special Social Security Fund of the Personnel of the Wool Industry.

In addition to the reimbursement plans mentioned, there are others that are governed by their own legislation, for example, products for *diabetes mellitus* that are reimbursed by the State like syringes, needles and lancets (100% reimbursed) and test strips (85%). In the manipulated drugs, this is reimbursed in 30% of the RRP [22].

## **Decentralization of the delivery of Hospital Medicines to the Community Pharmacy**

Many drugs need to be administered in the hospital environment due to a closer monitoring of adverse effects and a greater control of correct use and effectiveness, which leads to some prescription drugs being dispensed exclusively in the hospital environment [23,24].

In this pandemic situation, exceptional measures were created that restrict the patient displacement to the hospital, but at the same time ensured the continuity of their therapies, so the hospital pharmacies ensured the dispensing of drugs on an outpatient basis, but in other situations in which it was not possible to transfer the drugs in this way, the hospital pharmacies articulated with the community pharmacies and made it possible to dispense it [23,24].

Facilitating the access of hospital drugs to community pharmacies, there was an increase in adherence to therapy, greater convenience and greater access to therapy by the population, a measure applied to also reduce the risk of infection by avoiding the movement of patients to the hospital.

## **Covid-19 pandemic, an overview**

Since the year 2019, human population faced a global pandemic caused by a severe acute respiratory syndrome coronavirus 2 (or SARS-Cov-2), first identified in the Chinese city of Wuhan. For marketing purposes, it was abbreviated to “Covid-19 pandemic”

At the peak of their emotions, panic – as expected by new health situations – allowed the pharmacist to develop an opportunity to explore one of his most studied and trained skills: primary health counseling.

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The adoption of a healthy and preventive lifestyle can promote some doubts that can be solved by the pharmacist, who must be, especially at this time, updated with the latest training (either through the internet or some forms of media).

Faced with this consequent behavioral readaptation of society, measures to contain the spread of SARS-CoV-2 were implemented in community pharmacies for the protection of workers and patients, highlighting the following:

- Acrylic dividers;
- Use of protective masks (ideally FFP2 for the efficiency of 92%);
- Recurrent disinfection of the bench area, writing material and ATM terminal;
- Reinforce the safety distance between the patient and the service desk.

Ensuring personal safety is one of the important factors, allowing the continuation of society and its daily life within the greater spectrum of normality, understanding that those with chronic pathologies increase in the necessary social protection, as well as the elderly due to frailty (inherent to age and possible underlying medical conditions) thus creating a risk group.

The pandemic made it difficult for patients to go get drugs and who do not go to the pharmacy for fear of having contracted the virus.

In order to minimize these events, it is up to the pharmacist and the patient to respect social distancing and seek solutions to enable communication and appropriate pharmaceutical follow-ups to ensure support and follow-up therapy.

A disseminated information turns out to be like a "Telephone game", where information (whether it is transmitted by the media or personally) passes through a person who transmits it to another, often only a part, without any clarification of its veracity.

At a global scale, it increases misinformation, creates fake news and often encourages panic. As such, it is vital that pharmaceutical students (and professionals) study and be able to keep up to date to meet the information needs of the user. This preparation can come to be as simple as "a correct hand cleaning protocol".

The excessive acquisition of pharmaceutical products, which created inequality of inventory and rupture of products, harmed the pharmacy and then the patient at different scales.

To prevent this, a law was passed which prevents, with exceptions, the dispensation of more than 2 packages of the same drug in one month [25].

## **Counseling and dispensing, if required**

As the business that it is, the pharmacy has 2 lines of profit, which share equal characteristics: the “Prescription Drugs” and the “Not-Prescription Drugs”. From their designations, it is possible to deduce the difference: while the first requires medical authorization, the latter does not require a prescription.

## **Body care and Dermocosmetic**

Constantly expanding, cosmetical is an area to take into account in building a solid and profitable inventory. As the cellular aging process has not yet a proven cure, it is necessary to create alternatives to try to slow it down.

Analyzing the trends, there will be an inversion with the future geriatric population because they will grew up in an environment where skin care is an impetus of beauty and must be cared for and cherished.

Body hygiene falls into this classification, but with the diversified offer in large commercial superficies, the patient can choose to buy. These products contain substances with the purpose of cleaning, perfume, modifying the appearance or keeping the skin in a good condition to put on the outside of the human body, on the oral mucosa or on the teeth. All regulation is the responsibility of INFARMED.

FM as available the following brands:

- Avene®;
- Bexident®;
- Corega®;
- Ducray®;
- Elgydium®;
- Eludril®;
- Isdin®;
- Lactacyd®;
- Mustela®;

In order to have a wide range to meet the needs (current and future), there is a part dedicated to cleansing, skin hydration, anti-aging, sun protection and dry skin, and is also dedicated to oily skin.

## **Dietary Supplements**

It aims to complement the food region by providing nutrients (such as vitamins, fiber, proteins, carbohydrates and vitamins) missing by the type of diet followed or to be ingested in different amounts.

This supplements exist in the form of capsules, tablets, sachets of powder and drinkable ampoules, all following the regulation of the DGAV [26].

Although it doesn't require medical prescription, it is important to explain the designation "supplement", which is characterized by adding to the diet and not to replace something.

Some brands, such as Bioarga®, Bioactive®, Cerebrum®, Centrum®, Tonicê® and Valdispert®, as well as magnesium supplements (Magnesium OK® and Magnesio rapid®) are some of the ranges available [26].

## **Veterinary Drugs (MUV)**

Veterinary Drugs (MUV) are specific substances for animal administration, highlighting the main domestic groups, dogs, cats and rabbits, and are regulated by the DGAV.

It is one of the classes of products in the pharmacy that one must take into account the demographic situation of the pharmacy, in this case, containing a lot of young and old population, which predispose to the acquisition of animals such as cats and dogs.

FM has the following products:

- Contraception – Megecat® and Pilusoft®.
- Vaccination for Canine Disease (Parvovirus, Infectious Hepatitis and Leptospirosis) – Eurican®.
  - Internal deworming - Drontal® and Strongid® and Tenil Vet®.
- External deworming – Frontline®, Adavntix® and Advantage®.
- Increase appetite – Anima Strath®.
- Vaccination against Myxomatosis in Rabbits – Mixohipra®.
- Vaccination against viral hemorrhagic disease – Cylap HVP®.
- Fertility induction – PG 600®.

It is important to not forget to ask the age and weight of the animal in question in order to advise the best product and inform the method of administration.

If medical prescription is required, take into account the need for the presentation of the veterinary prescription in your availability.

## **Medical Devices**

This category includes any material and/or instrument for the purpose of diagnosis and/or treatment that is not obtained by means of a pharmacological, metabolic or immunological action, but by physical action [27].

FM as available [27]:

- Canadians;
- Compression socks;
- Compresses;
- Knee pads;
- Lancets;
- Pregnancy tests;
- Wrists;
- Thermometers;
- Syringes;
- Surgical gloves;
- Strips for glycemic determination.

There are 4 distinct classes by the time of contact with the human body, the possible risk of use, degree of invasion and anatomical part covered [27]:

- Class I - Low Risk;
- Class II<sub>a</sub> - Medium Risk;
- Class II<sub>b</sub> - Medium Risk;
- Class III - High Risk.

## **Special food and children dietetic products**

These products are vital for people whose assimilation or metabolic process is altered, and are also for people with physiological conditions who require a more controlled and

rigorous intake of certain nutrients, helping to uptake the nutritional needs and supplementing the current diet or serving as a substitute.

In FM, products from the Resource® and Temperel® ranges are available.

In addition to these products, there are some that can meet the needs of infants and children of young age (1 to 3 years), such as milks for premature infants, low birth weight newborns and special formulas for the treatment of some problems such as constipation, diarrhea or cramps.

The ranges available are NAN® Nutribén® and Aptamil®.

## **Health Services provided in the Pharmacy**

Nowadays to think that the pharmacy is no longer just a place of sale and dispense of medicines is not to take advantage of the evolution of technology and social needs.

So, to meet the future needs, FM offers other types of services such as:

Hearing assessment, done 1 time a month, the specialist goes to the pharmacy and in addition to evaluating the hearing, also performs maintenance of hearing aids;

Assessment of weight and height, useful for making the BMI, is made using an automatic digital device. For a correct determination, the user must be in an upright position with his gaze on the horizon. At the end of each measurement, the interpretation should always be made by the pharmacist and analyze with the range and reference and understand the reason if it is not in that same range in such, later the user taking into account the value. It is important to emphasize that high values are associated with concomitant and chronic diseases like diabetes, arterial hypertension and cardiovascular disease.

Blood pressure measurement, an automatic electronic device is used, determining systolic and diastolic blood pressure and the number of heart beats per minute. Before the measurement, the patient must be in a resting state for at least 5 minutes in order to stabilize blood pressure, and check if he has smoked or ingested alcoholic beverages or caffeinated beverages (e.g. coffee, ice tea, green tea, etc...) in the previous 30 minutes. After the execution of 2 results, and if these don't fit in the values and in the pharmaceutical assessment of the patient, one should ask the possibility of medication

and if positive which, evaluating the correct therapeutic adherence and adopt non-pharmacological measures (e.g. smoking cessation) the practice of physical exercises.

Monitorization of biochemical parameters (e.g. total cholesterol, capillary blood glucose, triglycerides, etc...).

It is important that the health professional always perform the previous (and others) assessment in a custom cabinet that offers comfort and privacy to the patient.

It is necessary to note that in blood glucose, the disinfection of the patient ring finger is required and it is important to ask the patient if he fulfilled the fasting process or how many hours ago he consumed the last bit of solid food, to make the determination as real as possible, with a small margin of error.

In the end, the professional should interpret the result and record it in a card given to the patient to follow the evolution of the situation in question and whenever there are values outside the recommended range considered or situations of a possible “*diabetes mellitus*” diagnostic, it should be referred to the doctor in order to prevent or delay the onset of complications, in particular micro-compliant studies.

The triglycerides and cholesterol values must be recurrently controlled for the risk of cardiovascular diseases and the general health measures must be taken into account for subsequent control, like the practice of physical exercise, an adequate and controlled diet with reduced intake of carbohydrates and lipid contents, giving predominance to a fiber and protein regime. Recently, studies on red rice have had their advantages in reducing these values if the uptake doesn't exceed the threshold.

## **Prescription Processing and Billing**

In order to speed up the accounting, in the early days of each month, the billing of the prescription for the end of the month is accounted for, receiving the reimbursement from the Portuguese state of all the contributions made during that month.

It is essential to thoroughly verify that all the elements of the medical prescriptions are correctly filled out and legible to avoid returns.

After a final check-up, the papers should be separated and organized by commercial entities, number of recipes and lot number, emphasizing that each lot is constitute by a maximum of 30 medical prescriptions and by the respective entry of lot identification where they contain information such as:

- Amount of revenues;
- Batch sequence number;
- Date;
- Drug RRP in each prescription;
- Full payment by the respective entity;
- Total contribution *per* patient.

In the next step, the batch summary and the monthly drug invoice containing the total amounts invoiced for the reimbursement schemes of each of the entities is printed.

In the case of the batch summary list, it is printed in duplicate for NHS and in quadruple for other entities.

### **Monthly Invoice**

It is printed in a checkerboard, with the original and duplicate sent together with the prescription.

The triplicate is sent to the ANF and the quadruplicated is stored in the internal archive of the FM.

### **NHS Batches**

The lots of revenues associated with the NHS are collected until the 5<sup>th</sup> day of each month by the post mail (CTT) that make the connection with the Invoice Conference Center (CCF) effective.

The remaining lots, involved by participating entities, are sent on the 8<sup>th</sup> day of each month, inclusive, to the ANF, which acts as an intermediary of the other participating entities.

### **Paperless prescriptions**

This type of prescriptions don't need the previous process because the existence of physical documentation is null and void, being automatically valid through Sifarma to the CCF, with the exception being if the entities verify that there is any error in the billing of the prescriptions, then they are returned to the FM, and upon receiving it has a maximum of 60 days for correction of the error/s and subsequent reshipment.

## **Professional Development Activities**

The presentations and knowledge of the Pharmacist Tânia Correia who has an exhaustive knowledge of MUV and who was kind enough to transmit it to me.

Also, Pharmacist João Paiva showed me how to work with all the aspects of the system, giving me more training skill. As for Technician Cristina Paiva, she showed me how to be kind in the balcony.

## **Final Considerations**

The execution of the “Curricular Internship”, with the evaluative weight of 30 UCTs is relevant for having directly put me in the job that I chose as future sustenance, offering me possibilities of learning that are not possible in a classroom, the typical Portuguese expression “put your hands in the dough”.

The social aspect, a characteristic little trained by the lack of exposure to the patient over these 5 years, becomes a school of new sensations, being each day a different applicability in situations and in people of different professions, by the fact of being an aging population with characteristic stubbornness and some lack of patience, it becomes important to clear the mind and offer the most adjusted advice with clarity, calm and practicality.

The biggest obstacle to the perfect execution of my future profession is related to the therapeutic adherence of the patient to the medication, something that I will have to perfect the techniques of approach and opinion, emphasizing the rational use of the medication.

I highlight, in farewell, a thank you of esteem and deep affection for the majority part of the Farmácia Modelar team, having created bonds of beautiful friendship and future companionship with the majority of the team.

***Tragopogon porrifolius*: phytochemical characterization and evaluation of the potential antitumor effect of the extract on the prostate cells**

**Professional Experience in Research and in Community Pharmacy**

# **Chapter N° 2 – *Tragopogon porrifolius*: phytochemical characterization and evaluation of the potential antitumor effect of the extract on the prostate cells**

## **General Introduction**

### **Contextualization of the objective of the study**

Plants have a crucial role in the development of medicine, primarily due to their ability to synthesize secondary metabolites with potentially significant biological activity. In traditional medicine, plants were used in various ways to treat many different ailments [1]. According to the World Health Organization, over 80% of the global population still depends on traditional and folk medicine, most of which is based on plant remedies [2].

A tumor (or cancer) is the term used to describe a disease that presents an uncontrolled cell proliferation, giving rise to new cells with uncontrolled apoptotic process. These new cells give rise to cell clusters that begin to compromise innate immunity [3]. They can be subdivided into benign tumors (cells that don't spread through the lymphatic or blood system to other tissues and rarely cause death) or malignant tumors (more aggressive cells that can spread throughout the body and even if removed can grow again and increase the risk of death). These diseases are the second leading cause of death worldwide, after cardiovascular disease [3].

There are previous studies done on *T. porrifolius* that reveal anti-tumor activity in breast carcinomas (MDA-MB-231) and in colorectal adenocarcinomas (Caco-2), so it is useful to understand whether this experimentally verified anti-tumor effect extends to other neoplasms, in this study, in the prostate [4].

This study aims to obtain further data regarding *Tragopogon porrifolius* extract and its possible anti-tumor effect in prostate neoplasms by *in vitro* essays comparing extract samples to PNT1A (normalized prostate cells) and PC3 (metastasized cells) cells.

## Plants in Antiquity

The strong connection between the Human Being and Nature makes possible our development as a sentient race and the consequent improvement of the quality of life through inventions, there being no technological advance that saves us without an environment that sustains us in our most basic functions, such as breathing and eating [5]. Plants are multicellular, eukaryotes (possess a defined nucleus) and autotrophs (produce energy through photosynthesis) organisms that are found in abundance throughout Planet Earth and that have been used over millennia by Egyptian, Indian, Greek and Roman ancestors as a source of food and for prevention of diseases, as well as building materials for survival purposes [1,6].

In order to preserve this knowledge, compilations (herbal manuals) were created, containing the uses and procedures of healing used in those times, evolving to the present day where much of this knowledge continues to be employed, despite having undergone updates coinciding with the evolutionary process [5]. The species in study, *Tragopogon porrifolius*, comes from the *Asteraceae* family, one of the largest families with proven benefits for the human health, as summarized in the figure 2 [6].



**Figure 2:** The proven effects of species from the *Asteraceae* family. Adapted from [6].

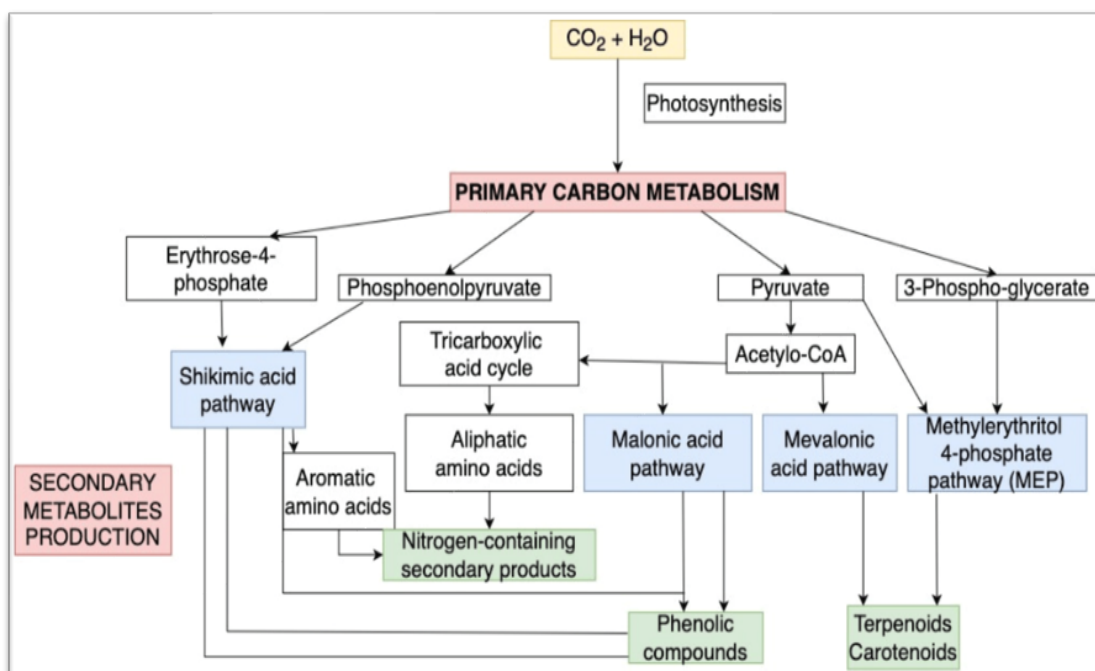
## The importance of Plants

Plants are often exposed to different stressors and unfavorable environmental conditions, which forces it to develop chemical defenses and synthesize secondary metabolites with primary protective functions. This biological effect is widely investigated by the pharmaceutical industry in order to create herbal medicines, called "Phytotherapeutic Medicines", to expand the range of therapeutic options used in the treatment of pathologies, through the experimentally verified effect [6,7]. Plants have two types of metabolisms [7]:

- Primary – Metabolic process essential to the survival of the organism (one example is the photosynthesis process, which originates energy);
- Secondary – Originates organic compounds that are not necessary for the survival of the plant, but that have important biological activity for experimental studies.

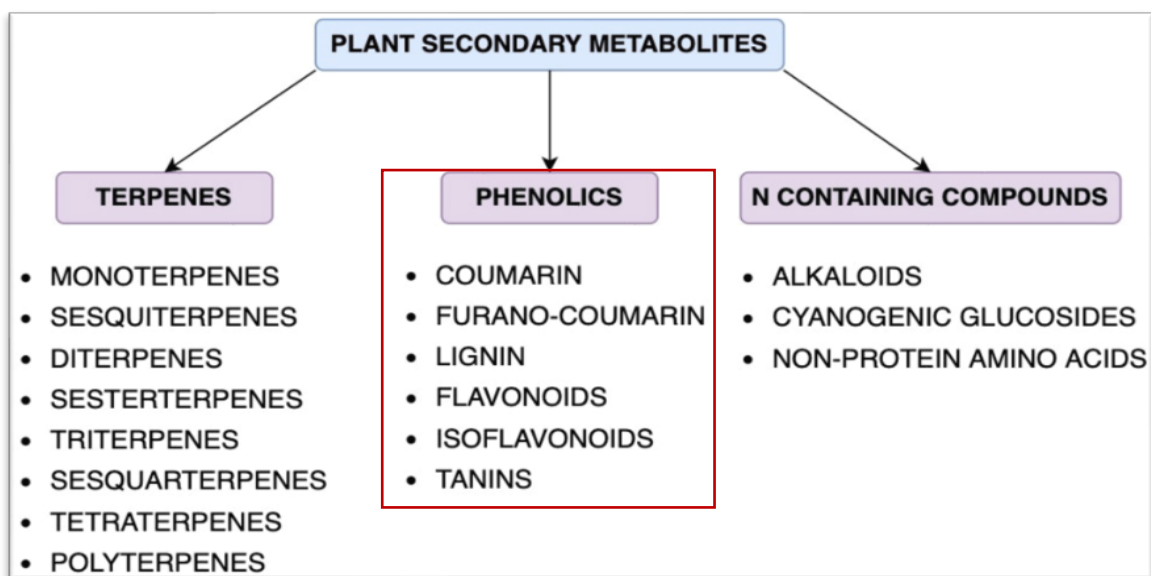
These secondary metabolites have their origin through metabolic pathways applied to the primary metabolites. They can be subdivided into three chemically distinct groups, shown in figure 3 [6,7]:

- Compounds containing nitrogen (example: Alkaloids – derivatives of amines);
- Phenolic Compounds ("x" hydroxyl groups connected to an aromatic hydrocarbon);
- Terpenes (junction of isoprenes).



**Figure 3:** Simplified scheme of primary and secondary metabolisms in the plant and consequent by-products originated. The primary metabolism begins with the junction reaction of carbon dioxide molecules with water catalyzed by photosynthesis that will give rise to 4 main by-products. Then, the secondary metabolism acts within the plant transforming the primary products into secondary **metabolites** by **4 cellular pathways**. Adapted from [7].

Groups of secondary metabolites have key compounds in their molecular structure that show their differences and allow sub-grouping the different species based on their molecular structure. The following classification in figure 4 simplifies this division, however, it can differ from other authors, especially regarding the phenolic compounds.



**Figure 4:** The secondary metabolites of plants grouped into three groups – "Compound containing nitrogen atom", "Phenolic Compound" and "Terpene". Adapted from [7].

The phenolic group (polyphenols), ubiquitous in plants, are one of the main subject in this paper due to their bioactive functions, specially the high antioxidants properties that some authors defend it can decrease the oxidative stress in cancer cells. [2,6,7]

## Antioxidant Features

The term “Antioxidant” is associated with substances that prevent or decrease the oxidation of rapidly oxidizable materials. Through ingestion of exogenous antioxidants (or dietary antioxidants), we can improve the immune system natural ability to protect against oxidizing agents. [6] This protection feature is crucial to our organism since if left unchecked, free radicals can accumulate and induce oxidative stress conditions, resulting in damage cellular functions, as seen in figure 5. [8]

The exogenous consumption of antioxidants supports the cellular defense systems that can be compromised do to age relative problems or even exhaustive exposure to oxidative stress. [8,9]

Extracts from plants of the *Asteraceae* family demonstrate free radical scavenging ability, which has been attributed to their phenolic compound content. The phenolic compounds act by improving the endogenous antioxidant system, chelating the metal ions and evading the formation of free radicals, reducing the Reactive Oxygen Species (ROS) and improving cell renewal [8,9].

The ROS are “captured” by phenolic compounds and also have a known doble physiological role acting as “builders and destroyers”, capable of affecting cellular activity and promoting cellular homeostasis [8]. These species are captured by phenolic compounds.

It then possible to infer that if an increase in ROS formation occurs that exceeds the antioxidant capacity of our immune biological system, then oxidative stress will occur, a process that damages cell structures and causes various pathologies, including cardiac, carcinogenic and age-related [8,9,10].



**Figure 5:** Simplified scheme for DNA damage caused by ROS. Copied from [10].

## The Asteraceae (Compositae) Family

The botanical family *Asteraceae* (or *Compositae*) is the largest and most diversified of the *Angiosperm* (the most diverse group within the kingdom *Plantae*), with an extensive and diverse taxonomy, presenting numbers that amount to more than 1600 genera and more than 2500 species documented [1,11]. It is estimated that, of all flowered species discovered, this family contributes for about 10%, covering all environments on this planet, with the exception of the ice-cold continent Antarctica [12,13]. Contrary to other families in *Angiosperms*, it is not limited to tropical climes [11,13].

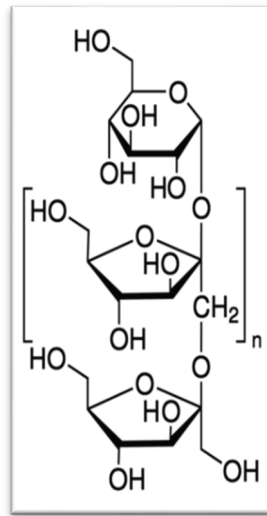
The *Asteraceae* family is widely investigated because several of its species contains molecules that contribute greatly to medical and food investigation [14,15]. The most unique aspect of this family is their head-like inflorescence branch system that scientists called “Capitulum”, presented in figure 6 [11].



**Figure 6:** Different *Asteraceae* flower head forms. (A) Typical flower head with phyllaries, ray and disc florets, (B) Radiate, (C) Discoid and (D) Ligulate flower head. Copied from [11].

Some scientists sub-divided this family into 3 subfamilies and 1 minor subfamily, but other authors present some classifications that range to about 13 families. Despite their wide diversity, most family members share a similar chemical composition: for example, all discovered species are good sources of inulin, a natural polysaccharide with strong prebiotic properties, whose structure can be seen in figure 7. As previously mention, they have strong antioxidant properties, but also some anti-inflammatory and antimicrobial activity, as well as diuretic and wound healing properties [16].

Some applications range from food crops (like *Lettuce* and *Safflower*), herbal and medicinal species (like *Calendula*) and as a form of ornaments (like *Gerbera* or *Dahlia*) [15].



**Figure 7:** Molecular structure of Inulin. Classified as a Fructan, a fructose polymer. Copied from [16].

The morphology of the *Asteraceae* plants is diverse, since some species are trees reaching more than 30 meters (like *Dasyphyllum excelsum* or *Vernonia arborea*), but the majority are like rosette-trees perennial or less annual herbs, ranging from 1–3 meters maximum with sunflower appearance [2,17]. The form of the leaves varies widely: while most are large, others are small and spiny, and some are nonexistent, with their function being taken over by a green stem.

### **The *Tragopogon* genus**

Since ancient times that the species belonging to the genus *Tragopogon* have been used in traditional medicine and consumed as vegetables all over the world [1,2,4]. Traditional Medicine is, according to the World Health Organization (WHO), the sum of practices, knowledge and techniques used in the prevention and improvement of mental and physical health [2].

In medicinal terms, there are proven effects of this species, namely anti-inflammatory, antioxidant and hepatoprotective, as well as gastrointestinal (helpful in constipation and vomiting) and also some healing properties [2,18,19].

This genus has similarities with the genera *Geropogon* and *Scorzonera* (the black Salsify), with annual, biennial or perineal species, as previously mention [18].

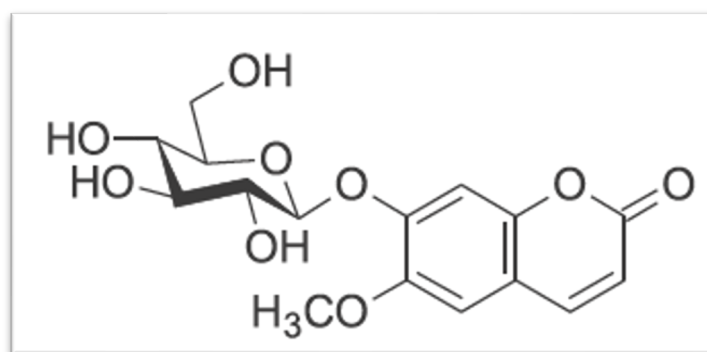
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Of the 110 species of the genus *Tragopogon* discovered and studied, only 12 were investigated for their bioactivity [2,18,20]. Those studies concluded the existence of generalities of components among the various species, highlighting:

- Bibenzyl derivatives;
- Benzylphthalide derivatives;
- **Coumarin (Scopoletin 7-O-glucoside)**;
- Dihydroisocoumarin derivatives;
- Esters of phenylpropanoic acids;
- Hydroxyphenylacetic acid derivatives;
- Flavonoids;
- Phenylmethane derivatives;
- Phenylpropane derivatives;
- Stilbenes;
- Spermine derivative;
- Terpenoids.

The coumarinic derivative – *Scopoletin 7-O-glucoside* – has a strong anti-inflammatory action and was identified in this family only in the *T. porrifolius*, presenting itself as a lactone, as seen in the figure 8 [1].



**Figure 8:** Molecular schematic of *Scopoletin-7-o-glucoside* obtained from *T. porrifolius*. Copied from [1].

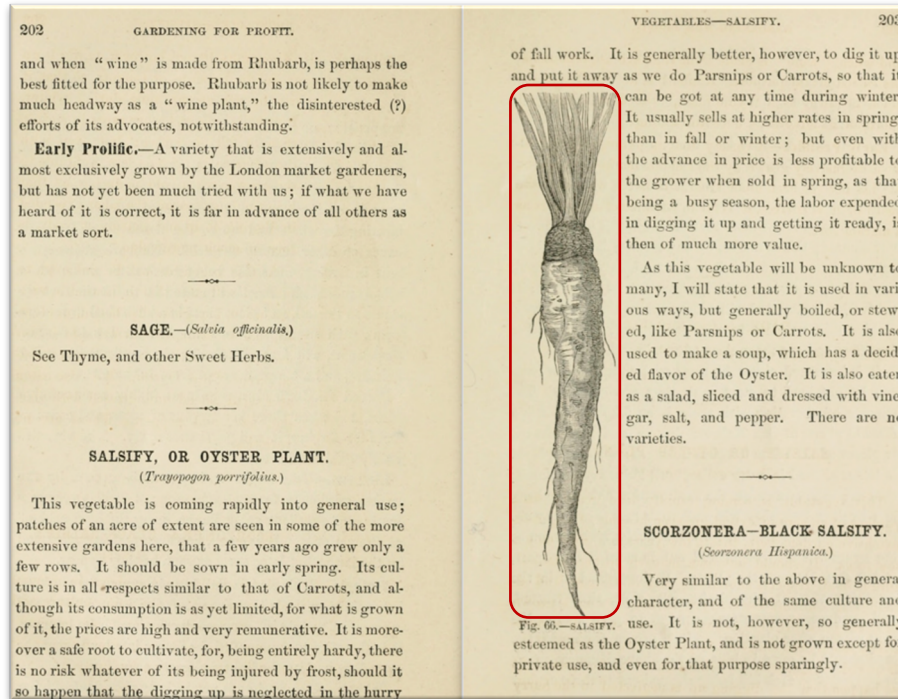
The antiquity of medicinal plants is interesting, mainly because of the superstitions and uses associated to the plants, since they could only assist the effects, it had on the human body and didn't have access to technological instrument [21].

Notably, the author Peter Henderson (birth: 1822; death:1890) was the first to wrote about *T. porrifolius* in 1867 in his book titled "*Gardening for Profit: A Guide to the*

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*Successful Cultivation of the Market and Family Garden*” presenting a picture depicting it in a “carrot-like way”, as shown in figure 9 [21].



**Figure 9:** *T. porrifolius* (red) as described by Peter Henderson on his book titled “*Gardening for Profit: A Guide to the Successful Cultivation of the Market and Family Garden*” released in 1867. Copied from [21].

### ***Tragopogon porrifolius*, or Salsify**

The *Tragopogon porrifolius* (or *White Salsify*) is the botanical designation for a biennial plant (means that it takes up to 24 months to fully complete a biological cycle) part of the *Asteraceae* family. It is native to the Mediterranean region, namely Africa (north), Asia and Europe (center), having been used by ancient civilizations in an attempt to prevent diseases. This species is described in great detail in Lebanese folklore [2].

Ancient accounts identify this plant in cookbooks existing since the 16 century in countries such as Germany, France and Italy. It can be consumed raw or cooked, even the roots [5].

There are the following 3 subspecies:

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- *T. porrifolius australis*;
- *T. porrifolius cupani*; family
- ***T. porrifolius porrifolius***.

The designations "Goat's Beard" (the appearance of the leaves) and "Vegetable Oyster" (the salty-dirt taste of the root) apply to this species [2,11].

This plant takes between 12 to 18 weeks from its sowing (planting the seed) until its harvest to be ready for consumption, which should be done in a soil with certain conditions, like good irrigation, sandy and with a slightly acidic hydrogen potential (pH) between 6.0 to 6.8. It can reach a height of 125 centimeters with purple leaves and has cream-colored roots, as can be seen in figure 10 where it also displays the characteristic capitulum [21,22]. All components of the plant are edible, including the flower.



**Figure 10:** The *T. porrifolius* plant, in aerial view of its flowers (left) and underground view of its roots (right). Copied from [21].

### **Chemical composition of *Tragopogon porrifolius***

The chemical composition of *T. porrifolius* highlights its excellent antioxidant properties due to the high presence of polyphenolic compounds.

Through phytochemical investigation, the compounds present in table 1 attribute the nutritional value to this species [2, 23, 24].

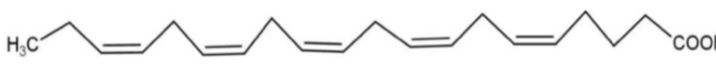
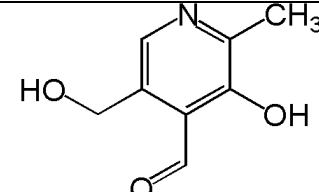
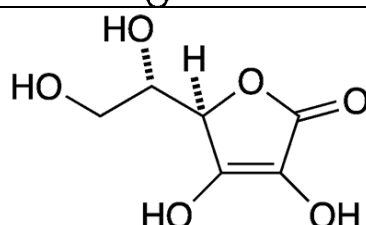
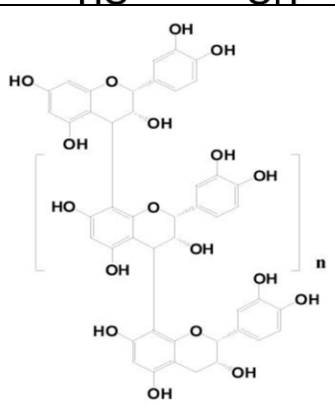
The information contained in table 2 to table 10 presented below summarizes the information of the components identified in the genus (*Tragopogon*) and in the specie under study (*porrifolius*) through specific studies carried out by other authors.

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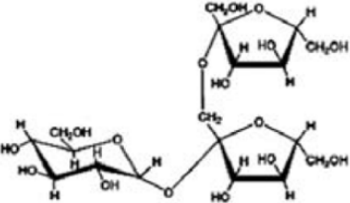
As an example, the Fructooligosaccharides (FOS) presented in table 2 gives the prebiotic qualities to Inulin.

**Table 2:** Components that attribute nutritional value to *T. porrifolius* and its general structures [2, 23, 24].

Component Names	General Structures
Saturated fatty acid	$  \begin{array}{ccccccc}  & & \text{O} & \text{H} & \text{H} & \text{H} & \\  & & \parallel &   &   &   & \\  \text{H} & - & \text{O} & - & \text{C} & - & \text{C} & - & \text{C} & - & \text{C} & - & \text{H} \\  & & & &   & &   & &   & &   & & \\  & & & & \text{H} & & \text{H} & & \text{H} & & & &   \end{array}  $
Essential fatty acid  Example:  Eicosapentaenoic Acid (EPA)	
Vitamin B-6	
Vitamin C  (Ascorbic Acid)	
Polyphenols  (Example: Procyanidins)	 <p style="text-align: center;">Procyanidins: n &gt; 0:</p>

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<b>Cont.</b>	
FOS  (Fructooligosaccharides)  Example: 1-Kestose	

The flavonoids are a group of natural substances with variable phenolic structures and a broad spectrum of biological activity. These natural products are well known for their beneficial effects on health being considered as an indispensable component in a variety of nutraceutical, an emerging new pharmaceutical area that studies the phytochemicals and its impact on life), pharmaceutical, medicinal and cosmetic applications because of their anti-oxidative, anti-inflammatory and anti-carcinogenic properties. [24].

Research on flavonoids received an added impulse with the discovery of the low cardiovascular mortality rate. Information on the working mechanisms of flavonoids is still not understood properly [24,25]. Flavonoids identified in *T. porrifolius* are listed in table 3.

**Table 3:** Flavonoids identified in *T. porrifolius* and respective bibliographic reference [19,23,24,25].

<b>Flavonoids</b>	
<b>Compound</b>	<b>Reference</b>
Apigenin	[23]
Luteolin	[23]
Quercetin	[23]
Quercetin 3- <i>O</i> - $\beta$ - <i>D</i> -glucoside	[24]
Rutin	[25]
Isorhamnetin 3- <i>O</i> -glucoside	[24]
Isovitexin	[19]
O-Xylosylisovitexin	[19]
Isoorientin	[23]
Swertisin	[19]

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<b>Cont.</b>	
Swertiajaponin	[19]
Luteolin 7-O-glucoside	[24]
Vitexin	[23,24]
Orietin	[23,24]
Vicenin-1	[19]
Vicenin-2	[19]
Isoschaftoside	[20]
Catechin	[25]

Triterpenoids are polyphenols with cyclic structures and exhibit cytotoxicity against certain tumor cells. Some triterpenoids have been synthesized by structural modifications of natural compounds. Some of these analogs are considered to have very good anti-inflammatory and anticarcinogenic proprieties [19]. Triterpenoids identified in *T. porrifolius* are exhibited in table 4.

**Table 4:** Triterpenoids Tragopogonsaponins and Tragopogonsaponin methyl ester identified in *T. porrifolius* and its bibliographic reference [13,23,26,27].

<b>Triterpenoids</b>	
<b>Compound</b>	<b>Reference</b>
$\beta$ -Amyrin	[23]
$\beta$ -Amyrin acetate	[23]
Lupeol	[23]
Echinocystic acid	[13]
Tragopogonsaponins A to R	[26]
Tragopogonsaponin Methyl Ester A to J, N, O, P and R.	[26]
Tragopogonoside Methyl Ester	
A to I	[27]

The stilbenoids are a family of phenolic compounds with a structure known as a “stilbene” (2 aromatic rings connected by a carbon chain with a double bond) classified as phytoalexins, antimicrobial compounds produced in plants to give protection against toxins. This compounds present with several biological activities, including anti-

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inflammatory, anti-diabetic and cardio- protector. Resveratrol is the most studied member [30]. Stilbenoids identified in *T. porrifolius* are listed in table 5.

**Table 5:** Stilbenoids identified in *T. porrifolius* and its bibliographic references [24,29,30].

Stilbenoids	
Compound	Reference
Resveratrol	[29]
$\alpha,\beta$ -Dihydorhaponticin	[30]
3'-O-Methyl-, $\alpha,\beta$ dihydorhaponticin	[30]
6"-O-(7,8-dihydrocaffeoyl)- $\alpha,\beta$ -dihydorhaponticin	[30]
3-O- $\beta$ -Glucopyranosyl-demethoxyca-nnabispiradienone	[24]

**Table 6:** Bibenzyl and Benzylphthalides derivatives identified in *T. porrifolius* and respective bibliographic references [24,28].

Bibenzyl and benzylphthalide derivatives	
Compound	Reference
Dihydroresveratrol	[28]
5,4'-Dihydroxy-3- $\alpha$ -L-rhamnopyranosyl-(1'' $\rightarrow$ 3')- $\beta$ -D-xylopyranosyloxybibenzyl	[28]
2-Carboxyl-5-hydroxy-3-methoxy-4'- $\beta$ -glucopyranosyl-oxybibenzyl	[28]
2-Carboxyl-3,4'-dihydroxy-5- $\beta$ -D-xylopyranosyloxybibenzyl	[28]
Tragopogonic acid	[28]
3-Caffeoyl-(9 $\rightarrow$ 5)- $\beta$ -apiosyl-(1 $\rightarrow$ 6)- $\beta$ -glucopyranosyloxy-5,4'-dihydroxy-3'-methoxybibenzyl	[24]
3-Caffeoyl-(9 $\rightarrow$ 5)- $\beta$ -apiosyl-(1 $\rightarrow$ 6)- $\beta$ -glucopyranosyloxy-4'-dihydroxy-5,3'-dimethoxybibenzyl	[24]
(S)-3-(4- $\beta$ -Glucopyranosyloxybenzyl)-7-hydroxy-5-methoxyphthalide	[24]
3-(4-methoxy-benzyl)-5,7-dimethoxyphthalide	[28]
1-Hydroxypinoresinol-1-O- $\beta$ -glucopyranoside	[28]
7- $\beta$ -Glucopyranosyloxy-(S)-3-(4-hydroxybenzyl)-5-methoxyphthalide	[24]
7-(1 $\rightarrow$ 6)- $\alpha$ -rhamnosyl- $\beta$ -glucopyranosyloxy-(S)-3-(4-hydroxybenzyl)-5-methoxyphthalide	[24]

The coumarins are phytoalexins and integrate the benzo-pyrones family (chromone) with a wide spectrum of biological activities, namely anti-inflammatory, antibacterial

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and antihypertensive. Most coumarins are thermally stable with a very good optical activity. [31] Coumarins identified in *T. porrifolius* are presented in table 6.

**Table 7:** Dihydroisocoumarins identified in *T. porrifolius* and its bibliographic reference [28,31].

<b>Dihydroisocoumarins</b>	
<b>Compound</b>	<b>Reference</b>
Thunberginol C	[31]
Scorzocreticin	[31]
Scorzocreticoside I	[31]
Hongkongenin	[31]
6-O-Methylscorzocreticin	[31]
6,8-Di-O-methylscorzocreticin	[31]
6-O-Methyl-scorzocreticoside I	[28]
Tragoponol	[31]
(S,E)-2,4-dihydroxy-6-(2-(2-hydroxy-6-(4-hydroxystyryl)-4-methoxybenzoyloxy)-2-(4-methoxyphenyl)ethyl) benzoic acid	[31]

**Table 8:** Phenylmethane derivatives identified in *T. porrifolius* and respective bibliographic reference [23,25,32].

<b>Phenylmethane derivatives</b>	
<b>Compound</b>	<b>Reference</b>
Benzoic acid	[32]
<i>p</i> -Hydroxybenzoic acid	[23]
<i>m</i> -Hydroxybenzoic acid	[23]
Protocatechuic acid	[23]
Gallic acid	[25]
Vanillic acid	[23]
Syringic acid	[23]
Salicylic acid	[23]
Gentisic acid	[23]

**Table 9:** Phenylpropane derivatives identified in *T. porrifolius* and respective bibliographic reference [23,29,30].

<b>Phenylpropane derivatives</b>	
<b>Compound</b>	<b>Reference</b>
<i>p</i> -Coumaric acid	[23]
Caffeic acid	[23]

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<b>Cont.</b>	
Ferulic acid	[23]
Sinapic acid	[29]
<i>p</i> -Dihydrocoumaric acid methyl ester	[30]

**Table 10:** Esters of phenylpropanoic acids identified in *T. porrifolius* and its bibliographic reference [20,24,32].

<b>Esters of phenylpropanoic acids</b>	
<b>Compound</b>	<b>Reference</b>
Chlorogenic acid	[20,24]
3,5- <i>O</i> -Dicaffeoylquinic acid	[24]
4,5- <i>O</i> -Dicaffeoylquinic acid	[24]
Rosmarinic acid	[32]

**Table 11:** Phytochemicals in small percentages (less than 1%) identified in *T. porrifolius* and respective bibliographic reference [20,23,24].

<b>Phytochemicals</b>	
<b>Compound</b>	<b>Reference</b>
<i>p</i> -Hydroxyphenylacetic acid	[23]
<i>o</i> -Hydroxyphenylacetic acid	[23]
<b>Scopoletin 7-<i>O</i>-glucoside</b>	[20]
Tetra- <i>N</i> -coumaroyl spermine	[24]

### Studied effects of *T. porrifolius*

The *Tragopogon porrifolius* pharmacological effects on human health can be attributed to their range of phytochemical compounds, including polyphenols, phenolic acids, flavonoids, acetylenes and triterpenes. As listed in the previous tables, there's a synergy of compounds that compose the molecular structure with such a distribution that *T. porrifolius* can act as a potent antioxidant and anti-inflammatory specie. Other examples not listed are Arctiin, a glycoside ligand with strong antioxidant, and antiproliferative activities and a variety of sesquiterpene lactones, metabolites that give the plants their bitter taste [33]. There are several species that share similar chemical compositions and consequently organic properties with the specie in study. The table 11 compiles some species that fit in the same category as *T. porrifolius* and compare their activities.

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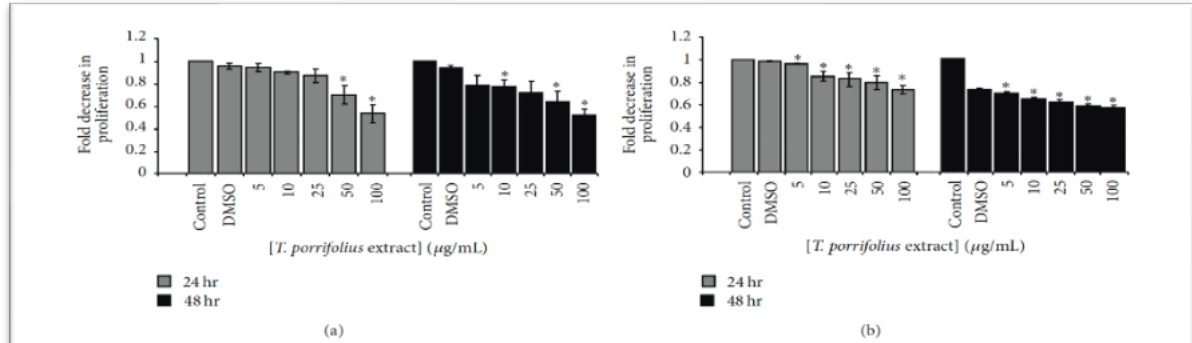
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It's possible to extract that *T. dubius Scop.* and *T. graminifolius DC.* have ethnopharmacological, phytochemical and pharmacological similarities in relation to *T. porrifolius*. The consumption of *T. porrifolius* in aqueous medium improves lipemia and increases the feeling of satiability in rats without adverse effects [23].

Name of taxa	Ethnopharmacological uses	Phytochemistry	Pharmacological properties
<i>T. buphthalmoides</i> (DC.) Boiss.	+	-	+
<i>T. dubius</i> Scop.	+	+	+
<i>T. graminifolius</i> DC.	+	+	+
<i>T. malicus</i> Nikitin	-	-	+
<i>T. mirus</i> Ownbey	-	+	-
<i>T. miscellus</i> Ownbey	-	+	-
<i>T. oligolepis</i> Hartvig & Strid	-	+	+
<i>T. orientalis</i> L.	-	+	+
<i>T. porrifolius</i> L.	+	+	+
<i>T. pratensis</i> L.	+	+	+
<i>T. reticulatus</i> Boiss. & Huet	+	-	+
<i>T. sinuatus</i> Avé-Lall.	+	+	+
<i>T. tommasinii</i> Sch.Bip.	-	+	+

**Table 12:** The taxonomy of several *Tragopogon* species. Adapted from [23].

Furthermore, in figure 11 it is demonstrated that *T. porrifolius* can inhibit, at some extent, the proliferation of cancer cells in some type of tumors.



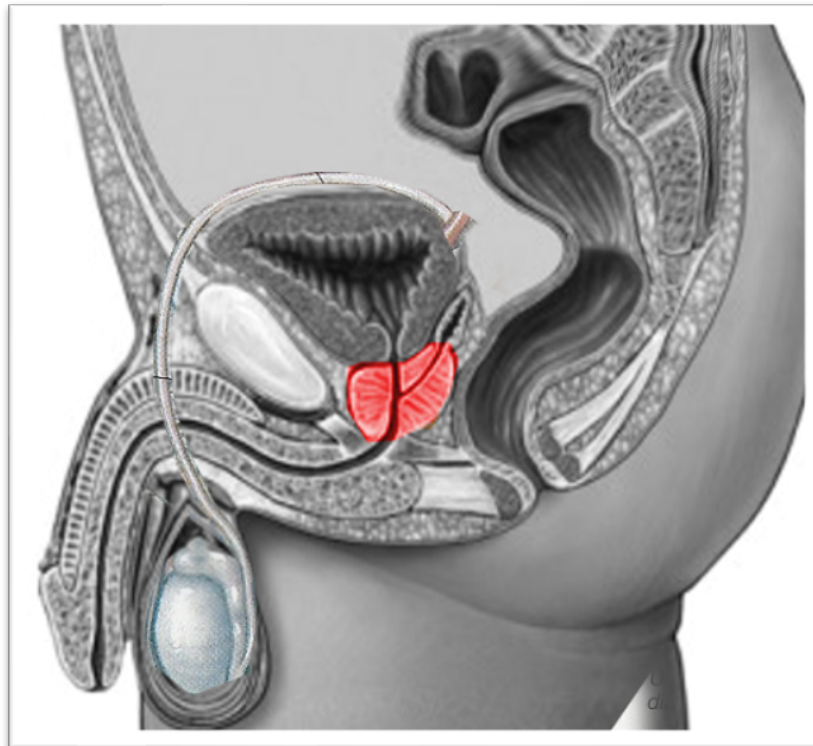
**Figure 11:** The effect of *T. porrifolius* on proliferation of MDA-MB-231 (a) and Caco-2 (b) cell lines in the presence of increasing concentration of the methanolic extract at 24 and 48 hours of treatment demonstrating the possibility of a time-dependent effect  $p < 0.05$  to the group that received only DMSO. Adapted from [34].

## Prostate

### Anatomy and physiology of the prostate

The prostate is a walnut-sized organ weighing between 15-20 grams [35]. It's located immediately below the urinary bladder between the pelvic diaphragm and the peritoneal cavity. Moreover, the prostate involves the urethra emerging from the ejaculatory ducts (Figure 12) [35, 36].

The prostate gland is considered an exocrine gland of the male reproductive system, being its main biological function the production of the prostatic fluid that plays an extremely important role in male fertility [35].



**Figure 12:** Localization of the prostate in a sagittal view of the pelvic cavity. The prostate is located below the bladder and surrounds the first portion of the urethra and the ejaculatory ducts. Identification of the prostate gland in red. [37]

### Anatomic division by John McNeal concepts

In the year 1968, John McNeal presented pioneering anatomic concepts that are still accepted today, namely the division of the prostate into 4 different zones – central zone, transition zone, peripheral zone and the fibromuscular stroma (Figure 13) [38].

The characteristics of each zone are summarized in the table 13. The zone considered the site of origin of the majority of prostate cancer cases is the peripheral zone [38].

**Table 13:** Characterization of the different zones of McNeal anatomy distribution [39].

<b>The Four McNeal Zones</b>			
<b>Designation</b>	<b>Description</b>	<b>Glandular tissue (%)</b>	<b>PCa incidence rates</b>
Peripheral (PZ)	Delimits the central zone and contains the urethral ducts	<u>70</u>	<u>68</u>
Central (CZ)	Surrounds the ejaculatory ducts	25	8
Transition (TZ)	Adaxial to urethra	5	24
Anterior Fibromuscular Stroma	Covers the transition zone and the distal urethra ventrally	NO	NO

## **Development**

The prostate gland starts its development in the embryonic phase and ends in the postnatal phase, being mainly regulated by the serum levels of testosterone [40]. From birth until puberty, the size of the prostate does not change; however, with the puberty this organ begins to grow and produce the prostatic fluid [40]. At the age of 30, prostates' size increases because of the hormonal changes characteristic of the aging process and may give rise to a condition designated as benign prostatic hyperplasia (BPH) that affects more than 50% of adult male population and may evolve to prostate cancer [36, 40].

## **At cellular level**

The extra-cellular matrix is constituted by non-cellular components and connective tissue. The hallmark histological characteristic is the fibromuscular stroma composed by mixed clusters of elastic fibers with smooth muscles cells [41].

The prostatic epithelium can be sub-divided into four main types of cells [41]:

- Basal cells, separate the lumen from the stroma;
- Neuroendocrine cells, release hormones and neuropeptides that promote the prostate growth;
- Secretory epithelial cells or luminal cells, responsible for the production of the prostatic fluid;
- Stem cells, quiescent reservoir that can differentiate into basal or luminal-like stem cells.

### **Prostate cancer, a general concept**

Prostate cancer is the 2<sup>th</sup> most common cancer worldwide and the 5<sup>th</sup> leading cause of death [42]. Prostate cancer is a clinically heterogeneous disease, has some patients can experience it as an indolent disease while others can show signs of aggressive disease with progression and metastasis [42,43]. This pathology may be asymptomatic in the begin, but as it progresses, a diversity of symptoms may be developed [43].

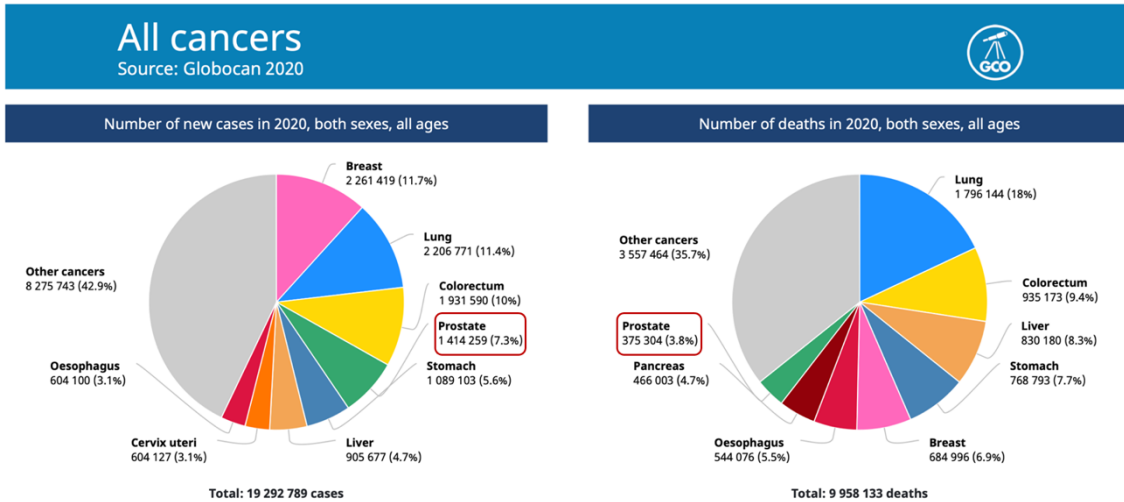
The diagnosis of prostate cancer involves methods such as digital rectal examination, prostate-specific antigen (PSA) analysis and prostate biopsies [43]. Initial prostate cancer diagnosis involves a blood test to measure PSA levels together with digital rectal examination [43]. To confirm diagnosis is required a biopsy. After histological analysis, prostate biopsies are graded following the *Gleason* pattern and a *Gleason* score is attributed [43].

Treatment options for localized prostate cancer include active surveillance, ablative radiotherapy and radical prostatectomy. For men with recurrent or metastatic prostate cancer, treatments like Androgen Deprivation Therapy (ADT), radiotherapy, and chemotherapy are administered. Combination therapy is often more effective, but it's important to note that prostate cancer remains incurable with current treatments [43].

### **Epidemiology**

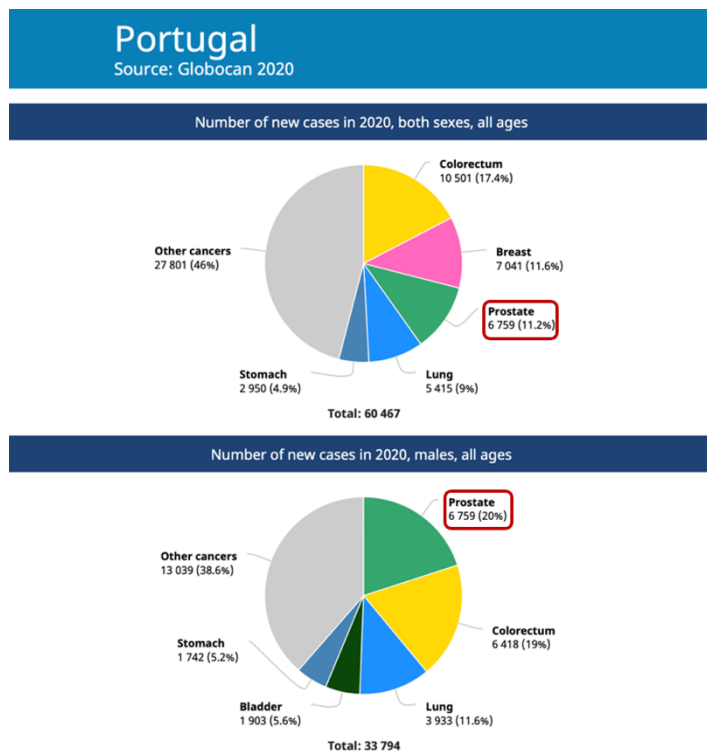
According to the World Cancer Research Fund International, in the year 2020, the confirmed diagnosis of prostate cancer ascend to over 1.4 millions new cases, as shown in figure 14 [44]. Also, it is estimated that PCa caused 375,304 cases of deaths among men worldwide of all ages [45].

International Agency for Research on Cancer  
World Health Organization



**Figure 13:** Study conducted in the year 2020 by WHO. Worldwide prostate cancer incidence is approximately 7.3%.

In the particular case of Portugal, the latest data estimated by Globocan (year 2020) indicate that prostate cancer represent the second oncological disease with higher incidence and the fifth in terms of mortality (figure 15). [46] Moreover, recent studies show that the mortality associated with prostate cancer seems to be steadily decreasing over time while the incidence increases [47].



**Figure 14:** Study conducted in the year 2020 by WHO in Portugal. In Portugal, prostate cancer incidence considering both sexes (up) and only the male population (below) is 11.2 and 20%, respectively.

## **Risk Factors**

The potential risk factors to prostate cancer can be divided into non-modifiable factors such as age, race, and family history and modifiable risk factors such as diet, physical activity, smoking, and obesity [48].

Ethnicity: South American and African-American males have a higher predisposition than non-Hispanic Whitemen, Asian and Middle Eastern men [49]. The justification may rely in the interplay between the genetic component and the environmental factors or in health care disparities [42].

Age: As age progresses so does the risk, rising rapidly after age 50. According to analytic studies, about 6 in 10 cases of prostate cancer are found in men older than 65 who have lower overall survival [42].

Family History: The risk of prostate cancer is increased for men with a family history of any cancer or prostate cancer in first-degree relatives [42]. Several other studies confirmed that family history is the most important risk factor compared to age and ethnicity in the development of prostate cancer [50].

Overweight: Studies confirmed a positive correlation between excessive weight and prostate cancer incidence [42, 52].

Smoking: According to the International Agency for Research on Cancer (IARC), more than 60% of chemicals that are present in a cigarette are carcinogens of class I and class II. [42] Some studies concluded a positive association between smoking and prostate cancer recurrence and mortality [54].

Diet: The nutrients present in the food are extremely important in prostate cancer onset and progression and may have different effects:

Lipids: Lipids, including fats and steroids, serve various functions in the body, including energy storage, signaling, and structural components of cell membranes. However, a high-fat diet has been associated with an increased risk in several types of cancer, including prostate, breast, and colon cancers.

Animal products: Studies have confirmed a positive correlation between the consumption of animal fats and a higher incidence and mortality rate in prostate cancer

patients [55]. There is some controversy regarding the direct correlation between the consumption of saturated fats and trans fats and the increase of prostate cancer risk. A study performed by the World Research Cancer and Fund showed that the consumption of, at maximum, 500 grams of red meat per week was negatively correlated with a high risk of prostate cancer [55,56].

In this context, environmental and artificial contamination in animal diets, as well as the presence of omega-3 unsaturated fatty acids in fish oil are to be considered [42]. There are also some studies suggesting that carbohydrates, specifically sucrose molecules may not have a direct correlation with an increased risk of prostate cancer; however, a significant correlation was made between dairy intake of milk and calcium and advanced-stage prostate cancer, especially in whole milk, characterized by high percentages of fat [56, 57].

Alcohol beverage: This type of drink contains ethanol and can act as a drug, capable of compromising both physical and psychologically human beings. A definitive study showed that high levels of alcoholism is linked to a higher risk of prostate cancer development [58]. This risk is not specific to any particular type of alcoholic beverage. Furthermore, alcohol consumption has a direct connection to the severity of prostate cancer, potentially promoting the growth of tumors and speeding up the progression to metastatic prostate cancer [58].

Lycopene: Studies have formulated a hypothesis (largely supported by animal experimentation in rodent animals) that lycopene is an anticancer bioactive compound. It's a carotenoid pigment is responsible for the reddish color in foods (like tomatoes and watermelon) that possess anti-inflammatory and antioxidant properties that help prevent oxidative stress and has a protective effect against prostate cancer [61, 62, 63]

## **Development, an overview**

In a general overview, there's two distinct stages in the development of human prostate cancer: androgen-dependent adenocarcinoma and androgen-independent adenocarcinoma (or castration-resistant) [65]. Androgens are the main sex hormones involved in male reproductive activity. The primary androgens are androstenedione and testosterone, followed by dehydroepiandrosterone (DHEA), dehydroepiandrosterone

sulfate (DHEA-S), and dihydrotestosterone (DHT). They serve as the principal regulators of normal prostate function and control the growth and proliferation of prostate cancer cells [35, 65].

Testosterone, the most abundant androgen, is converted irreversibly into 5- $\alpha$ -dihydrotestosterone (DHT) by the action of 5 $\alpha$ -reductase. DHT binds to androgen receptors, promoting the continuous development of the prostate gland and controlling cell growth and apoptosis. Initially, prostate cancer is driven by androgens and androgen receptors, making it highly sensitive to hormonal therapy in the early stages. However, as the disease progresses, some prostate cancer cases may become resistant to hormone therapy. [35, 36, 65]

Over time, prostate cancer cells can undergo mutations that lead to complex sensitizations. This evolution ultimately reaches a point where androgen receptor activity persists even in the absence of androgens, allowing cancer to continue growing. This stage is referred to as hormone-refractory prostate cancer, where hormonal therapy loses much of its effectiveness. This resistance is achieved through a significant amplification of androgen receptor expression and sensitivity. Consequently, these receptors continue to function and promote cell proliferation even when androgen levels are greatly reduced, sometimes by up to 80% [66]. In some cases, prostate cancer cells can increase their own production of 5 $\alpha$ -reductase to maintain high levels of DHT within tissues, enhancing their functionality [65, 66].

Additionally, androgen receptors can also be activated through different mechanisms, such as phosphorylation by mitogen-activated protein kinases (MAPKs) [66].

Prostate cancer involves the activation of phosphoinositide-3-kinase (PI3K), an enzyme associated with cell growth and proliferation. This is accompanied by downregulation and deletion of the tumor suppressor phosphatase and tensin homolog (PTEN), which plays a crucial role in controlling cell growth [71]. Upregulation of MAPKs and mutation (like deletion) in other tumor suppressors like NKX-3.1 contribute to uncontrolled cell growth [67]. Cancer cells release growth factors as they mutate, further stimulating their growth.

## **Cancer care, importance of the nutritional treatment**

In cancer care, a multidisciplinary team works to create a treatment plan depending on each tumor, stage, side effects and the patient general health. In some cases, local surgery can be required having in count the symptoms: for example, if the urinary tract is compromised, a transurethral resection of the prostate can be used to relieve symptoms where a bilateral orchiectomy works as an hormonal therapy by surgically removing the testicles. Chemotherapy has become a synonymous word for cancer, but there's also alternative therapies like immunotherapy, hormonal therapy (androgen-deprivation) and also drugs like Docetaxel or Enzalutamide that act as targeted therapy, blocking the effect of testosterone. It is important to remember that each drug is used at a different stage of the tumor. It has been proven that drugs like Aspirin, Metformin and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs with anti-COX-2 activity) that work by reducing the inflammatory activity as well as angiogenesis can improve prostate cancer care. Besides treatment, in prostate cancer, active surveillance is most vital and regular testing will be required.

Nutraceuticals are products derived from food sources with potential health benefits beyond basic nutritional value [80]. Some studies have explored the potential role of nutraceuticals in preventing and treating prostate cancer, but it is important to note that more research is needed to definitively establish their effectiveness [79, 80] They are typically used as complementary approaches to conventional medical treatments and considered to be effective, at some extent, in prostate cancer [80]:

Selenium: Selenium is a trace element found in some foods and is believed to have antioxidant properties. Some studies have suggested that selenium supplements may reduce the risk of prostate cancer, especially in areas where soil selenium levels are low. However, excessive selenium intake may be harmful [80].

Vitamin E: Vitamin E is fat-soluble antioxidant and some studies have investigated its potential role in reducing the risk of prostate cancer. However, results have been mixed and excessive vitamin E supplementation may have adverse effects [80].

Lycopene: As previously mentioned, some studies have suggested that a diet rich in lycopene may be associated with reduced risk of prostate cancer development [61, 80]

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Green Tea Extract: Green tea contains compounds called polyphenols, mainly EpiGalloCatechin Gallate (EGCG), which have antioxidant properties. Some studies have explored the potential of green tea extract in inhibiting the growth of prostate cancer cells. While the results are promising, more research is needed to determine its effectiveness [80]

Fish Oil (omega-3 fatty acids): Omega-3 fatty acids, found in fish oil, have anti-inflammatory properties and may help in the fight against prostate cancer [80].

Curcumin: Curcumin is a compound found in turmeric; a spice commonly used in Indian cuisine. It has anti-inflammatory and antioxidant properties and has been studied for its potential anti-cancer effects, including in prostate cancer. Some studies have shown promising results in cell cultures and animal models, but more research is needed [80].

Pomegranate Extract: Pomegranate contains compounds such as polyphenols and ellagic acid, which have been investigated for their potential role in inhibiting the growth of prostate cancer cells [80]. Some studies have shown positive effects, but more research is needed to confirm these findings.

It is vital to emphasize that although nutraceuticals may have potential benefits, they should not be used as a substitute for standard medical treatments for prostate cancer, such as surgery, radiation therapy, chemotherapy, or hormone therapy. Additionally, lifestyle factors such as a healthy diet, regular exercise and maintaining a healthy weight are also essential components for prostate cancer prevention and treatment.

As exposed, these supplements can have an effect on prostate cancer but is also important to discover new therapeutic tools. Since antioxidants have a proven effect on cell proliferation inhibition and *T. porrifolius* presents vast quantities of antioxidants (polyphenol's) in its molecular structure, this root may proposed as a nutritional treatment in prostate cancer.

## Chapter nº 3 – Materials and Methods

### Materials

The roots of *T. porrifolius* used for the study were acquired in the section "Vegetables and Roots" in a local supermarket on October 21, 2021 in the morning. The root was placed in a plastic bag inside a thermal bag to try to keep the temperature and humidity conditions as homogeneous as possible.

### Sample Preparation

After disinfecting the work surface with 70% alcohol and washing the root with distilled water, the root was cut into tiny pieces and transferred to a porcelain crucible and put inside an incubator at 25 °C and 60% humidity to optimize the drying process of the root (vital to remove the adjacent water molecules) for three days. The sample was seen two times a day (one in the morning and the other in the afternoon) until it reached the desired dehydrated consistency. Afterwards, the sample was transferred to a mortar and with a pestle it was milled until obtaining a yellowish powder (due to the impurities still present) and transferred to a volumetric flask of 50 mL. Then, a methanolic solution (in a 70/30 ratio) was added to promote the extraction of compounds. After a two minute rest, the flask was placed in an ultrasound bath for 6 hours with occasional agitation.

Subsequently, the flask was removed from the bath and the extract was filtered using *Whatman* No. 1 vacuum paper, a process repeated 3 times to ensure maximum optimization of the extraction. The solvent was removed by vacuum pressure in a rotavapor under a temperature of 38.0 to 40.0 °C, a process repeated for 1 day until the residue was slightly moist with mucous appearance and amber coloration.

The next day, the residue was removed from the balloon with a spatula and placed in *falcons* covered with cellophane paper (with holes to allow the evaporation process) in a desiccator where it remained for 2 days until complete drying was ensured, giving rise to a powder with cream white color. In this moment, the residue was weighed to calculate the yield of the reaction, following this equation:

$$\text{Yield}(\eta) = \frac{\text{mass}_{\text{AfterEvaporation}}}{\text{mass}_{\text{Initial}}} \cdot 100 \quad (2)$$

Finally, the *falcons* were stored at negative temperatures ( $-20\text{ }^{\circ}\text{C}$ ) to maintain the conditions and use in future work.

### **Determination of Total Phenolic compounds**

This method aims to determine the content of phenolic compounds present in the methanolic extract.

Through a colorimetric method using *Folin-Ciocalteu* reagent it is possible to measure the reducing capacity of the species under study using gallic acid as a standard compound. The polyphenols present in *T. porrifolius* catalyzed through a redox reagent *form* a spectrophotometrically quantifiable blue complex. This method through UV/VISIBLE spectrophotometric analysis is easy to perform, fast and low cost.

Then, re-suspend the powder from the *T. porrifolius* sample in methanol at the concentration of 100 mg/mL – original concentration. Then, filter the extract again and perform a dilution in the ratio of 1:5 (1 part of original concentration in 5 parts of methanol) – diluted extract.

To 50  $\mu\text{L}$  of the standard solutions or methanolic solutions of the extracts (2.5 mg/mL) were added 450  $\mu\text{L}$  of distilled water and 2.5 mL of 0.2N Folin-Ciocalteu reagent (dilution with distilled water). After, it was left to react for 5 minutes at room temperature and then added 2.0 mL of aqueous sodium carbonate solution (75 g/L). They were placed in a bath at 30  $^{\circ}\text{C}$  for 90 minutes, shaking intermittently and at the end of this incubation the absorbances were read at 765 nm. The blank solution was prepared by replacing gallic acid with methanol and the tests were carried out in triplicate. Total phenols were expressed as “mg of gallic acid equivalents per gram of extract (mg GAE/g extract)”.

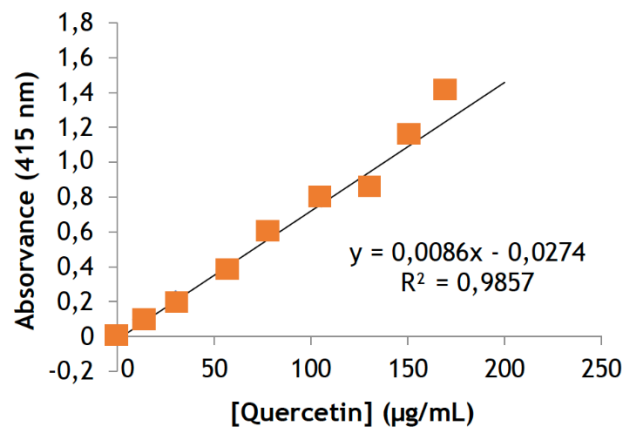
### **Determination of Total Flavonoid compounds**

It was determined using the colorimetric method described by *Chang et. al* using Quercetin (10-100 mg/L) as a standard compound.

As previously done, the original concentration and a dilution was carried out in the ratio of 1:20 (1 part of the original concentration in 20 parts of methanol) – diluted extract.

To 500 µL of standard solutions and methanolic extract solutions (2.5 mg/mL), it was added 1.5 mL of methanol, 0.1 mL of aqueous aluminum chloride solution (10%), 0.1 mL of aqueous solution of potassium acetate (1M) and 2.8 mL of distilled water.

After, it was left to react for 30 minutes at room temperature and then the absorbances were read at 415 nm. The blank solution consisted of replacing the sample by methanol and the tests were carried out in triplicate. Total Flavonoids were expressed as “mg of quercetin equivalents per gram of extract (mg QE/g extract)”, as seen in figure 16.



**Figure 15:** Calibration curve of Total Flavonoids using “Quercetin” as reference.

## Determination of Antioxidant activity

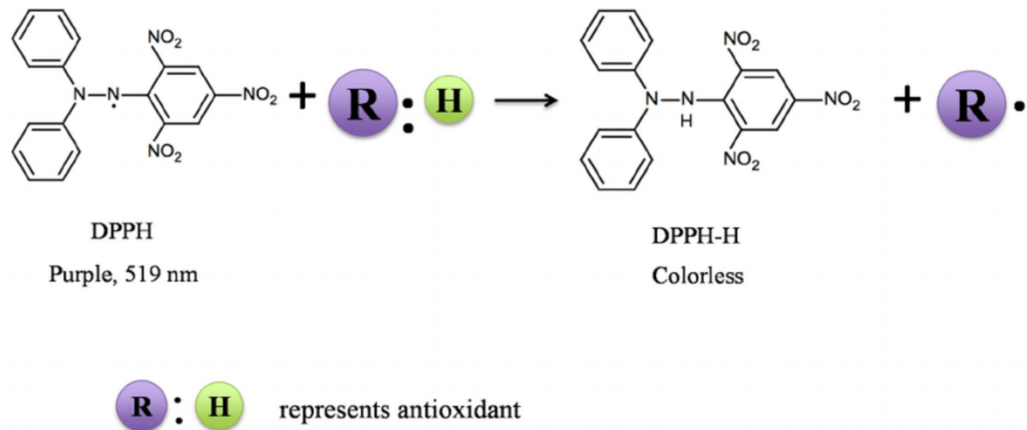
### DPPH colorimetric assay.

This assay assists in the determination of the antioxidant potential of the extract under study through the reduction of free radicals existing in the DPPH. This is an indirect evaluation because the antioxidant capacity of the extract under study is greater the lower the amount of non-reduced DPPH.

The molecule 2,2-diphenyl-1-picrylhydrazil ("DPPH") is used to measure antioxidant properties by eliminating the DPPH radical by antioxidants to give rise to a stable free radical that reacts with hydrogen atoms. The electron of the nitrogen atom in DPPH is reduced by joining with a hydrogen atom to form a hydrazine (DPPHH). This reaction is simplified in figure 17.

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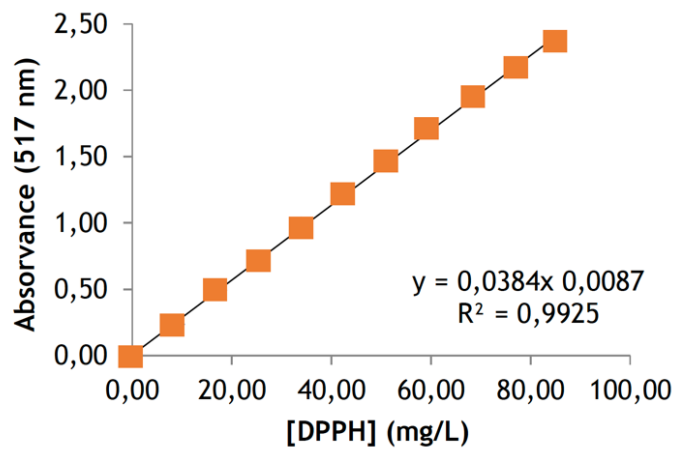
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**Figure 16:** Neutralization of DPPH (violet color) in DPPHH (yellow color). Adapted from [18]

In order to measure the antioxidant activity of the *T. porrifolius* extract, it was required to prepare dilutions of the same, namely 25, 50, 100, 150, 200 and 250  $\mu\text{g}/\text{mL}$ .

For this method procedure, a calibration curve was constructed, outlined in figure 18 with DPPH methanolic solutions.



**Figure 17:** Calibration curve of methanolic solutions of DPPH.

The standard reference compound was Gallic Acid. This compound was previously studied for its antioxidant activity, which allows to obtain the efficient concentration of extract that can reduce at least half (50%) of the initial concentration of DPPH (the  $\text{IC}_{50}$ ) and later calculate the antioxidant activity index (AAI).

From the previous range, three methanolic solutions (0.080, 0.1242 and 0.200 mM) were arranged and for each of these concentrations and for the different concentrations of the extract, we added 0.1 mL of sample and 3.9 mL of DPPH and left to incubate for

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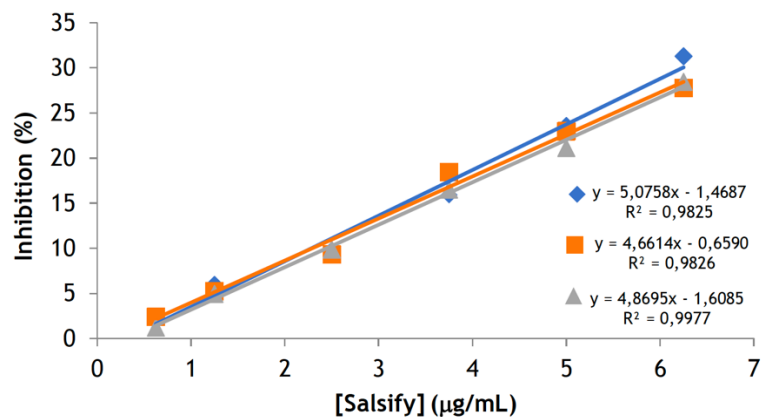
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90 minutes in total dark at room temperature. A blank solution contained only methanol, and a control solution was also made by replacing the 0.1 mL of sample with 0.1 mL of methanol. The tests were carried out in triplicate and the absorbances were read at 517 nm.

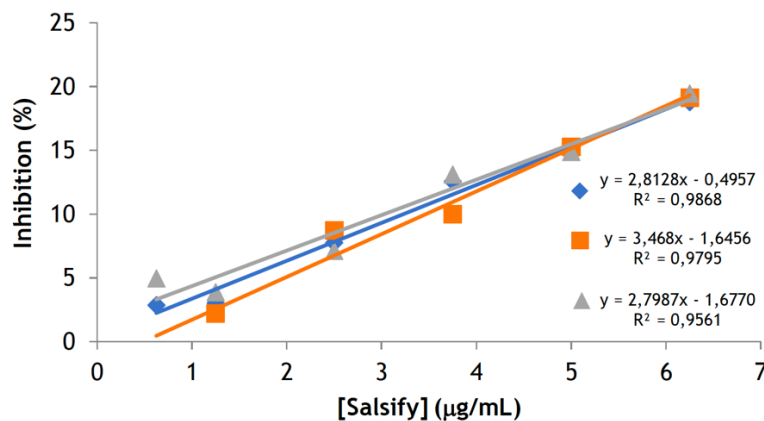
The IC<sub>50</sub> was calculated graphically using calibration curves of extract concentration versus corresponding radical scavenging activity and the data is exposed in table 14. The radical scavenging activity was calculated by the formula (3):

$$\text{Inhibition (\%)} = \frac{\lambda_{\text{Control}} - \lambda_{\text{Sample}}}{\lambda_{\text{Control}}} \cdot 100 \quad (3)$$

Thus, for each concentration of DPPH, the percentage of inhibition was determined and the calibration curves of the *T. porrifolius* concentration against radical scavenging activity are demonstrated in the following 3 figures, figure 19 ([DPPH] = 0,080 mM), figure 20 ([DPPH] = 0,124 mM) and figure 21 ([DPPH] = 0,200 mM).



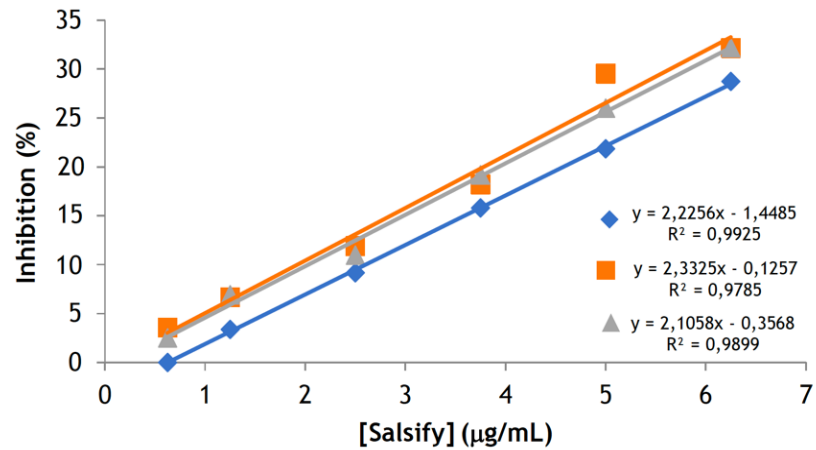
**Figure 18:** Calibration curve for [DPPH] = 0,080 mM in *T. porrifolius* extract.



**Figure 19:** Calibration curve for [DPPH] = 0,124 mM in *T. porrifolius* extract.

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**Figure 20:** Calibration curve for  $[DPPH] = 0,200 \text{ mM}$  in *T. porrifolius* extract.

**Table 14:** The  $IC_{50}$  value obtained for the *T. porrifolius* extract using data from the calibration curves for each DPPH concentration.

<b>[DPPH] = 0,080 mM</b>		
<b>Essay</b>	<b><math>IC_{50}</math></b>	<b>Average <math>\pm</math> Standard deviation</b>
1	10,989	11,833 $\pm$ 1,623
2	13,521	
3	10,989	

<b>[DPPH] = 0,124 mM</b>		
<b>Essay</b>	<b><math>IC_{50}</math></b>	<b>Average <math>\pm</math> Standard deviation</b>
1	17,023	16,333 $\pm$ 1,265
2	14,989	
3	16,987	

<b>[DPPH] = 0,200 mM</b>		
<b>Essay</b>	<b><math>IC_{50}</math></b>	<b>Average <math>\pm</math> Standard deviation</b>
1	22,256	23,486 $\pm$ 1,056
2	19,356	
3	28,847	

<b><math>IC_{50}</math> [DPPH] = 0,080 mM, 0,124 mM and 0,200 mM</b>	
<b>Total Average <math>\pm</math> Standard deviation</b>	<b>17,551 <math>\pm</math> 4,105</b>

Following these results, it's important to incorporate them in an equation to express the final antioxidant activity present in this *T. porrifolius* extract, resulting in the Antioxidant Activity Index (AAI), calculated from the following formula (4):

$$\text{AAI} = \frac{[\text{DPPH}]_{\text{Final}} (\mu\text{g/mL})}{\text{IC}_{50} (\mu\text{g/mL})} \quad (4)$$

### Method using $\beta$ -Carotene/Linoleic acid system

The  $\beta$ -carotene/linoleic acid system, which consists of the discoloration (oxidation) of  $\beta$ -carotene induced by oxidative degradation products of linoleic acid, estimates the ability relative to antioxidant compounds present in plant extracts to scavenge the radical linoleic acid peroxide ( $\text{LOO}^\circ$ ), which oxidizes the  $\beta$ -carotene present in the plant. This mechanism is a phenomenon mediated by hydroperoxide radicals resulting in the oxidation of linoleic acid by oxygen. The free radical of linoleic acid, formed after the capture of a hydrogen atom from one of its methylene-dialyl groups, attacks highly unsaturated  $\beta$ -carotene molecules and once they lose their double bonds by oxidation, in the absence of antioxidants, the compound loses its chromophore which can be observed spectrophotometrically.

After preparing the  $\beta$ -carotene solution (20 mg/mL in chloroform), 500  $\mu\text{L}$  were added to 40  $\mu\text{L}$  of linoleic acid, 400  $\mu\text{L}$  of *Tween* 40 and 1 mL of chloroform. The chloroform was then evaporated in a rotary evaporator for 5 minutes submerged in 45  $^\circ\text{C}$  and then 100 mL of distilled water saturated with  $\text{O}_2$  was added to form an emulsion. Then, 5 mL of the emulsion were added to 300  $\mu\text{L}$  of *T. porrifolius* extract. The solutions were stirred and left to react for about 60 minutes at 50  $^\circ\text{C}$ . All tests were carried out in duplicate and absorbances were read at 470 nm at origin time (0 minute) and after 60 minutes.

After acquiring the absorbance values, the percentage of inhibition was calculated using the formula (5):

$$\text{Inhibition (\%)} = \frac{\lambda_{\text{Sample}}^{t=1 \text{ hour}} - \lambda_{\text{Control}}^{t=1 \text{ hour}}}{\lambda_{\text{Control}}^{t=0 \text{ hours}} - \lambda_{\text{Control}}^{t=1 \text{ hours}}} \cdot 100 \quad (5)$$

The evaluation of antioxidant activity through the  $\beta$ -carotene/linoleic acid system was compared with the control standard BHT (butylated hydroxytoluene) and, for this, methanolic solutions were prepared with concentrations ranging from 5, 50, 100, 250, 500 and 1000  $\mu\text{g/mL}$ .

As such, the same concentration range was applied to the methanolic *T. porrifolius* extract solution.

## Cell lines

The use of cell lines is a methodology that allows the development of cells in simulated physiological conditions and in a controlled environment that facilitates scientific investigation due to the reproducibility associated with the data under study.

In the present study, two prostate cell lines were used. A non-neoplastic PNT1A (human post-pubertal prostate epithelial cell line) and a neoplastic PC3 line considered aggressive (derived from metastasis presented in bone). The PC3 cells present as very aggressive in late stages of the disease and as being androgen-independent. Both lines were purchased from the European Collection of Cell Cultures (ECACC) located in England. Those characterize are shown in table 15 as well as figure 21, that shows classical examples for each line as a good morphology.

The study began when the cells got the desired confluence and in order to prepare the cell suspension to be used in the essay, we started by removing the medium of the medium-sized culture vial and adding 3.0 mL of a solution containing Trypsin (0.05%) leaving it to act for approximately 5 minutes, in order to highlight the cells of the vial. Then, 7.0 mL of medium was added, thus neutralizing the action of trypsin. The resulting mixture of cells suspended in medium and trypsin was subsequently transferred to a 15.0 mL falcon tube and centrifuged at 250 RCF (relative centrifugal acceleration). The supernatant was discarded and the *pellet* of cells deposited at the bottom of the *falcon* was resuspended with a 10.0 mL of RPMI-1640 medium. From the resulting solution, 10.0 L was removed  $\mu$ into an *ependorf* tube and 10.0  $\mu\text{L}$  of tryptane blue was added, which will signal the viable cells existing in the count carried out in a Neubauer chamber.

The following equation (6) determines the concentration of viable cells per mL of solution:

$$[\text{Cells}_{\text{Viable}}] = \overline{\text{Cells}_{\text{Visible}_{\text{Quadrant}}}} \cdot 2_{(\text{Dilution Factor})} \cdot 10^4_{(\text{Neubauer chamber})} \quad (6)$$

### Cell proliferation/viability essay (MTT)

The original *T. porrifolius* solution was prepared by dissolving and homogenizing 50 mg of *T. porrifolius* extract in a mixture of 0.15 mL H<sub>2</sub>O and 0.15 EtOH. With those data, is possible to calculate the mass concentration required for the essay by applying the equation (7):

$$C = \frac{m(\text{mg})}{V(\text{mL})} = \frac{50}{0.15 \cdot 0.15} = 167 \text{ mg/mL} \quad (7)$$

All other 6 *T. porrifolius* preparations (concentration range 10, 50, 100, 1000 and 2000 µg/mL) were prepared by successive dilutions in culture medium.

Before the essay *per say*, the cell lines were prepared to ensure the best possible conditions, closely resembling *in vivo* conditions. Specifically, the PNT1A and PC3 cell lines were cultured in RPMI 1640 medium (Gibco, Invitrogen/Life Technologies in England) supplemented with 1.0% streptomycin (Gibco, Invitrogen/Life Technologies in England) and 10.0% fetal bovine serum (Biochrom AG in Germany). These cells were maintained in an incubator at 37 °C with 5.0% CO<sub>2</sub> until they reached a confluence of over 70%, which took approximately 2 weeks. “Passages” were performed as needed to control confluence. After reaching the desired density and morphology, a specific number of cells were seeded for each cell line: 4000 for PNT1A and 3000 for PC3.

For every essay, 3 sets of 96-well plates were used maintaining the same conditions (act as triplicates). The plates were sterilized in a laminar flow chamber before use. Figure number 22 schematizes the conditions of each well used for each essay. In columns 3 to 8 of the plates, 100 µL of each concentration of the extract were added, with concentrations ranging from 10 to 2000 µg/mL of *T. porrifolius*, creating an increasing gradient of concentration. Column 12 contained 100 µL of a 2% SDS solution, which acted as the positive control to ensure there was no expected cell proliferation.

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A medium column separated this from the other columns to minimize any potential for vaporization. The remaining columns were filled with 100  $\mu$ L of Phenol Red medium. Each well had a final volume of 200  $\mu$ L after successive dilutions of the extract. The medium used was RPMI-1640.

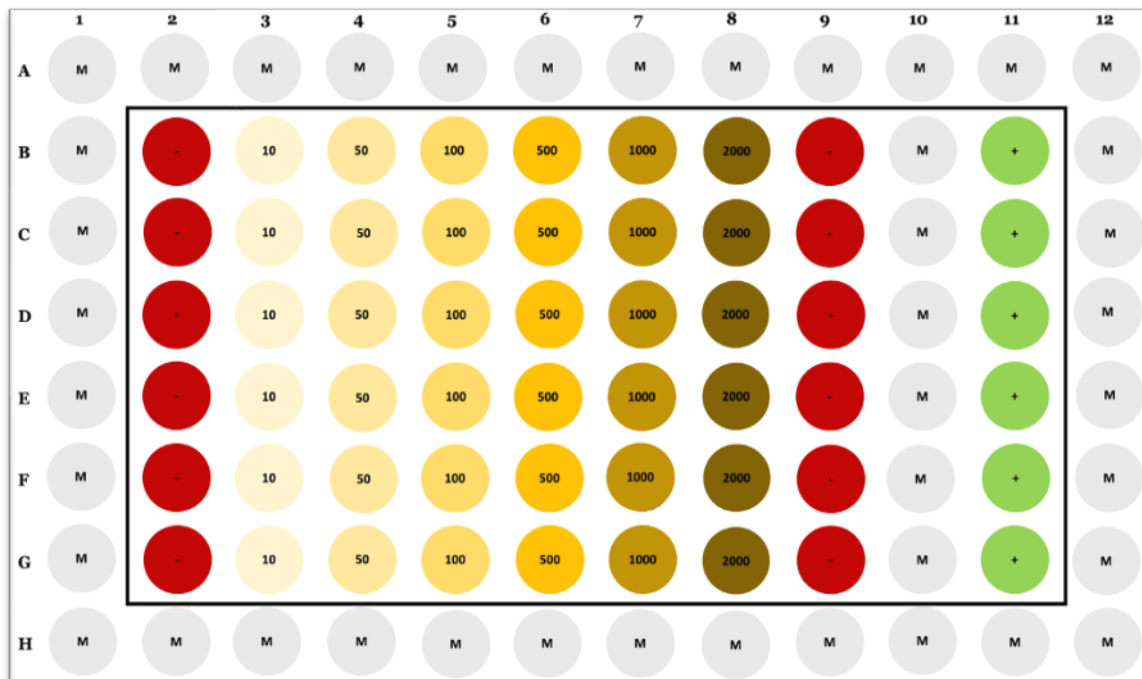
The viability MTT assay was carried out at 24, 48, and 72 hours of culture.

The MTT reagent (3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide), a yellow mono-tetrazolium salt that when reduced by active cells forms formazan, a water-insoluble molecule colored purple.

Briefly, the culture medium was removed and added 200  $\mu$ L of phenol red-free medium along with 10  $\mu$ L of MTT solution (Sigma-Aldrich in USA). The culture plates were then incubated in the dark for 4 hours, wrapped in foil.

After this period, the medium was discarded and the remaining formazan crystals (indicative of viable cells) were dissolved in 200  $\mu$ L of dimethyl sulfoxide (DMSO).

To ensure homogeneity, all the plates were wrapped in aluminum foil, agitated for 5 minutes, and measured the absorbance at 570 nm in the xMark™ Microplate Absorbance Spectrophotometer (BioRad in USA). This step is crucial as the absorbance value is directly proportional to the number of viable cells in the study group.



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**Figure 21:** Schematic plaque of 96 wells used in this study, with the range of concentrations (10, 50, 100, 500, 1000 and 2000 µg/mL). **(-)** - Negative Control to ensure the viability of the experimental treatment, as this column is not subject to any experimental treatment with *T. porrifolius* extract so no results are expected. **(+)** - Positive Control to ensure the viability of the experimental treatment, as this column is not subject to any experimental treatment with *T. porrifolius* extract but is exposed to a 2% SDS solution, which causes cell destruction. **(M)** – Medium with phenol red to prevent volatilization of compounds. **Column 10** - separates a negative control from a positive one in order to prevent the volatilization of the DMSO into the positive control and change the results, thus serving as a means of control for the possibility of volatilization.

## Statistical analysis

In order to transform visual results into quantifiable ones, the software “GraphPad Prism v6.00” was used. A  $p < 0,05$  values were deemed to be statistically significant and, considering significance: (\*)  $p < 0,05$  , (\*\*),  $p < 0,01$ , (\*\*\*)  $p < 0,001$  and (\*\*\*\*)  $p < 0,000$ . The experimental data is shown as “mean  $\pm$  SEM”.

## Chapter N° 4 - Results and Discussion

### Plant extraction

The dried *T. porrifolius* was extracted with methanol using an ultrasound bath facilitating the transfer of compounds from this material to the extraction solvent. This type of extraction can also break down cell walls resulting in greater compound extraction efficiency in a reduced extraction time, providing better yield results.

The yield value obtained for the *T. porrifolius* extract was  $26.52 \pm 3.17\%$ . The comparison with yields obtained in other studies is not possible as there may be significant differences, especially when different extraction conditions and solvent polarities are used, as they influence extraction efficiency.

### Determination of Total Phenolic compounds

Phenolic compounds are among the most prevalent secondary metabolites in the Plantae kingdom, acting mainly as phytoalexins (anti-infective properties), and protective agents against the ultraviolet spectrum. They are an important part of plants due to their free radical scavenging capacity. Total Phenolic concentration can be used as a rapid screening test of antioxidant activity and was determined using the *Folin-Ciocalteu* method. The *T. porrifolius* extract presents a total phenolic compound value of  $198.426 \pm 3.822$  mg GAE/g. Another study with extractions using other solvents like water and ethanol/water (80/20), showed different results,  $102.9 \pm 3.9$  mg GAE/g and  $145. \pm 3.9$  mg GAE/g, respectively [4]. The results vary depending on the extraction solvent, sample source, and methodology used [4]. As a comparison, the phenolic content for *Portulaca oleracea* (purslane), a leaf crop, was  $216.96 \pm 0.87$  mg GAE/g, as determined by Aryal *et al* [81].

In rough comparison, methanol and water were found to extract more phenolic compounds than water and ethanol.

The variations in results can be attributed to multiple factors, including sample preparation, climatic conditions, and sample collection. Seasonal variations also affect phenolic compound levels.

## Determination of Total Flavonoid compounds

Flavonoids are the most common and widely distributed group of phenols in plants and have been shown to be highly effective in eliminating most types of oxidizing molecules, which may be involved in DNA damage and tumor promotion. The total flavonoid content of this extract was  $36.185 \pm 1.543$  mg QE/g. In the previous study, the highest total flavonoid content was obtained with ethanol (80%) registering at  $28.500 \pm 0.200$  mg QE/g and was three times greater than the extraction with water ( $14.700 \pm 0.300$  mg QE/g). [4] As a comparison, a study conclude that this content for *Portulaca oleracea* (purslane), a leaf crop, was  $39.38 \pm 0.57$  mg QE/g [83].

Additionally, some authors found that absolute ethanol is more effective for extracting flavonoids compared to water, however in this particular case, an extraction in methanol/water is preferred. This highlights the importance of solvent selection in the extraction process.

## Determination of Antioxidant Activity

### DPPH method

The DPPH radical is widely employed for assessing the efficacy of free radical scavenging due to its straightforward reaction. This particular radical is stable and exhibits a distinctive absorption peak in the range of 515 to 517 nm. A reduction in the absorption of the DPPH solution occurs when antioxidant extracts or standard solutions convert DPPH into its reduced hydrazine form.

From the concentration versus % inhibition graphs, it was possible to calculate the  $IC_{50}$  for the *T. porrifolius* extract. This parameter helps determine the concentration required to achieve 50% inhibition of DPPH radicals. Lower  $IC_{50}$  values indicate higher antioxidant capacity of the sample under study.

The obtained data demonstrate that, concerning radical scavenging activity, the *T. porrifolius* extract exhibits greater potential at  $17,551 \pm 4,105$   $\mu$ g/mL.

As mentioned, the DPPH radical has widespread use in evaluating free radical scavenging activity because of the ease of this reaction. This free radical is stable and has a characteristic absorption maximum between 515 and 517 nm. Decreased absorption of

DPPH solution occurs when antioxidant extracts or standard solutions reduce DPPH to its reduced form of hydrazine.

As far as AAI goes, based on the mean obtained by the 3 essays in table 15 it presents a relatively strong activity of  $1,231 \pm 0,213$ , value between the established range from 1.0 to 2.0. Taking into account that there is evidence that phenolic compounds have antioxidant activity, this results are promising.

AAI values are within the following reference ranges for antioxidant capacity:

- Weak (< 0.50);
- Moderate (0.50 – 1.0);
- **Strong (1.0 – 2.0)**;
- Very strong ( $\geq 2.0$ ).

### **Method using $\beta$ -Carotene/Linoleic acid system**

This study focused on evaluating the antioxidant activity of crude methanolic extracts, primarily employing the  $\beta$ -carotene/linoleic acid system method. This method assesses the inhibition of lipid peroxidation induced by free radicals. The findings indicated that the *T. porrifolius* extract exhibited anti-peroxidation properties with a lower antioxidant activity compared to the standard BHT (Butylated Hydroxytoluene), as shown in figure 23.

This can lead to a consideration as the potential of alternative solvents such as ethanol. For the same concentration of extract and standard (BHT), at a concentration of 500  $\mu\text{g/mL}$ , the  $\beta$ -carotene bleaching activity of *T. porrifolius* was  $8.23 \pm 9,25\%$ , a much lower value than that of BHT at  $87.23 \pm 0.91\%$ . It is also important to highlight that *T. porrifolius* only begins to show signs of inhibition around 500  $\mu\text{g/mL}$ , leading to the consideration that in addition to being a time-dependent extract, it is also dose-dependent.

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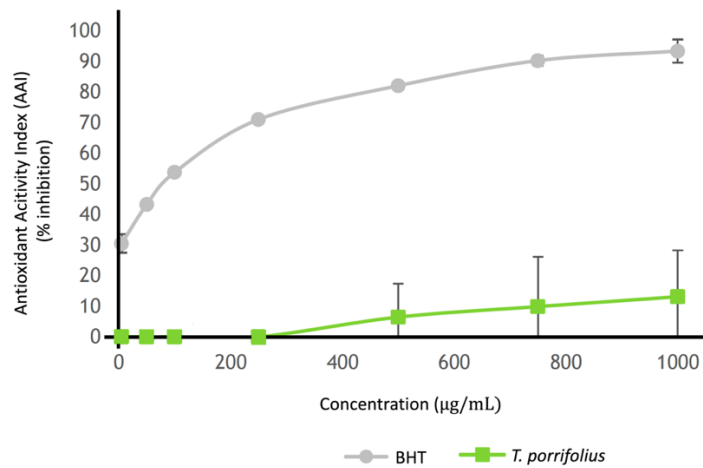


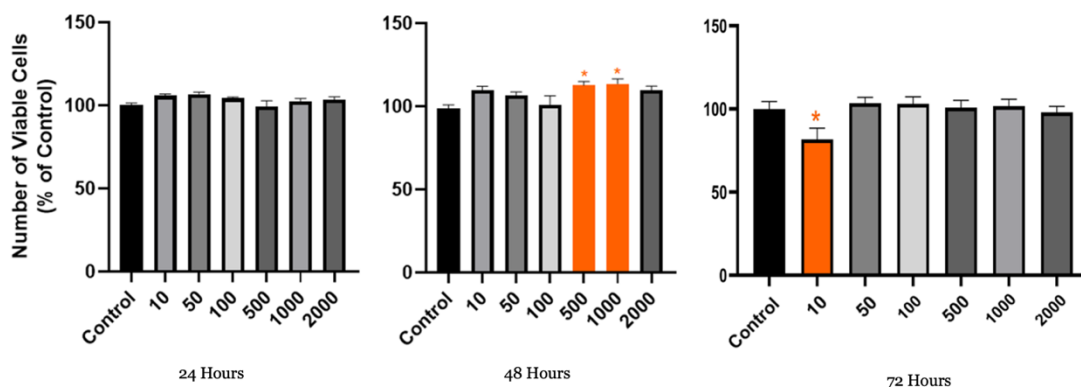
Figure 22: AAI of the BHT standard and the T. porrifolius extract.

## Cytotoxic Activity

The assessment of the extract's impact on cell viability was conducted indirectly through the MTT assay. A lower percentage of MTT reduction indicates lower cell viability and, consequently, a greater apoptotic effect of the extract. An extract is classified as “cytotoxic” if its effect results in a reduction of at least 30% or if MTT reduction is less than 70% when compared to the control.

### PNT1A

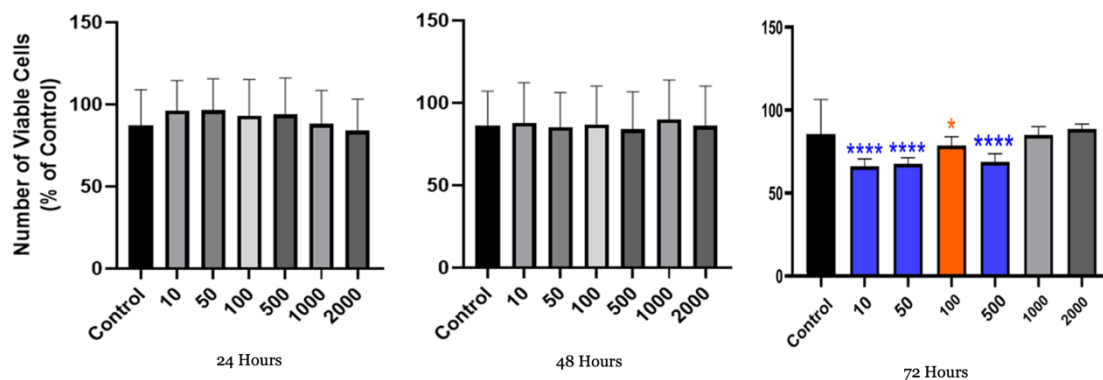
In this non-neoplastic prostate cancer cell line (PNT1A) as expected in figure 24 shows that significant reductions didn't occur. At 24 hours the extract did not produce any quantifiable stimulus when compared to the control. At 48 hours, there's no reduction of viable cells, quite the contrary, however 2 outliers at 500 and 1000 µg/mL. After 72 hours of exposition, the range from 50 to 2000 µg/mL maintains the same as the control, with the group exposed to 10 µg/mL being considered as an exception, however a promising one, since no visible and empiric reduction occur at higher range concentrations.



**Figure 23:** Impact of *T. porrifolius* extract at various concentrations (10, 20, 100, 500, 1000 and 2000  $\mu\text{g}/\text{mL}$ ) on the survival of non-cancerous human prostate epithelial PNT1A cells over different durations (24, 48, and 72 hours), as assessed through the MTT assay. The results are presented as a percentage relative to the control group, with error bars representing the mean  $\pm$  standard error of the mean ( $n \geq 6$ ).

### PC3

The viability of neoplastic cells (PC3) is visibly expressed in figure 25. As previously explained, this extract appears to have a time and dose-dependent manners, confirmed by the 24 hours assay were, as expected, no inhibition occurred. After 48 hours of treatment, there was no significant difference in viability compared to the control group. However, after 72 hours a visible reduction in proliferation occurred in the groups exposed to 10, 50, and 500  $\mu\text{g}/\text{mL}$ . The group exposed to 100  $\mu\text{g}/\text{mL}$  didn't show this tendency, however a reduction did occur. The lowest proliferation was observed at 10  $\mu\text{g}/\text{mL}$ . There was no significant reduction in proliferation when using 2000  $\mu\text{g}/\text{mL}$  of *T. porrifolius* extract. This means that *T. porrifolius* does possess an effect in PC3 cells and can inhibit proliferation even in small concentrations range.



**Figure 24:** Impact of *T. porrifolius* extract at various concentrations (10, 20, 100, 500, 1000 and 2000  $\mu\text{g}/\text{mL}$ ) on the survival prostate cancer cells PC3 over different durations (24, 48, and 72 hours), as assessed through the MTT assay. The results are presented as a percentage relative to the control group, with error bars representing the mean  $\pm$  standard error of the mean ( $n \geq 6$ ).

## Chapter N<sup>o</sup> 5 - Conclusions

The present dissertation had 2 main objectives: the confirmation of the possible antioxidant effect of a *Tragopogon porrifolius* (or *T. porrifolius*) extract in prostate cells viability, namely non-neoplastic prostate epithelium cells – PNT1A and neoplastic androgen-independent (and also, more aggressive) PC3 cells.

With the analysis of the DPPH method and the  $\beta$ -carotene/linoleic acid system for determining antioxidant activity, it can be concluded that the two methods are in agreement in the results obtained for these extract, that presents a relatively strong antioxidant activity. In relation to the standards used (gallic acid for DPPH and BHT for  $\beta$ -carotene), both in one method and in the other, the extract has a relatively weaker antioxidant activity than these. Since literature says that phenolic compounds are involved in antioxidant activity and that this compound presents in its molecular structure, this points has been proved.

As prostate cancer *per say*, an uncontrolled proliferation of prostate cells is a condition commonly seen in the male population as they age, and is associated with the development of prostate cancer. In terms of cellular viability, the viability of PNT1A wasn't affected and the PC3 cells produced a notable reduction in proliferation even at lower concentrations being in accordance with previous studies [1, 4]. This proves that further testing should be an option to this plant as a therapeutical tool and adjuvant in food sources to patients with prostate cancer and should be evaluate in apoptotic ways to show how the present inhibition can occur.

## Chapter N° 6 - Future Prospects

As seen through this document, there is limited information available on the potential of *Tragopogon porrifolius* in relation to prostate cancer.

*T. porrifolius* is a plant known for its edible roots and has been used in traditional medicine for various purposes, but its specific effects on prostate cancer prevention or treatment have not been extensively studied or documented. As demonstrated by this assay, it possess potential for becoming a therapeutical tool for initial prostate screening, however further studies regarding range concentrations and other cell lines are vital to acquire definitive conclusions. One option would be to test *T. porrifolius* against other tumor cell line to verify it's effects. In order to definitively prove that consumption of *T. porrifolius* or ingestion of supplements are both beneficial for prostate cancer prevention and attenuation, the necessity to assay the bioavailability of these compounds, post-digestion will remain a crucial aspect.

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*Tragopogon porrifolius*: phytochemical characterization and evaluation of the potential antitumor effect of the extract on the prostate cells

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## Chapter Nº 8 - Publications

# Effect of a Salsify (*Tragopogon porrifolius*) Extract on Human Prostate Cells Viability

P.G.P. Rocha, L.R.S. Fonseca, A.P. Duarte, S. Socorro.

 [apcd@ubi.pt](mailto:apcd@ubi.pt)  [ssocorro@fcsaude.ubi.pt](mailto:ssocorro@fcsaude.ubi.pt)

CICS-UBI, Health Sciences Research Centre, University of Beira Interior (UBI), Covilhã, Castelo-Branco, Portugal.

## Introduction

*Tragopogon porrifolius* is the botanical designation for Salsify, a biennial plant native of the Mediterranean region and used by ancient civilizations to prevent infections.

Previous studies suggested that *T. porrifolius* displays anticancer effects in human breast and colon cancer cells by regulating cell viability, proliferation and oxidative status.

The effect of Salsify in prostate cancer (PCa) remains unexplored.

## Objectives

- 1) To perform the phytochemical characterization of the *T. porrifolius* extract.
- 2) To analyze the effects of the *T. porrifolius* extract in human prostate cells (PNT1A and PC3) viability as a first indication to further studies covering the cancer hallmarks.

## *T. Porrifolius* Extract



## Experimental Design

Hydroalcoholic mixture (70 – 30)  
Ultrasound  
Solid-Liquid extraction



## Chemical Characterization



### DPPH

- Total phenolic compounds;
- Inhibitor of radical polymerization (FRP);
- Violet in solution (DPPH);
- Yellow when neutralized;
- Colorimetric method;
- Read at 520 nm.

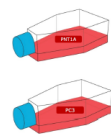
### $\beta$ -carotene

- Total flavonoid compounds;
- The most relevant carotenoid in humans;
- Food coloring agent;
- Strong antioxidant.

## Treatment of neoplastic (PC3) and non-neoplastic (PNT1A) human prostate lines



10 – 2000  $\mu$ g/mL  
48-72 Hours



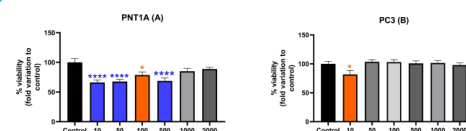
MTT

Cell Viability

## Results

**Table 1** - Overall chemical characterization and antioxidant potential of the *T. porrifolius* extract.

Compounds	Quantity
Phenols	72,67 $\pm$ 5,03 mg Gallic acid/Dry matter
Flavonoids	32,56 $\pm$ 0,24 mg Quercetin/Dry matter
DPPH	IC <sub>50</sub> = 12,14



**Figure 1** - Viability of PNT1A (A) and PC3 (B) in human prostate cells cultured with a concentration range of *T. porrifolius* extract for 72 hours. Viability was determined by the MTT assay. Results are expressed as % of the control (n=6). \*p<0,050; \*\*\*\*p<0,000

## Conclusions

Chemical analysis of *T. porrifolius* extract demonstrated its enrichment in phenolic compounds, which was underpinned by a significant antioxidant activity demonstrated by an IC<sub>50</sub> value of 12,14 in the DPPH assay. Exposure to the *T. porrifolius* extract for 72 hours decreased the viability of PNT1A and PC3 cells at least for some concentrations in the 10 to 500  $\mu$ g/mL range. Although preliminary, the results obtained indicate that *T. porrifolius* can modulate the viability of human prostate cells, and likely inhibit PCa cell growth.

Also, these findings support investigating the effects over the hallmarks of cancer, namely, proliferation, apoptosis, oxidative damage and glycolytic metabolism. This work is ongoing.

