ESCOLA
SUPERIOR
DE SAÚDE
POLITÉCNICO
DO PORTO

P.PORTO

M

MESTRADO
FARMÁCIA

Quantification of phenolic compounds in *Melilotus indicus* and *Pterospartum tridentatum*

José Pedro Santos Cruz

10/**2022**

ESCOLA
SUPERIOR
DE SAÚDE

Quantification of phenolic compounds in *Melilotus indicus* and *Pterospartum tridentatum*

Autor

José Pedro Santos Cruz, Porto, Portugal

Orientadores

Professora Doutora Patrícia Carla dos Santos Correia, Centro de Investigação em Saúde e Ambiente (CISA), Escola Superior de Saúde (ESS), Instituto Politécnico do Porto (IPP), Porto, Portugal

Professora Doutora Ana Isabel de Freitas Tavares de Oliveira, Centro de Investigação em Saúde e Ambiente (CISA), Escola Superior de Saúde (ESS), Instituto Politécnico do Porto (IPP), Porto, Portugal

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Mestre em **Farmácia** – Ramo em **Tecnologia do Medicamento e de Produtos de Saúde** pela Escola Superior de Saúde do Instituto Politécnico do Porto.

Agradecimentos

No capítulo dos agradecimentos, queria demonstrar a minha gratidão para todos os que me apoiaram nesta longa e reconfortante jornada. Aos meus pais e à minha família, pelo apoio e amor incondicional, aos meus amigos que apesar da distância demonstraram sempre prontidão em ajudar, às orientadoras Professoras Doutoras Patrícia Correia e Ana Isabel Oliveira que foram incansáveis e pacientes em todo o trabalho realizado e às minhas colegas de investigação, Liliana e Mariana, que em grande espírito de equipa e entrega, levamos o barco a bom porto. Um agradecimento ao corpo docente do Mestrado em Farmácia, à Escola Superior de Saúde do Porto e ao Centro de Investigação em Saúde e Ambiente. E, a todos aqueles que direta ou indiretamente tiveram o seu contributo nesta caminhada. As barreiras tornaram-se desafios e os objetivos um grande desenvolvimento pessoal.

"Tudo é ousado para quem a nada se atreve."

Fernando Pessoa

Resumo

Melilotus indicus e *Pterospartum tridentatum* são duas plantas com alto teor de compostos fenólicos. Os objetivos deste estudo são compilar os métodos publicados para a determinação de compostos fenólicos nas plantas em estudo e a validação de um método para a determinação destes compostos em extratos das mesmas. Foi realizada uma revisão clássica em três bases de dados de estudos publicados desde 2000 para a determinação de compostos fenólicos nas plantas de interesse. A validação de um método de análise destes compostos por cromatografia líquida de elevada eficiência, contemplou parâmetros como linearidade, precisão, exatidão, limites de deteção e quantificação, seguida de uma análise preliminar em extratos. A literatura relata a análise de vinte e oito a vinte e nove compostos, respetivamente nos extratos de *M. indicus* e *P. tridentatum*. Foram estipuladas curvas de calibração para hesperidina, ácidos cafeico e clorogénico, com limites de quantificação inferiores a 2,98 $\mu g.mL^{-1}$. A precisão apresentou valores inferiores a 15% e a exatidão variou entre 90 e 114%. Uma análise preliminar quantificou rutina, ácidos clorogénico e cafeico, e identificou rutina, quercetina-3-glucósido, hesperidina, ácidos gálico, clorogénico e cafeico, nos extratos de *M. indicus*, e quantificou rutina, hesperidina, ácidos gálico e clorogénico, nos extratos de *P. tridentatum.*

Palavras-chave: *Melilotus indicus; Pterospartum tridentatum*; validação; quantificação; compostos fenólicos

Abstract

Melilotus indicus and *Pterospartum tridentatum* are two plants with high content of phenolic compounds. The objectives of this study are to compile the published methods for the determination of phenolic compounds in the plants under study, and the validation of a method for the determination of these compounds in extracts of them. A classical review was carried out in three databases of studies published since 2000 for the determination of phenolic compounds in the plants of interest. The validation of a method for the analysis of these compounds by high performance liquid chromatography contemplated parameters such as linearity, precision, accuracy, limits of detection and quantification, followed by a preliminary analysis in extracts. The literature reports the analysis of twenty-eight to twenty-nine compounds, respectively in *M. indicus* and *P. tridentatum* extracts. Calibration curves were stipulated for hesperidin, caffeic and chlorogenic acids, with quantification limits below 2.98 $\mu g.mL^{-1}$. The precision showed values below 15% and the accuracy ranged between 90 and 114%. A preliminary analysis quantified rutin, chlorogenic and caffeic acids, and identified rutin, quercetin-3-glucoside, hesperidin, gallic, chlorogenic and caffeic acids, in *M. indicus* extracts, and quantified rutin, hesperidin, gallic and chlorogenic acids, in *P. tridentatum* extracts.

Keywords: *Melilotus indicus; Pterospartum tridentatum*; validation; quantification; phenolic compounds

Table of contents

Abbreviations, acronyms, and symbols

°C	Degrees Celsius
%	Percentage
σ_{lab}	Standard deviation of the laboratorial concentration
µg	Micrograms
µL	Microlitres
µm	Micrometres
ACN	Acetonitrile
b	y-axis intercept
C18	Octadecyl
CA	Caffeic acid
C_{exp}	Mean of the expected concentrations
CH	Chosen wavelength
CLA	Chlorogenic acid
C_{lab}	Mean of the laboratorial concentrations
C_{re}	Concentration obtained using the peak area of each compound directly in the correspondent calibration equation
CV	Coefficient of variation
D1	Detected below linearity range and limit of quantification
D2	Detected above linearity range
DAD	Diode array detector
ESI	Electrospray Ionisation
g	Grams
GA	Gallic acid
GC	Gas chromatography
h	Hours
HBA	Hydroxybenzoic acids
HCA	Hydroxycinnamic acids
HESP	Hesperidin
HPLC	High performance liquid chromatography
ICH	*International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use*
IR	Infrared

LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
m	Mass
MeOH	Methanol
min	Minutes
mg	Milligrams
mL	Millilitres
mm	Millimetres
MS	Mass spectrometric detector
N/D	Not detected
N/S	Not specified
n.a.	Not available
NAR	Naringin
nm	Nanometres
PAD	Photodiode array detector
PC	Phenolic compounds
QUE	Quercetin
Q3G	Quercetin-3-glucoside
R	Pearson correlation coefficient
RP	Reverse phase
R_t	Retention time
RUT	Rutin
S	Slope of the calibration curve
S_b	Standard deviation of the y-axis intercept of the calibration curve
S_e	Standard error of the slope
S_s	Standard deviation of the slope
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
UHPLC	Ultra-High performance liquid chromatography
UV	Ultraviolet
UV-B	Ultraviolet B
Vis	Visible
v/v	Volume per volume

List of tables

List of figures

1. Introduction

1.1. The impact of plants on health

Plants and their benefits have accompanied humanity since the beginning, in food, medicine and to survival (Šantić *et al.*, 2017; Sen & Samanta, 2014). Khan (2014) suggests that communities in the prehistoric era used the medicinal effects of plants. Moreover, ancient civilizations also categorised medicinal plants, such as: the Atharvaveda in traditional Indian medicine – a sacred text of Hinduism that described medicinal treatments (2000 BC); the Eber Papyrus – one of the oldest medical treatises of the Egyptian culture (1550 BC); Hippocrates (460–337 BC) considered the father of medicine, identified about 400 medicinal substances from plants; as well as Mithridates VI (133 BC – 63 BC), king of Pontus, in Hellenic culture, tried to develop a universal antidote for poisoning (Cheng, 2001; Griffin & D'Arcy, 1996; Khan, 2014). This medicinal plant knowledge was shared between generations and, in present times after being scientifically confirmed, some of those plants are included in the official pharmacopoeias (Šantić *et al.*, 2017).

The demand for natural products, especially those obtained from plants, has taken a crucial role in the development of alternative approaches to conventional medicines (Borges *et al.*, 2016). Medicinal plant means any plant or plant-derived substance, with minimal or no industrial processing, used for therapeutic purposes (Tilburt, 2008). According to World Health Organization data, the search for herbal treatments combined with conventional medicines has increased significantly in recent years (WHO, 2021).

Phytochemicals, namely secondary metabolites, are "bioactive compounds from different parts of plants such as seeds, cereals, vegetables, fruits, leaves, roots, spices and herbs" (Ferreira & Abreu, 2007, p. 36), may have multiple therapeutic purposes (e.g. antioxidant, antimicrobial, anti-inflammatory, antitumour). The combination of different compounds has represented a significant increase in beneficial effects when compared to individual use, such as α-tocopherol which, in association with kaempferol, has a higher inhibitory power of lipid oxidation caused by free radicals (Albuquerque *et al.*, 2021; Phan *et al.*, 2018). Previous studies have demonstrated the versatility of these compounds in acute and chronic diseases, and approximately 20% of known plants have been used in pharmaceutical trials with positive health outcomes (Altemimi *et al.*, 2017; Phan *et al.*, 2018). Some phytochemicals, such as chlorogenic acid (CLA) and naringin (NAR), are known for antioxidant and free radical scavenging activities, while kaempferol has been suggested to increase insulin sensitivity and prevent complications associated with diabetes (Ahangarpour *et al.*, 2019; Alkhalidy *et al.*, 2018; Altemimi *et al.*, 2017; Bacanli *et al.*, 2019). On the other hand, some bioactive compounds such as caffeic acid (CA) is described to mediate inflammation through proteins, others like hesperidin (HESP) to inhibit histamine release during an allergic reaction (Choi

et al., 2018; Deshetty *et al.*, 2020; Zhu *et al.*, 2018). Furthermore, phytochemicals such as quercetin (QUE) and rutin (RUT) are also responsible for preventing or decreasing the penetration of ultraviolet (UV) radiation into skin tissues and, consequently, reducing to a great part the endogenous reactions responsible for oxidative stress, inflammation, and deoxyribonucleic acid damage (Dobrikova & Apostolova, 2015; Kamel & Mostafa, 2015; Skarupova *et al.*, 2020). Another application of bioactive compounds from plants (e.g., luteolin) is the maintenance of neuronal balance and the treatment of cognitive disorders (Kumar & Khanum, 2012; Nabavi *et al.*, 2015).

At this point, nearly 10000 phytochemicals have been identified, while others remain unknown (Al-Ishaq *et al.*, 2020). These compounds have in their constitution primary metabolites (e.g. chlorophylls, proteins and amino acids) and secondary metabolites (such as phenolic compounds, carotenoids, alkaloids, nitrogen and organosulfur compounds) (Ferreira & Abreu, 2007; Roy & Datta, 2019). Phenolic compounds (PC) are one of the most studied metabolites, with *in vitro*, *in vivo,* and clinical trials demonstration of evidence (Kumar & Khanum, 2012; Lattanzio, 2013), and are the target of the present study.

1.2. Phenolic compounds: classification and biological activities

Approximately, more than 8000 PC have been identified, however, there is no agreement about their classification (Nollet & Gutierrez-Uribe, 2017; Santana-Gálvez & Jacobo-Velázquez, 2018). According to the different types of classification, the following methods have been used most frequently: a) flavonoid – non-flavonoid, b) number of aromatic rings, c) carbon skeleton, and d) base chemical structure (Santana-Gálvez & Jacobo-Velázquez, 2018).

Some authors such as Ferreira & Abreu (2007) and Roy & Datta (2019) defend that PC can be subdivided into different classes, phenolic acids, flavonoids, stilbenes, coumarins and tannins, while others such as Albuquerque *et al.* (2021) and Takó *et al.* (2020) include lignans as an additional group (Figure 1.1). Chemical structure and biological activity are the main factors in the differentiation between the several groups (Roy & Datta, 2019; Takó *et al.*, 2020).

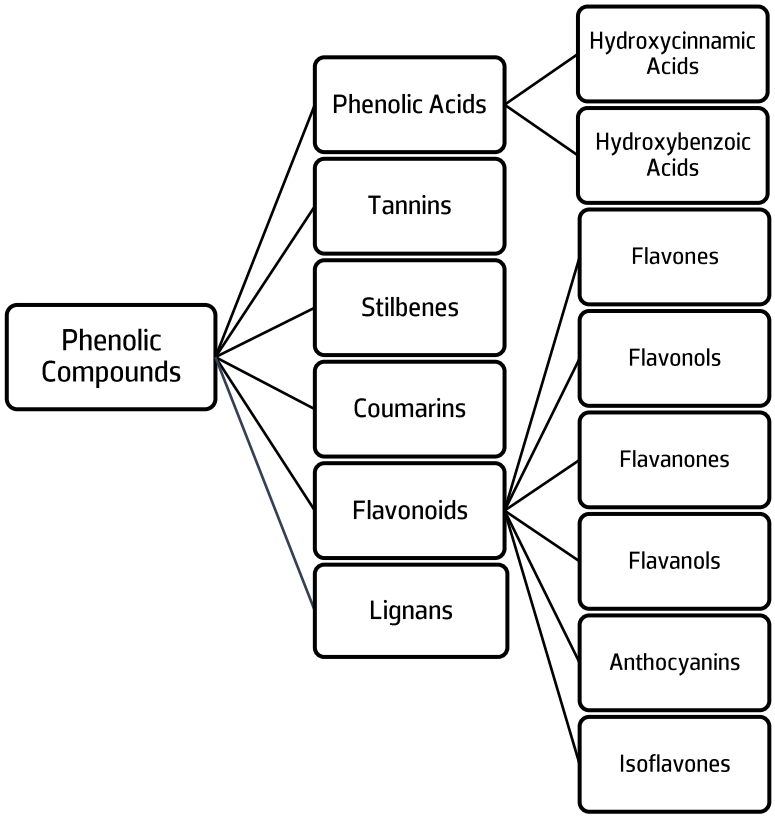


Figure 1.1 – Classification model for phenolic compounds adapted from Roy & Datta (2019) and Takó *et al.* (2020).

PC show beneficial biological activities for the organism such as antibacterial, anti-inflammatory, antigenotoxic, hepatoprotective, and antimutagenic (Lyu *et al.*, 2020). In addition, they exhibit a protective capacity against a wide range of oxidation and metabolic damage (Kumar & Khanum, 2012).The concentration of these compounds depends, simultaneously, on several factors such as the type of plant, the season, the growing and harvesting conditions and storage (Arnoso *et al.*, 2019).

Phenolic acids are subdivided into two groups: hydroxycinnamic acids (HCA) and hydroxybenzoic acids (HBA), and show an acidic profile as a result of the carboxyl group present in their chemical structure (Haminiuk *et al.*, 2012). CA, ferulic, ρ-coumaric, and sinapic acids are the most frequent of the HCA, which derived essentially from cinnamic acid (Bento-Silva *et al.*, 2020; Kumar & Goel, 2019). On the other hand, HBA, derived from benzoic acid, are most found as ρ-hydroxybenzoic, protocatechuic, vanillic, gallic (GA) and syringic acids (Albuquerque *et al.*, 2021; Bento-Silva *et al.*, 2020; Kumar & Goel, 2019). *Melilotus indicus* and *Pterospartum tridentatum* are two plants present in the portuguese flora that will be the target of this study. The compounds present in Table 1.1, are the most relevant phenolic acids in these plants, as well as their described bioactivities. As a result of their numerous applications, phenolic acids have received special scientific interest due to their therapeutic, cosmetic, and food properties (Kumar & Goel, 2019; Prince *et al.*, 2011).

Table 1.1 – Phenolic acids and their biological activities.

Main Class	Subclass	Phenolic compound	Bioactivities	Reference
Phenolic Acid	Hydroxycinnamic acids	CA	Antioxidant, anti-inflammatory, antimicrobial, antitumour, antimetastatic, antiviral, UV-B protective activities.	(Albuquerque *et al.*, 2021; Kumar & Goel, 2019; Skarupova *et al.*, 2020)
		CLA	Antioxidant, antibacterial, antiviral, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic neuroprotective, antimicrobial, antidiabetic activities.	(Gil & Wianowska, 2017; Kumar & Goel, 2019; Naveed *et al.*, 2018)
	Hydroxybenzoic acids	GA	Antioxidant, antimicrobial, anti-inflammatory, antitumour, neuroprotective, anticarcinogenic activities.	(Albuquerque *et al.*, 2021; Fernandes & Salgado, 2016; Li *et al.*, 2017)

UV-B – Ultraviolet B; CA – Caffeic acid; CLA – Chlorogenic acid; GA – Gallic acid.

Flavonoids, one of the most frequent secondary metabolites found in plants, have been characterised and subdivided into several subclasses, including flavones, flavanols, flavonols, flavanones, isoflavones and anthocyanidins (Ferreira & Abreu, 2007; Rosa *et al.*, 2019; Roy & Datta, 2019). These metabolites exhibit two aromatic rings (A and B) and a heterocyclic ring (C) (Figure 1.2) as a similar feature (Hosseinzadeh *et al.*, 2020; Rosa *et al.*, 2019; Tuenter *et al.*, 2020). The main differences between subclasses are in the saturation degree and oxidation of the C-ring and the C-ring carbon position that attaches to the B-ring (Hosseinzadeh *et al.*, 2020; Tuenter *et al.*, 2020).

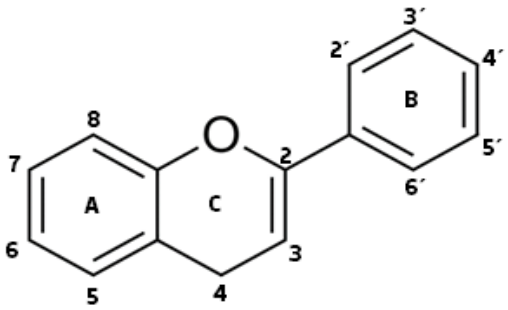


Figure 1.2 – Basic structure of flavonoids adapted from Rosa *et al.* (2019).

In the past years, flavonoids have been the subject of increasing interest because of their potential beneficial effects, including anti-inflammatory activity, protection of the cardiovascular system, regulation of neuronal function, prevention of age-related neurodegeneration and/or antioxidant activity (Altemimi *et al.*, 2017; Haminiuk *et al.*, 2012; Kumar & Khanum, 2012; Liu *et al.*, 2019; Tuenter *et al.*, 2020). The biological activity is directly related to the configuration, substitution and number of hydroxyl groups present, which will give a greater or lesser antioxidant activity (Albuquerque *et al.*, 2021). Table 1.2 describes the most relevant flavonoids present in *M. indicus* and *P. tridentatum* and their biological activities.

Table 1.2 – Flavonoids and their biological activities.

Main Class	Subclass	Phenolic compound	Bioactivities	Reference
Flavonoids	Flavones	Apigenin	Antioxidant, anti-inflammatory, antiviral, antiproliferative, antitumour, antineurodegenerative, antidepressant activities and promoting of dermal density and elasticity.	(Albuquerque *et al.*, 2021; Lee *et al.*, 2015; Liu *et al.*, 2019; Salehi *et al.*, 2019)
		Luteolin	Antioxidant, anti-inflammatory, antitumour, neuroprotective, antiallergic activities and activating of dopamine transporters.	(Albuquerque *et al.*, 2021; Manzoor *et al.*, 2019; Seelinger *et al.*, 2008)
	Flavonols	QUE and derivatives (e.g., RUT)	Antioxidant, anti-inflammatory, antitumour, cardioprotective, immunoprotective, UV protective, antihypertensive, antihypercholesterolemic, antiatherosclerotic, anti-obesity, anticarcinogenic, cytoprotective, neuroprotective, antithrombotic, and antiallergic activities.	(Albuquerque *et al.*, 2021; Kumar & Khanum, 2012; Ramzan, 2015; Skarupova *et al.*, 2020; Ulusoy & Sanlier, 2020)
		Kaempferol	Antioxidant, anti-inflammatory, antitumour, cardioprotective, antiangiogenic, antiadipogenic, antimicrobial and antidiabetic activities.	(Albuquerque *et al.*, 2021; Byun *et al.*, 2012; Liao *et al.*, 2016; Tatsimo *et al.*, 2012)
	Flavanones	HESP	Antioxidant, anti-inflammatory, antitumour, antiallergic, antihypertensive, neuroprotective, anticarcinogenic, hepatoprotective, nephroprotective, gastroprotective, analgesic, insulin-sensitizing and immunomodulatory activities.	(Albuquerque *et al.*, 2021; Li & Schluesener, 2017; Skarupova *et al.*, 2020; Stevens *et al.*, 2019)
		NAR	Antioxidant, anti-inflammatory, anticarcinogenic, antibacterial, gastroprotective, antimutagenic, hepatoprotective, immunoprotective, cardioprotective and antiviral activities.	(Albuquerque *et al.*, 2021; Chen *et al.*, 2016; Stevens *et al.*, 2019)
	Isoflavones	Genistein	Antioxidant, estrogenic, stimulating of collagen biosynthesis, wound healing, UV-B protective, cardioprotective and antitumour activities.	(Albuquerque *et al.*, 2021; Chaves, 2015; Pedro *et al.*, 2013; Skarupova *et al.*, 2020; Thangavel *et al.*, 2019)

UV-B – Ultraviolet B; QUE – Quercetin; RUT – Rutin; HESP – Hesperidin; NAR – Naringin.

1.3. Quantification of phenolic compounds

To support these potential human health benefits, a detailed profiling of these phytochemical compounds is required. Prior to this characterisation, harvesting, preparation and extraction of the extracts is carried out to improve their quality in the process (Fischer *et al.*, 2011; Ignat *et al.*, 2011). After sample preparation, a chemical analysis is required and, for that, analytical techniques are used for the separation, identification and quantification of the compounds in study (Bagetta *et al.*, 2012). According to the literature review, several analytical techniques have been reported for the analysis of plant-derived

compounds, including thin-layer chromatography (TLC), gas chromatography (GC), supercritical fluid chromatography and high performance liquid chromatography (HPLC) (Bagetta *et al.*, 2012; Santos & Magalhães, 2020).

Among the analytical methods reported for the quantification of PC in *M. indicus* and *P. tridentatum*, GC coupled with mass spectrometry detector (MS) (Saleem *et al.*, 2021), HPLC coupled with diode array detector (DAD) (Aires *et al.*, 2016; Roriz *et al.*, 2014), HPLC-MS (Gomaa *et al.*, 2015) or HPLC coupled with UV-visible detector (Vis) (Luís *et al.*, 2011) are most commonly used.

HPLC (Figure 1.3), is one of the most used efficient techniques in several natural products analysis, and it has been recognised since the 1980s for its ability to separate, identify and quantify efficiently (Bagetta *et al.*, 2012; Santos & Magalhães, 2020; Waters Corporation, s.d.).

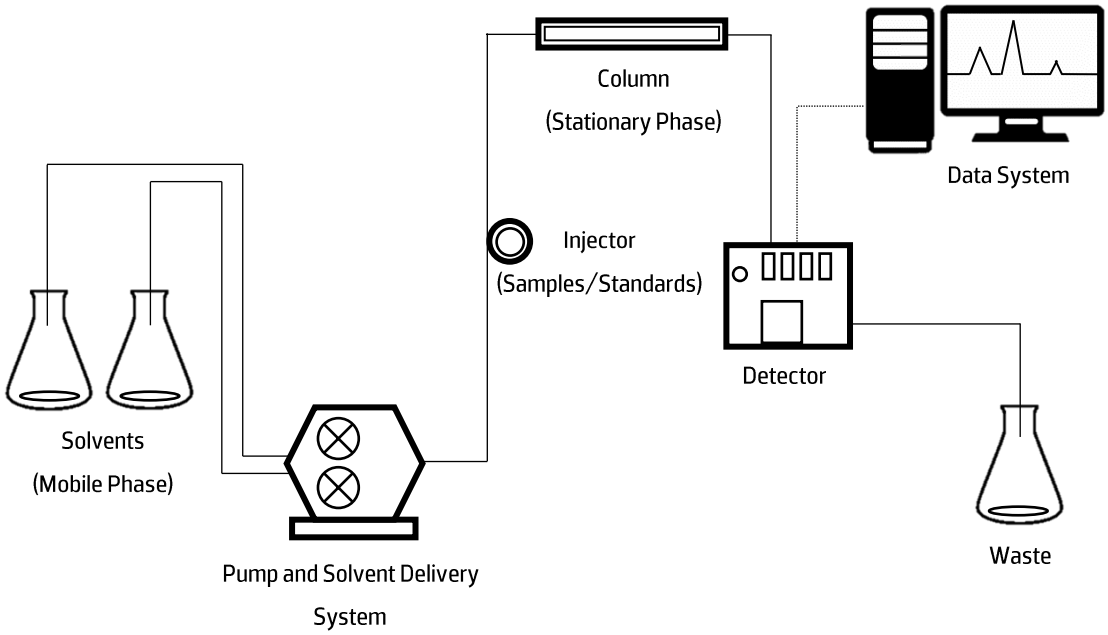


Figure 1.3 – Scheme of High Performance Liquid Chromatography equipment adapted from Waters Corporation (s.d.).

Chromatographic techniques have several advantages including their "high reproducibility, good linear range, ease of automation and its ability to analyse the number of constituents of the herbal preparation" (Bagetta *et al.*, 2012, p. 110). However, when the chromatography result gives a chromatogram with closely peaks and a high degree of scanning complexity, there is a need to optimise the process (Bagetta *et al.*, 2012). Therefore, using preparative chromatographs, changing the stationary and mobile phases, and replacing the column, could increase their resolution, speed and reproducibility (Bagetta *et al.*, 2012; Hakkimane & Guru, 2017). The introduction of HPLC-DAD could be an advantage for this technique, since it increases detection, sensitivity, and reduces analysis time and cost (Bagetta *et al.*, 2012; Haminiuk *et al.*, 2012; Santos & Magalhães, 2020). Regarding the stationary phase, the reverse

phase (RP) presents advantages in the separation of PC due to its low cost, less pollution and higher stability compared to normal phase (Haminiuk *et al.*, 2012; Mattonai *et al.*, 2019; Peraman *et al.*, 2014; Santos & Magalhães, 2020). In reverse phase, the stationary phase consists mostly of a porous hydrophobic silica gel support, with lower polarity than the mobile phase, where elution is faster for less hydrophobic compounds, while the retention time (R_t) is higher for more hydrophobic compounds (Dias *et al.*, 1999; Hakkimane & Guru, 2017; Haminiuk *et al.*, 2012). In addition, the most frequently used stationary RP for the separation of PC is Octadecyl (C18) silica (Aires *et al.*, 2016; Dias *et al.*, 1999; Gomaa *et al.*, 2015; Haminiuk *et al.*, 2012). Since, the chemical structure of PC is very similar to each other, a gradient elution is normally used, so the mobile phase runs in a binary system (Haminiuk *et al.*, 2012), using a polar solvent (usually ultrapure water acidified with formic, acetic, or phosphoric acids) and a less polar solvent (acetonitrile (ACN) or methanol (MeOH)) (Dias *et al.*, 1999; Haminiuk *et al.*, 2012). On the other hand, there is not a universal maximum absorbance in the UV and visible regions for the identification and quantification of the different PC. Therefore, it is necessary to determine the optimum absorbance of each compound (Haminiuk *et al.*, 2012).

1.4. Validation of an analytical method

When a new analytical method is developed, or there is a change to some parameter or objective in existing methods, the validation of the method becomes unequivocal to ensure the quality and reliability of its results (EMA, 1995; Ermer & Miller, 2005; FDA, 2015). In 1990, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) emerged with the mission to harmonise international regulatory guidelines for new drugs and other analytical areas, between Europe, the United States and Japan (Ermer & Miller, 2005; ICH, s.d.). However, these standards should not be considered as simple checklists, but as basic premises for the method validation and safety of the analytical process (Ermer & Miller, 2005). These requirements should be identified by the operator, ensuring appropriate acceptance and validation criteria (Shabir *et al.*, 2007).

The criteria generally evaluated in a HPLC method validation, for the identification and quantification of compounds, are described in Table 1.3.

Table 1.3 – Validation criteria frequently evaluated in identification and quantification tests and the minimum number of determinations required (Ermer & Miller, 2005).

Analytical Process	**Validation criteria**	**Minimum number**
Identification	Specificity	Not applicable
Quantification	Linearity	5
	Range	Not applicable
	Accuracy	9[b]
	Precision Repeatability Intermediate precision Reproducibility[a]	6 or 9[c] (2 series)[d]
	Limit of detection	Dependent on approach
	Limit of quantification	

[a] considered in the context of standardising an analytical procedure; [b] a minimum of three concentrations in triplicate is required; [c] it requires a minimum of six determinations at 100% of the test concentration or nine determinations within the validated range, with three concentrations in triplicate; [d] a minimum of two series of determinations are required, changing the operator, the equipment, the measurement day, or a combination of all factors, and the minimum number of tests for each series is the same as described in repeatability.

Specificity is considered the basis of any analytical procedure (Ermer & Miller, 2005). This criteria allows the evaluation of the unequivocal presence of a certain compound, when it is expected the presence of others in the sample (e.g. impurities) (Ermer & Miller, 2005; ICH, s.d.). In opposition to ICH, many analytical organizations argue that specificity is the culmination of selectivity, where the results obtained are exclusive to the compound under study (Ermer & Miller, 2005; Paz, 2018).

Linearity permits the evaluation of the direct proportionality between the results obtained and the concentration of the compound, within a certain range (Ermer & Miller, 2005; ICH, s.d.). To do this, it is necessary to verify the model calibration using calibration curves, constructed with five or more concentrations of the standard in study (Ermer & Miller, 2005; Paz, 2018; Singh, 2013). Linearity should be based on the curve equation (which in most cases results in a linear calibration), correlation and linear regression analysis. The expression is given, according to equation (1) (Araujo, 2009; Paz, 2018):

$$y = S(x)+b \qquad (1)$$

where y are the y-axis values (instrumental signal), S is the slope of the calibration curve, x are the x-axis values (mass or concentration of the standards), *and* b is the y-axis intercept of the calibration curve.

The analysis of the correlation between two quantitative variables, x and y is done according to the Pearson correlation coefficient (R), and its value, to be considered acceptable and adequate, should be 0.995 or higher (Kumar *et al.*, 2015; Paz, 2018). As far as regression is concerned, it uses the least-squares adjustment to find the line that better matches the experimental results (RELACRE, 2000).

Range refers to the interval between the maximum and minimum concentration of the compound, including limit concentrations, at which adequate precision, accuracy and linearity results are found

(Araujo, 2009; Ermer & Miller, 2005; ICH, s.d.; Paz, 2018). Thus, the range requires validation of other parameters of the analytical process (Ermer & Miller, 2005).

Precision measures the degree of closeness (degree of dispersion) of the results obtained in multiple samples of the same compound, in specified conditions (ICH, s.d.). To evaluate precision, three levels can be considered, including repeatability, intermediate precision and reproducibility (Ermer & Miller, 2005). Repeatability is the ability of the analytical method to be precise within a short period of time, while maintaining the same experimental conditions (same sample/standard, same day, same operator, same equipment, same laboratory) (Araujo, 2009; Ermer & Miller, 2005; Paz, 2018). Intermediate precision determines if the results obtained are precise when experimental variables are changed, such as operator, day of measurement, equipment, using the same sample and laboratory (ICH, s.d.; Paz, 2018). The reproducibility of an analytical method is commonly evaluated in interlaboratory tests (Araujo, 2009; ICH, s.d.)

Accuracy evaluates the closeness between the experimental value obtained and the value taken as the conventional or reference value (Araujo, 2009; ICH, s.d.). In quantification tests, the minimum number of determinations required to assess the accuracy of the analytical method is nine, with three replicates of three different concentrations, within the specified range (Ermer & Miller, 2005). Accuracy can be determined using several approaches, including: determination of the compound concentration in the sample, using the standard addition method; determination of the percent recovery by measuring the analyte at known analytical concentrations, in blank matrix samples; comparison of results obtained with certified reference materials; and comparison of the results of one or more different analytical methods (Araujo, 2009; ICH, s.d.; RELACRE, 2000).

Limit of detection (LOD) allows to determine the lowest amount of compound that the method can detect, but not necessarily quantify (Araujo, 2009; Ermer & Miller, 2005). It is necessary to consider the noise of the equipment and, thus, determine the minimum reliable compound concentration that the method can detect (Araujo, 2009; Paz, 2018). Usually, the LOD represents a signal two to three times the noise of the equipment (Araujo, 2009; ICH, s.d.). LOD can be determined according to the equation (2) (ICH, s.d.):

$$LOD = 3 \times S_b/S \qquad (2)$$

using the standard deviation of the y-axis intercept of the calibration curve (S_b), and the slope of the calibration curve (S).

Limit of quantification (LOQ) is the lowest quantity of a compound in a sample that the method can quantify, with acceptable levels of precision and accuracy (Araujo, 2009; Ermer & Miller, 2005). In contrast

to the previous criteria, the LOQ corresponds to a signal ten times the noise of the equipment (Araujo, 2009; ICH, s.d.), and can be determined according to the equation (3) (ICH, s.d.):

$$LOQ = 10 \times S_b / S \qquad (3)$$

where, S_b is the standard deviation of the response-axis intercept of the calibration curve, and *S is* the slope of the calibration curve.

In addition to these main parameters, the sensitivity of the analytical method can be determined by interpreting the slope of the calibration curve. Thus, the greater the slope, the more sensitive the method is, due to the higher sensitivity to discriminate small variations in concentration (RELACRE, 2000).

The objectives of this study are to compile published methods for the determination of phenolic compounds in aqueous and methanolic extracts of *M. indicus* and *P.tridentatum* and to validate a HPLC-DAD method for the detection and quantification of CA, CLA and HESP in these extracts.

2. Analytical methods for the detection and quantification of phenolic compounds in *Melilotus indicus* and *Pterospartum tridentatum*

Abstract

The use of plants for therapeutic purposes has been the result of scientific advances over the last few years. *Melilotus indicus* and *Pterospartum tridentatum* belong to the third family of flowering plants, the Fabaceae. For biological, pharmaceutical and cosmetic applications, a full characterisation of the plants and their bioactive compounds is initially required. The aim of this review is to gather information, compiling the several detection and quantification methods, the phenolic compounds identified and their quantities present in these plants. A literature search was carried out in PubMed, Science Direct and SciELO using the following isolated and combined keywords "*Melilotus indicus*", "*Pterospartum tridentatum*", "Phenolic compounds", "Detection", "Quantification", selecting studies published since 2000 that referred analytical methods for the detection and quantification of phenolic compounds in *M. indicus and P. tridentatum*. For *M. indicus*, three methods were found in the literature, only two of which quantified phenolic compounds. A total of twenty-eight compounds were identified, such as catechin and dihydroquercetin, while twenty-one were quantified, namely chlorogenic and caffeic acids. The range of quantification varied from 0.16 ± 0.02 (hesperidin) to 826.60 ± 21.10 (quercetin-3-O-rutinoside) $\mu g.g^{-1}$ dry weight. For *P. tridentatum*, eight methods were found for the characterisation of phenolic compounds. A total of twenty-nine compounds were identified such as rosmarinic acid and 7-methylorobol, while twenty-one were quantified, e.g., genistein and quercetin-3-O-rutinoside. Quantification ranged from 53.2 ± 0.2 (sissotrin) to 32200.0 ± 2600.0 (vanillic acid) $\mu g.g^{-1}$ dry weight. These studies showed different methods that identify and quantify phenolic compounds in *M. indicus* and *P. tridentatum* extracts, particularly high performance liquid chromatography, and were able to identify several classes of phenolic compounds, including hydroxycinnamic and hydroxybenzoic acids, flavonols, flavones, isoflavones and flavanones. However, it is necessary to develop new methods to improve the separation, identification and quantification of phenolic compounds.

Keywords: *Melilotus indicus*, *Pterospartum tridentatum*, phenolic compounds, detection, quantification

2.1. Introduction

Over the years, several plants have been studied with the purpose of obtaining compounds of therapeutic interest. The Fabaceae is considered the third family of flowering plants with approximately 19500 referenced species and 750 genera, to which *M. indicus* and *P. tridentatum* belong (Batanony *et al.*, 2020; Roriz *et al.*, 2014).

The genus *Melilotus* has more than 18 annual and biennial species studied (Ahmed *et al.*, 2012; Kanzana *et al.*, 2021; Yan *et al.*, 2017). *M. indicus,* also commonly known as "anafe-menor, coroa-de-rei, trevo-de-cheiro, trevo-de-namorado", is one of the small-sized annual species (about 50cm high), with light yellow flowers and it can be found widely in the Asian and European continent (Ahmed & Al-Refai, 2014; Saleem *et al.*, 2021; Talavera *et al.*, 2000). Flowering period is between March and August (Ahmed *et al.*, 2012). In Portugal, it is generally distributed along the coast, but can also be found in the interior of the central regions of the country (Figure 2.1)(Lourenço *et al.*, 2021; Sociedade Portuguesa de Botânica, s.d.-a). This plant offers several potential beneficial effects such as analgesic, antioxidant, insecticide, emollient, astringent, antibacterial, anticoagulant, and anti-inflammatory (Ahmed & Al-Refai, 2014; Bashir *et al.*, 2022; Saleem *et al.*, 2021). However, in high doses, *Melilotus* species are considered poisonous (Abd El-Hafeez *et al.*, 2018). Additionally, these species are used in the agricultural sector as green manure and to fix nitrogen in the saline soils (Kanzana *et al.*, 2021).

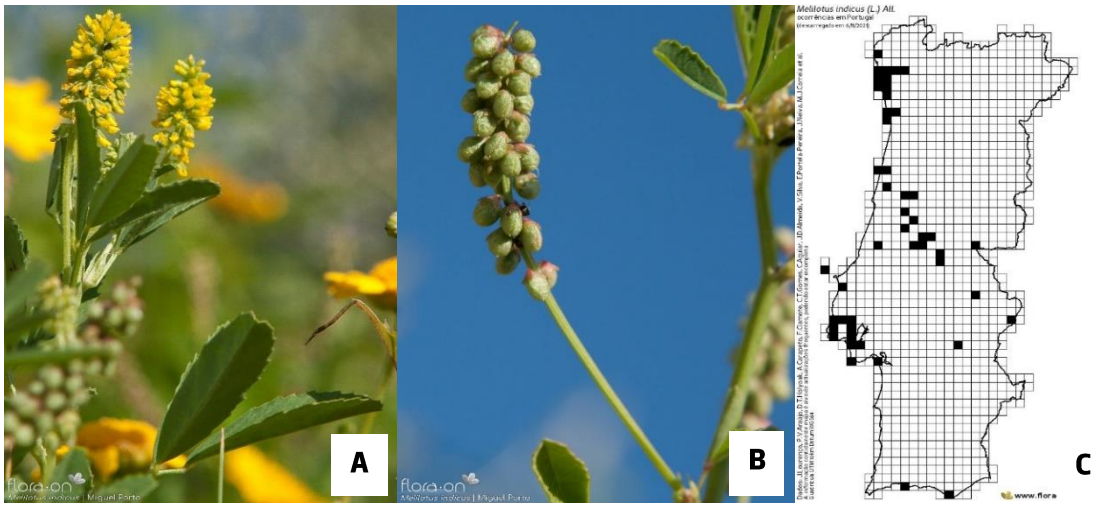


Figure 2.1 – Photographs and distribution map of *Melilotus indicus* in Portugal.

A) Photograph of the *M. indicus* plant, taken by (Porto, s.d.-a);
B) Photograph of *M. indicus* fruits, taken by (Porto, s.d.-b);
C) Distribution map of *M. indicus* in Portugal with 54 records by different Flora-On: Flora de Portugal Interactiva collaborators (Lourenço *et al.*, 2021).

P. tridentatum, commonly known as "carqueja" and previously known as *Chamaespartium tridentatum* and *Genistella tridentate*, belongs to the subfamily Papilionoideae (Balanč *et al.*, 2016; Ferreira

et al., 2012; Gonçalves *et al.*, 2020), it is a small endemic shrub which grows spontaneously in North Africa and the Iberian Peninsula (Aires *et al.*, 2016; Luís *et al.*, 2011; Roriz *et al.*, 2014). In Portugal, *P. tridentatum* is essentially distributed along the coast in the centre–south of the country, and it is homogenous in the north and centre (Figure 2.2) (Sociedade Portuguesa de Botânica, s.d.-b).

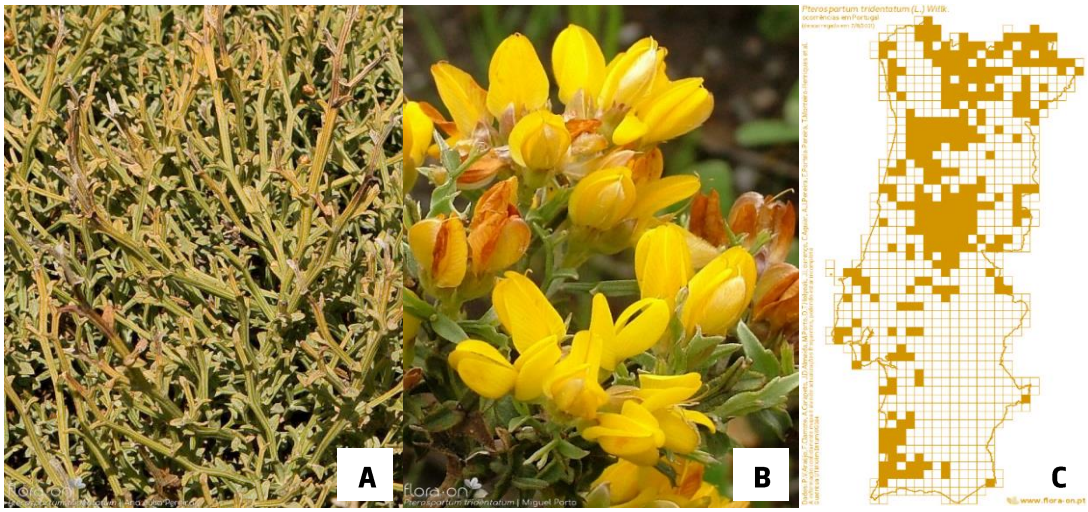


Figure 2.2 – Photographs and distribution map of *Pterospartum tridentatum* in Portugal.

A) Photograph of the native vegetation of *P. tridentatum*, taken by (Pereira, s.d.);
B) Photograph of the *P. tridentatum* plant, taken by (Porto, s.d.-c);
C) Distribution map of *P. tridentatum* in Portugal with 1106 records by different Flora-On: Flora de Portugal Interactiva collaborators (Araújo *et al.*, 2021).

Its flowers are usually used to treat diseases related to the respiratory system, as well as for their depurative and hypoglycaemic effect (Paulo *et al.*, 2008; Roriz *et al.*, 2014; Vitor *et al.*, 2004). Traditionally, the use of this plant is associated to the treatment of various diseases related to the cardiovascular, including hypertension, hypercholesterolaemia and diabetes; respiratory such as colds, pneumonia and bronchitis; and gastrointestinal systems, like gastric and intestinal problems, liver and bladder complications (Gonçalves *et al.*, 2020). The presence of phytochemical compounds with biological activity showed, in studies published by Aires *et al.* (2016) and Roriz *et al.* (2014), an antioxidant and antimicrobial profile of *P. tridentatum*.

The biological effects described in both plants may be related to the presence of polyphenols, commonly named PC, secondary metabolites of plant extracts (Aires *et al.*, 2016; Gonçalves *et al.*, 2020; Saleem *et al.*, 2021). To improve scientific knowledge and for further possible therapeutic and/or cosmetic applications of these and other plants, it is necessary to perform a full characterisation of their compounds. The aim of this review is to gather information, compiling the several detection and quantification methods in *M. indicus* and *P. tridentatum*, the PC identified and their quantities.

2.2. Methods

A literature search was carried out in PubMed, Science Direct and SciELO using the following isolated and combined keywords: "*Melilotus indicus*", "*Pterospartum tridentatum*", "Phenolic compounds", "Detection" "Quantification". From this search, scientific articles published between 2000-2022 were analysed. The selection criteria were studies referring analytical methods for the detection and quantification of PC in *M. indicus* and *P. tridentatum* and their amounts.

2.3. Results and Discussion

Initially, twenty-four articles were considered for analysis. Subsequently, thirteen were excluded since they did not analyse the identification and quantification of the PC of the plants in study. The review of the selected articles is presented below.

2.3.1. Analysis of phenolic compounds in *Melilotus indicus*

For *M. indicus*, three methods were found in the literature for the detection and quantification of PC, which identified a total of twenty-eight compounds (Table 2.1).

The method used by Ahmed & Al-Refai (2014) employed various chromatographic techniques, including HPLC-UV/Vis, HPLC coupled with infrared detector (IR), and HPLC-MS/MS, for the separation and identification of eight PC. It identified six flavonols (QUE, quercetin 3-O-β-D-glucopyranoside, quercetin 3-O-α-L-rhamnopyranoside, dihydroquercetin, isorhamnetin 3-O-β-D-glucopyranoside and isorhamentin 3-O-β-D-rutinoside) and two flavanols (catechin and epicatechin). These compounds were not quantified. The method described by Gomaa *et al.* (2015), the HPLC-UV/Vis and HPLC-ESI/MS were effective to identify and quantify thirteen PC. These compounds consisted of six flavonols (kaempferol, kaempferol-7-O-glucoside, QUE, RUT, myricetin and hyperoside), two flavones (apigenin and luteolin), three isoflavones (daidzein, daidzin and genistin) and two flavanones (HESP and NAR). This method showed that the most common compound was RUT with 826.60 ± 21.10 $\mu g.g^{-1}$ dry weight, while HESP was the component that showed the lowest quantified amount with 0.16 ± 0.02 $\mu g.g^{-1}$ dry weight. Flavonols were the dominant class of flavonoids in ripe seeds. In Bashir *et al.* (2022) method, the quantification was performed by HPLC-UV/Vis. In this study, a total of nine phenolic compounds were identified and quantified, including: one flavonol (QUE), four HCA (CA, CLA, *p*-coumaric and *m*-coumaric acids) and four HBA (syringic, GA, vanillic and benzoic acids). QUE was the phenolic component present in

the highest amount with 7.63 μg.g^{-1} dry weight, while GA and *m*-coumaric acid showed the lowest amount with 0.36 μg.g^{-1} dry weight. Phenolic acids were the dominant PC in an ethanolic extract (Table 2.1).

Table 2.1 – Content of the phenolic compounds identified and quantified in *Melilotus indicus* extracts by three analytical methods.

Method (Reference)	HPLC- UV/Vis, HPLC-IR and HPLC-MS/MS (Ahmed & Al-Refai, 2014)	HPLC-UV/Vis and HPLC-ESI/MS (Gomaa *et al.*, 2015)	HPLC-UV/Vis (Bashir *et al.*, 2022)
Plant part	Aerial parts	Ripe seeds	N/S
Plant extract	Ethanolic	Methanolic	Ethanolic
Wavelengths	N/S	310 and 380 nm	280 nm
Mobile phase	N/S	A: deionized water: acetic acid (98:2) and B: MeOH	A: water and acetic acid (94:6) and B: ACN
Phenolic compounds	**Detection**	**Quantification (μg.g^{-1} dry weight)**	
Apigenin	-	4.60 ± 0.61*	-
Benzoic acid	-	-	5.42
CA	-	-	1.44
Catechin	x	-	-
CLA	-	-	5.92
Daidzein	-	44.90 ± 4.16*	-
Daidzin	-	0.79 ± 0.07*	-
Dihydroquercetin	x	-	-
Epicatechin	x	-	-
GA	-	-	0.43
Genistin	-	5.52 ± 0.45*	-
HESP	-	0.16 ± 0.02*	-
Hyperoside	-	42.30 ± 5.10*	-
Isorhamentin 3-O-β-D-glucopyranoside	x	-	-
Isorhamentin 3-O-β-D-rutinoside	x	-	-
Kaempferol	-	2.30 ± 0.22*	-
Kaempferol-7-O-glucoside	-	0.79 ± 0.05*	
QUE	x	1.30 ± 0.13*	7.63
RUT	-	826.60 ± 21.10*	-
Quercetin 3-O-β-D-glucopyranoside	x	-	-
Quercetin 3-O-α-L-rhamnopyranoside	X	-	-
Luteolin	-	2.40 ± 0.29*	-
Myricetin	-	0.76 ± 0.04*	-
NAR	-	0.79 ± 0.06*	-
Syringic acid	-	-	1.59
Vanillic acid	-	-	5.62
m-coumaric acid	-	-	0.36
p-coumaric acid	-	-	0.56

*Results in terms of mean ± standard deviation; N/S – Not specified; x – Detected; CA – Caffeic acid; CLA – Chlorogenic acid; GA – Gallic acid; HESP – Hesperidin; QUE – Quercetin; RUT – Rutin; NAR – Naringin.

To compare the different methods, it will be necessary to obtain more data concerning the identified compounds and their amounts. Furthermore, a change in the mobile phase may influence the quantities obtained as well as the separation of the compounds (Bagetta *et al.*, 2012; Hakkimane & Guru, 2017). In addition, the plant part, solvent and extraction method may have some influence, since there is a diversity in the bioactive compounds and solubility properties present in plants, and thus, there is no ideal solvent for maximum extraction of all biological components (Gonçalves *et al.*, 2020; Mahdi-Pour *et al.*, 2012). On

the other hand, the use of additional wavelengths could identify and quantify other polyphenols, such as Aires *et al.* (2016) who identified and quantified biochanin A and ferulic acid at 320 nm and apigenin at 370 nm.

2.3.2. Analysis of phenolic compounds in *Pterospartum tridentatum*

For *P. tridentatum*, eight methods were found in the literature, where four of them identified PC (Table 2.2), while the other four were able to quantify PC (Table 2.3). For the analysis of the results only the main PC of each method were considered. Twenty-nine compounds were identified for PC detection methods (Table 2.2), while twenty-one were quantified (Table 2.3).

Concerning detection methods, Vitor *et al.* (2004) used HPLC- Electrospray Ionisation (ESI)/MS and was able to identify five PC in flowers. Of these compounds, four were isoflavones (5,5′-dihydroxy-3′-methoxy-isoflavone-7-O-β-glucoside genistin, prunetin and sissotrin) and one flavonol (quercetin-3-O-glucoside(Q3G)). Paulo *et al.* (2008) identified nine PC, in flowers, using the HPLC-ESI/MS method. The aqueous extract contained six isoflavones (5,5′-Dihydroxy-3′-methoxy-isoflavone-7-O-β-glucoside, 7-methylorobol, genistein, genistin, prunetin and sissotrin) and three flavonols (myricetin-6-C-glucoside, Q3G and RUT). The study by Ferreira *et al.* (2012) evaluated the aqueous extract using the ESI/MS method. This, identified five PC in flowers, being two flavone (luteolin-O-glucuronide and luteolin-O-(O-acetyl)-glucuronide), two flavonols (isorhamnetin-O-hexoside and pentahydroxy-flavonol-di-O-glucoside) and one HCA (rosmarinic acid). Pedro *et al.* (2013) performed the identification of phenolic compounds using various chromatographic techniques, including HPLC-DAD and LC-ESI/MS. This study identified six compounds in the aqueous extract, including three isoflavones (biochanin A, genistein-8-C-glucoside and sissotrin) and three flavonols (myricetin-6-C-glucoside, Q3G and taxifolin-6-C-glucoside) (Table 2.2).

Table 2.2 – Content of the phenolic compounds identified in *Pterospartum tridentatum* extracts by four analytical methods.

Method (Reference)	HPLC–ESI/MS (Vitor *et al.*, 2004)	HPLC–ESI/MS (Paulo *et al.*, 2008)	ESI/MS (Ferreira *et al.*, 2012)	HPLC–DAD and LC–ESI/MS (Pedro *et al.*, 2013)
Plant part	Flowers	Flowers	Flowers	N/S
Plant extract	Aqueous	Aqueous	Aqueous	Aqueous
Wavelengths	254 nm	N/S	N/S	200–500 nm
Mobile phase	A: MeOH: ACN (95:5) B: water with 0.1% TFA	0.1% aqueous formic acid: ACN (80:20)	N/S	A: 0.05% TFA B: MeOH
Phenolic compounds	**Detection**			
5,5′–Dihydroxi–3′–methoxy–isoflavone–7–O–β–glucoside	x	x	–	–
7–Methylorobol	–	x	–	–
Biochanin A	–	–	–	x
Genistein	–	x	–	–
Genistein–8–C–glucoside	–	–	–	x
Genistin	x	x	–	–
Isorhamnetin–O–hexoside	–	–	x	–
Luteolin–O–glucuronide	–	–	x	–
Luteolin–O–(O–acetyl)–glucuronide	–	–	x	–
Myricetin–6–C–glucoside	–	x	–	x
Pentahydroxy–flavonol–di–O–glucoside	–	–	x	–
Prunetin	x	x	–	–
Q3G	x	x	–	x
RUT	–	x	–	–
Rosmarinic acid	–	–	x	–
Sissotrin	x	x	–	x
Taxifolin–6–C–glucoside	–	–	–	x

N/S – Not specified; x – Detected; TFA – Trifluoroacetic acid; Q3G – Quercetin–3–O–glucoside; RUT – Rutin.

In quantification methods, the process used by Luís *et al.* (2011) was HPLC–UV/Vis. This study quantified PC in stems, leaves and flowers. In stems and leaves, a total of eight polyphenols were quantified, including four HCA (CA, CLA, *p*–coumaric and ferulic acids), three HBA (vanillic, syringic and ellagic acids) and one flavonol (Q3G). Vanillic acid was the phenolic component present in highest amount with 32200.0 ± 2600.0 $\mu g.g^{-1}$ dry weight, while ferulic acid and Q3G presented low amounts with 7400.0 ± 800.0 and 6600.0 ± 300.0 $\mu g.g^{-1}$ dry weight, respectively. Phenolic acids were the dominant PC in stems and leaves. The same method was used to quantify in flowers. In this case, the method quantified two HCA (CA and ferulic acid), three HBA (vanillic, syringic and ellagic acids) and one flavonol (Q3G), in a total of six PC. In contrast, ferulic acid was the dominant compound in the flower extract, having a concentration of 22200.0 ± 600.0 $\mu g.g^{-1}$ dry weight, whereas CA and syringic acid showed lower amounts with 3100.0 ± 300.0 and 1900.0 ± 400.0 $\mu g.g^{-1}$ dry weight, respectively. HPLC–UV/Vis was unable to detect and quantify CLA and p–coumaric acid in the flower extract. These results showed that the method quantified more PC and higher amounts in stems and leaves, but the greatest amounts of ferulic and ellagic acids, and Q3G were quantified in flowers. Roriz *et al.* (2014) performed the quantification of PC in flowers

by HPLC-DAD/MS. This method identified thirteen polyphenols, namely six flavonols (Q3G, RUT, quercetin deoxyhexosyl-hexoside, quercetin O-hexoside, dihydroquercetin and myricetin-6-C-glucoside) and seven isoflavones (5,5′-dihydroxy-3′-methoxy-isoflavone-7-O-β-glucoside, genistin, genistein, biochanin A, methylbiochanin A, prunetin and sissotrin). Dihydroquercetin was the phenolic component present in highest amount with 3873.6 ± 34.1 $\mu g.g^{-1}$ dry weight, while RUT and sissotrin presented low amounts, having a concentration of 125.7 ± 1.3 and 53.2 ± 0.2 $\mu g.g^{-1}$ dry weight, respectively. In Aires *et al.* (2016) method, the quantification was performed by HPLC-UV/DAD. This study identified and quantified nine PC. These compounds consisted of an HCA (ferulic acid), three flavonols (Q3G, RUT and dihydroquercetin), four isoflavones (genistin, genistein, biochanin A and sissotrin) and one flavone (apigenin). Like the previous method, dihydroquercetin was the dominant compound in the analysed extract, but with a higher amount of 21760.0 ± 30.0 $\mu g.g^{-1}$ dry weight. On the other hand, apigenin and ferulic acid presented lower amounts with 440.0 ± 2.0 and 270.0 ± 2.0 $\mu g.g^{-1}$ dry weight, respectively. Although dihydroquercetin was the component in greatest amount, isoflavones were the predominant class. Liquid chromatography (LC) coupled with UV/DAD detectors, used by Gonçalves *et al.* (2020), identified and quantified four phenolic components in flowers, of which two flavonols (Q3G and RUT) and two isoflavones (genistin and genistein). Genistein was the phenolic component that showed the highest quantified amount in the ethanolic extract, with a concentration of 8640.0 ± 90.0 $\mu g.g^{-1}$ dry weight, while genistin showed the lowest amount with 1780.0 ± 200.0 $\mu g.g^{-1}$ dry weight (Table 2.3).

Table 2.3 – Content of phenolic compounds quantified in *Pterospartum tridentatum* extracts by four analytical methods.

Method (Reference)	HPLC -UV/Vis (Luís *et al.*, 2011)		HPLC-DAD/MS (Roriz *et al.*, 2014)	HPLC–UV/DAD (Aires *et al.*, 2016)	HPLC-UV/DAD (Gonçalves *et al.*, 2020)
Plant part	Stems and leaves	Flowers	Flowers	N/S	Flowers
Plant extract	Methanolic		Methanolic	Methanolic	Ethanolic
Wavelengths	280 nm		280- 370 nm	280, 320 and 370 nm	280 and 350 nm
Mobile phase	A: water with acetic acid B: ACN/solvent A (6:4; V/V)		A: 0.1% formic acid in water B: ACN	A: water with 1% of TFA B: ACN with 1% TFA	A: 2% acetic acid in water B: 0.5% acetic acid in water and ACN (50:50; V/V)
Phenolic compounds	**Quantification ($\mu g.g^{-1}$ dry weight)***				
5,5′-Dihydroxy-3′-methoxy-isoflavone-7-O-β-glucoside	-	-	94.1 ± 0.9	-	-
Apigenin	-	-	-	440.0 ± 2.0	-
Biochanin A	-	-	433.5 ± 2.0	2890.0 ± 4.0	-
CA	18200.0 ± 3000.0	3100.0 ± 300.0	-	-	-
CLA	17800.0 ± 600.0	N/D	-	-	-
Dihydroquercetin	-	-	3873.6 ± 34.1	21760.0 ± 30.0	-
Ellagic acid	9700.0 ± 1800.0	10400.0 ± 400.0	-	-	-
Ferulic acid	7400.0 ± 800.0	22200.0 ± 600.0	-	270.0 ± 2.0	-
Genistein	-	-	167.1 ± 3.4	12010.0 ± 30.0	8640.0 ± 90.0
Genistin	-	-	162.9 ± 3.0	16750.0 ± 40.0	1780.0 ± 200.0
Methylbiochanin A	-	-	105.4 ± 1.8	-	-
Myricetin-6-C-glucoside	-	-	1316.7 ± 2.3	-	-
Prunetin	-	-	372.6 ± 2.6	-	-
Q3G	6600.0 ± 300.0	9300.0 ± 700.0	963.2 ± 4.3	1230.0 ± 10.0	5880.0 ± 30.0
RUT	-	-	125.7 ± 1.3	1580.0 ± 30.0	2100.0 ± 3.0
Quercetin deoxyhexosyl-hexoside	-	-	100.1 ± 1.5	-	-
Quercetin O-hexoside	-	-	474.7 ± 2.8	-	-
Sissotrin	-	-	53.2 ± 0.2	1370.0 ± 2.0	-
Syringic acid	8300.0 ± 800.0	1900.0 ± 400.0	-	-	-
Vanillic acid	32200.0 ± 2600.0	4200.0 ± 900.0	-	-	-
p-coumaric acid	16800.0 ±3200.0	N/D	-	-	-

*Results in terms of mean ± standard deviation of three replicates; N/D – Not detected; N/S – Not specified; CH – Chosen wavelength; CA – Caffeic acid; CLA – Chlorogenic acid; Q3G – Quercetin-3-O-glucoside; RUT – Rutin.

Comparing the four methods that quantified the compounds (Table 2.3), the study that identified and quantified more PC was HPLC- DAD/MS from Roriz *et al.* (2014). Only the method of Ferreira *et al.* (2012) was unable to identify Q3G (Table 2.2), but it is possible to confirm the presence of this compound in the aqueous, methanolic and ethanolic extracts. Also, the compounds genistein and genistin are susceptible to be detected in all the extracts analysed. The HPLC-UV/Vis method showed interesting values of PC, however, the introduction of more wavelengths beyond 280 nm could quantify more classes, as demonstrated by the methods of Aires *et al.* (2016), who quantified isoflavones at 320 nm, and Roriz *et*

al. (2014), who quantified flavones in a range between 350 and 370 nm. The selection of the extraction solvent may have influenced the results obtained, since less polar solvents cause the release of PC (methanolic and ethanolic extracts), while solvents such as water allows the extraction of more polar components (aqueous extracts) (Gonçalves *et al.*, 2020). It should be noted that a gradient elution was used in all methods to achieve a good separation (Gomaa *et al.*, 2015). Flowers are the plant parts most frequently used for the identification and quantification of phenolic compounds. Analysis of other plant parts could identify and quantify different compounds, as demonstrated by Luís *et al.* (2011) when analysing stems, leaves and flowers.

2.4. Conclusion

As a conclusion, there are different methods that have identified and quantified PC in *M. indicus* and *P. tridentatum* extracts, particularly using HPLC. These methods identified different classes, namely HCA and HBA, flavonols, flavones, isoflavones and flavanones in *M. indicus*, and HCA and HBA, flavonols, flavones and isoflavones in *P. tridentatum*. These bioactive compounds may be directly connected with the biological potential of these plants, showing, among other, antioxidant and antimicrobial activities. In *M. indicus*, RUT was the most common compound, with 826.60 ± 21.10 $\mu g.g^{-1}$ dry weight in the methanolic extract, while QUE with 7.63 $\mu g.g^{-1}$ dry weight, was in the ethanolic extract. In *P. tridentatum* was vanillic acid, with 32200.0 ± 2600.0 $\mu g.g^{-1}$ dry weight, in methanolic extract, while genistein with 8640.0 ± 90.0 $\mu g.g^{-1}$ dry weight, was in the ethanolic extract. However, for *M. indicus* extracts new methods need to be developed to compare identified and quantified PC.

As future investigation, it might be interesting to perform a RP-HPLC method using the optimal wavelengths for each compound, in methanolic and aqueous extracts of these plants, to identify and quantify more classes of PC. On the other hand, the use of different plant parts and extraction solvent and/or extraction methods may reveal other results.

3. Quantification of phenolic compounds in *Melilotus indicus* and *Pterospartum tridentatum* with a High Performance Liquid Chromatography method

Abstract

Melilotus indicus and *Pterospartum tridentatum*, plants frequently present in portuguese flora, contain secondary compounds with potential biological activity, such as phenolic compounds, carotenoids, alkaloids, nitrogen and organo-sulphur compounds. Phenolic compounds, one of the most studied secondary metabolites, have improved the quality of life due to their potential therapeutic effects, being used as anti-inflammatory, antimicrobial, antimicrobial and antioxidant agents. The purpose of the present study was the identification and quantification of phenolic compounds (caffeic acid, chlorogenic acid and hesperidin) in aqueous and methanolic extracts from *Melilotus indicus* and *Pterospartum tridentatum*. A high performance liquid chromatography method described in the literature was adapted, optimised and validated, while another study, performed in the same research team, did the same for other four compounds (gallic acid, quercetin-3-glucoside, rutin and naringin). Parameters validated were linearity, range, sensitivity, limits of detection and quantification, precision and accuracy. Five point calibration curves were determined for all three analytes, showing good linearity parametres, such as correlation coeficient higher then 0.999. The limits of detection were lower then 0.09 $\mu g.mL^{-1}$, while the limits of quantification were no greater then 2.98 $\mu g.mL^{-1}$. Precision showed values lower than 14.9% and accuracy, expressed as recovery, ranged from 90.2 to 114.1%. The validated method allowed a preliminary identification and quantification, of caffeic acid (106 $\mu g.g^{-1}$ dry weight) in the aqueous extract of *M. indicus*, while in the methanolic extract, chlorogenic acid (110 $\mu g.g^{-1}$ dry weight), caffeic acid (96 $\mu g.g^{-1}$ dry weight) and rutin (5678 $\mu g.g^{-1}$ dry weight). In *P. tridentatum*, three compounds were detected but not quantified: caffeic acid and hesperidin in the aqueous extract, and quercetin-3-glucoside in the methanolic extract. But regarding this last extract, gallic acid (702 $\mu g.g^{-1}$ dry weight), chlorogenic acid (488 $\mu g.g^{-1}$ dry weight), rutin (848 $\mu g.g^{-1}$ dry weight) and hesperidin (10370 $\mu g.g^{-1}$ dry weight) were also quantified. However, further studies are necessary, namely to confirm the detection and quantification of these compounds on the analysed plant extracts.

Keywords: quantification, phenolic compounds, *Melilotus indicus, Pterospartum tridentatum*, method validation

3.1. Introduction

Over the years, bioactive compounds from plants have improved the quality of life, not only in food but also in cosmetic and medicinal products (Mighri *et al.*, 2019; Vu *et al.*, 2018). Of these compounds, PC stand out as the phytochemicals with the highest phytotherapeutic interest, demonstrated by their antioxidant, antihypertensive, antimicrobial and antitumour properties (Albuquerque *et al.*, 2021; Kumar & Goel, 2019). In plants, PC are used not only for their antioxidants properties, but also as antifeedants, pollinators, phytoalexins, plant pigments, and protective agents against UV light (Mighri *et al.*, 2019). Some of those PC are HCA (CA and CLA), HBA (GA), flavanones (HESP and NAR) and flavonols (QUE, Q3G and RUT), the selected compounds of interest to the present investigation.

For the application of these and other compounds in the therapeutic context, a sequence of preliminary studies is necessary to identify and quantify each substance of interest. An efficient identification and quantification of bioactive compounds begins with the choice of the analytical method used and its validation (Fernandes & Salgado, 2016). There are several analytical methods published in the literature in the last ten years, about the detection, quantification and method validation, of at least three of the interest PC (CA, CLA and GA, NAR, QUE, Q3G, HESP and RUT), as described in Table 3.1. The parameters analysed for the validation of the methods were the linearity range, the regression equations, R, LOD and LOQ. Many methods are used to identify and quantify compounds in plants, of which HPLC is one of the most widely used. Its specifications allow for improved quality and accuracy in the analysis of extracts as well as validation criteria (Fernandes & Salgado, 2016; Magwaza *et al.*, 2016). The stationary phase frequently used for these studies is RP-C18 or octyl, 250 mm, but in some Ultra-High Performance Liquid Chromatography (UHPLC) methods smaller columns of 150 and 100 mm are utilised (Fernandes & Salgado, 2016). On the other hand, HPLC-MS/MS has gained importance, since it allows the identification of compounds with similar structures, without the need for comparison with standards (Bataglion *et al.*, 2014). The use of different types of detectors (e.g., UV, DAD and photodiode array detector (PAD)) increases the quality of the results and, advanced techniques such as ESI are used to make the method more sensitive but, on the other hand, more expensive and complex (Bataglion *et al.*, 2014; Skendi *et al.*, 2017).

From the studies described (Table 3.1), a total linearity range from 0 to 4730 $\mu g.mL^{-1}$ can be observed. The most sensitive method was developed by Mighri *et al.* (2019) for the higher slopes of the calibration curves, where a small variation in concentration translates into a great variability in the instrumental response. Almost all calibration curves present a y-axis intercept different from 0, except for Magwaza *et al.* (2016) and Vu *et al.* (2018) studies, that forced the calibration curve to intercept the origin. The value of R ranged between 0.9747 and 0.9999, being most of them above 0.999 that is the value considered to be the minimum acceptable for a good correlation (Kumar *et al.*, 2015; Ribani *et al.*, 2004).

Table 3.1 –Analytical methods for quantification of phenolic compounds in plants and respective validation parameters.

		Validation criteria					
Plant Method (Reference)	**Phenolic Compound**	**Linear range ($\mu g.mL^{-1}$)**	***S***	***b***	**R**	**LOD ($\mu g.mL^{-1}$)**	**LOQ ($\mu g.mL^{-1}$)**
Vitis spp HPLC-UV (Canas *et al.*, 2015)	CA	0.99–50	35827.52	-673.96	0.9999	0.30	0.99
	CLA	0.27–5	34191.55	- 486.43	0.9999	0.08	0.27
	GA	0.25–8	39893.22	-2450.14	0.9998	0.08	0.25
Oryza sativa L. HPLC-DAD (Irakli *et al.*, 2012)	CA	0.0016–0.032	0.077	-0.150	0.9995	0.00116	0.00352
	GA		0.051	-0.055	0.9995	0.00041	0.00126
	HESP		0.018	-0.042	0.9995	0.00099	0.00299
	QUE		0.010	0.023	0.9995	0.00110	0.00334
	Q3G	0.0016–0.024	0.018	-0.030	0.9985	0.00124	0.00376
	RUT	0.0016–0.032	0.021	-0.049	0.9990	0.00118	0.00359
Inula species HPLC-DAD (Gökbulut *et al.*, 2013)	CA	0.25-515	50521	-10.69	0.9996	0.075	0.250
	CLA	0.22-103	20692	-64.979	0.9989	0.067	0.220
	QUE	0.28-400	33078	-21.421	0.9997	0.085	0.280
	RUT	0.20-500	13776	-12.522	0.9999	0.060	0.200
Citrus reticulata HPLC-DAD (Magwaza *et al.*, 2016)	CA	5-150	62.73	0.00	0.9998	1.35	4.51
	CLA		33.83	0.00	0.9999	1.48	4.92
	HESP		30.84	0.00	0.9997	5.02	16.72
Origanum vulgare ssp., Hirtum, Thymus capitatus, Satureja thymbra, Melissa officinalis, Rosmarinus officinalis HPLC-DAD (Skendi *et al.*, 2017)	CA	0.5–20	70.15	-13.17	0.9993	0.08	0.25
	CLA		37.03	-1.49	0.9997	0.03	0.09
	GA		66.10	-2.33	0.9999	0.03	0.08
	QUE		4.79	-2.03	0.9989	0.14	0.43
	RUT	0.5–30	7.35	2.98	0.9998	0.04	0.13
Cerasus avium L. HPLC–ESI-MS/MS (Bursal *et al.*, 2013)	CA	0–0.1	4.1981	0.0831	0.9975	0.0002	0.0006
	GA	0–0.3	2.236	-0.046	0.9980	0.0001	0.0004
	QUE	0–0.3	0.245	-0.0001	0.9960	0.0003	0.0011
Juglans nigra L. HPLC – ESI-MS/MS (Vu *et al.*, 2018)	CA	0.1-50	373017	0.00	0.9854	0.04	0.12
	CLA		52360	0.00	0.9995	0.61	2.04
	GA		43081	0.00	0.9915	0.06	0.21
	NAR		27832	0.00	0.9955	0.04	0.12
	QUE		320950	0.00	0.9899	0.04	0.14
	Q3G		23215	0.00	0.9884	0.18	0.59
	RUT		2360	0.00	0.9834	0.21	0.69
Cephalaria species HPLC-ESI-MS/MS (Sarikahya *et al.*, 2019)	CA	n.a.	0.2543	0.0169	0.9798	0.001	0.005
	HESP		0.0536	0.0116	0.9747	0.0008	0.004
	QUE		0.0076	-0.0017	0.9950	0.002	0.01
	RUT		0.0030	-0.0036	0.9899	0.01	0.05
Sarcocornia ambígua HPLC–ESI-MS/MS (Bertin *et al.*, 2014)	CA	240–4730	209619	22547	0.9988	0.07	0.24
	CLA	230–4640	326704	-979.78	0.9992	0.07	0.23
	QUE	240–4730	35802	-1838.9	0.9994	0.07	0.24
Mauritia flexuosa L.f. UHPLC–ESI-MS/MS (Bataglion *et al.*, 2014)	CA	0.02–1	0.0975	-1.5589	0.9980	0.00008	0.0002
	CLA		0.0078	0.0921	0.9990	0.00006	0.0002
	QUE		0.0037	-0.0132	0.9995	0.00003	0.0001
Ephedra alata HPLC-PAD-ESI/MS (Mighri *et al.*, 2019)	CA	0.05-1.0	5883180	57492.4	0.9996	0.384	1.165
	CLA	0.05-20.0	10857700	- 207598	0.9993	0.031	0.093
	GA	0.05-7.6	2935900	48326.5	0.9999	0.102	0.308
	RUT	0.05-20.0	12608300	- 84599.3	0.9995	0.172	0.521
	NAR	0.05-5.0	24058000	71578	0.9986	0.001	0.002
	QUE	0.05-2.0	15792500	- 101425	0.9992	0.233	0.705

n.a. – not available; CA – Caffeic acid; CLA – Chlorogenic acid; GA – Gallic acid; HESP – Hesperidin; QUE – Quercetin; Q3G – Quercetin-3-O-glucoside; RUT – Rutin; NAR – Naringin.

In fact, in the study of Vu *et al.* (2018) only one of the seven calibration curves, comprises these criteria, while in Bursal *et al.* (2013) and Sarikahya *et al.* (2019) studies none of the presented R values are acceptable. QUE showed the lowest LOD and LOQ in the study of Bataglion *et al.* (2014), with 0.00003 and 0.0001 $\mu g.mL^{-1}$, respectively. All methods were able to identify CA in the extracts analysed, while QUE was not detected in the *Vitis* spp. extract. The potential antioxidant, anti-inflammatory and antimicrobial activity of these extracts may be associated with the presence of CA in their composition. (Albuquerque *et al.*, 2021; Kumar & Goel, 2019)

The main objectives of this study are to develop, optimise and validate an analytical method for the identification and quantification of PC and, subsequently, to apply it for the analysis of the PC present in aqueous and methanolic extracts of the plants *M. indicus* and *P. tridentatum*, by RP-HPLC-UV/DAD. Considering all bioactive compounds previously discussed, the present study will give main interest to the metabolites referenced in the literature, particularly GA, CA, CLA, Q3G, RUT, HESP and NAR. Based on the research presented here, the chosen analytical method was from Iraki *et al.* (2012), since it had similar characteristics to those available for this investigation, namely the equipment (HPLC-DAD) and the stationary phase (C18 Lichrolut) with similar dimensions (250 mm × 4.6 mm x 3 µm) and temperature (25°C) available. The mobile phase used was the same, composed of a gradient of ACN, MeOH and acidified water (acetic acid at 0.5% v/v). In addition, it was able to identify several phenolic compounds, five of interest for the study and it had good calibration results as well as precision (0.8-12.9%) and accuracy (84.3-102.8%).

3.2. Methods

3.2.1. Solvents e Reagents

All chemicals used were of analytical grade. CA and CLA were obtained from Sigma-Aldrich® (China). HESP purchased from Sigma-Aldrich (Spain). MeOH (HPLC 99.8% grade) was from Fisher Chemical® (Belgium). Glacial acetic acid (98%) and ACN (grade for HPLC) was from VWR® Chemicals (France). Water was ultra-purified using Smart2Pure 3 water purification system from ThermoScientific®.

3.2.2. Plant material

M. indicus and *P. tridentatum* were collected in June and July 2019, in Esmoriz (Ovar, Portugal) and Valongo (Porto, Portugal), respectively. The identification of the species was performed by a specialist from the Faculty of Sciences of the University of Porto and attributed a voucher ID.

3.2.3. Preparation of plant extracts

Preparation of the plants and their extraction process were carried out previously by Barbosa (2021). The plants were cleaned and dried at 40°C for 48h. *M. indicus* extracts were prepared from 15g powdered material of dried aerial parts in 75 mL of water (aqueous extract) or MeOH: water (80:20, V/V) (methanolic extract) and submitted in the respective solvent for 24h at room temperature and in the dark. *P. tridentatum* extracts were prepared from 15g powdered material of dried aerial parts in 150 mL of water (aqueous extract) or MeOH: water (80:20, V/V) (methanolic extract), with stirring for 3h, at room temperature and in the dark. Subsequently, the samples were filtered through a Whatman® No. 1 paper, the methanolic extracts were concentrated for drying, under vacuum at 40°C in a rotary evaporator (VWR®, IkaRV8), all extracts were freeze-dried and stored at -80°C (VIP™ Series, MDF-U53V), for further analysis (Barbosa, 2021; Kumar & Goel, 2019).

3.2.4. RP-HPLC-UV/DAD analysis

The components under analysis from each sample were identified by comparing their R_t with those obtained from standard compounds, while for quantification a calibration curve was constructed for each available phenolic standard, based on the UV signal. HPLC-UV/DAD was used for the detection of PC, in a range of 190 to 450 nm, to obtain a complete absorption spectrum of phenolic acids and flavonoid compounds. After analysis of the whole spectrum obtained for each compound, quantification was performed at the optimal wavelengths, depending on the compound under analysis: 280 nm (for HESP) and 320 nm (for CLA and CA). The analysis of PC was adapted from Irakli *et al.* (2012) and performed using a HPLC-UV/DAD with a quaternary pump and autosampler (Jasco® LC 4000 series, pump PU-4080-LG, autosampler AS-4050), coupled to PAD (Jasco® MD-4010), using as stationary phase a RP Silica-bonded C18 column (LiChroCART® 250-4, RP-18, 250 mm × 4 mm x 5 μm), at 25°C. The mobile phase was a gradient elution (Table 3.2), where eluent A was acidified water (acetic acid at 0.5% v/v), B was MeOH and C was ACN, with a flow rate of 1.000 mL.min^{-1} and an injection volume of 20 μL. All samples were analysed in triplicate and the results were expressed in μg.g^{-1} dry weight of plant extract.

Table 3.2 – Elution gradient selected for the analysis of aqueous and methanolic extracts of *Melilotus indicus* and *Pterospartum tridentatum*.

Time (min.)	Eluent A (%)	Eluent B (%)	Eluent C(%)
0	90	5	5
5	85	10	5
30	80	10	10
38	70	0	30
50	40	0	60
55	90	5	5
60	90	5	5

3.2.5. Development and validation of the analytical method

Once the elution gradient of the RP-HPLC-UV/DAD method was established, validation parameters for linearity, range, sensitivity, LOD, LOQ, precision and accuracy in standards were carried out according to ICH guidelines (ICH, s.d.).

Linearity and range

For calibration, individual stock solutions of each standard compound were prepared at a concentration of 333.3 μg.mL^{-1} (0.0005g in 1.5 mL) in mobile phase (acidified water (acetic acid at 0.5% v/v), methanol and ACN (90:5:5; v/v/v). Subsequently, several mixed standard solutions were prepared by successive dilutions at eleven different concentrations (47.61; 23.81; 11.90; 5.95; 2.98; 1.49; 0.74; 0.37; 0.19; 0.09 and 0.05 μg.mL^{-1}). Concentrations 47.61, 11.90, 2.98, 0.37 and 0.05 μg.mL^{-1} were injected in a total of 6 injections for the analysis of precision and accuracy in standards, and the remaining concentrations in triplicate. For each compound analysed, a calibration curve was constructed separately, according to equation (1):

$$y = S(x)+b \qquad (1)$$

where *y* are the y-axis values (peak area), *S* is the slope of the calibration curve, *x* are the x-axis values (concentration of the sample), *and b* is the y-axis intercept of the calibration curve.

For the fitting of the calibration curve, it was applied the linear least squares regression. The linearity criteria were: R greater then 0.999, it is considered an ideal fit of the data to the regression line (Kumar *et al.*, 2015; Ribani *et al.*, 2004), the standard error of the slope by equation (4) and inclusion of zero in confidence interval of the ordinate intercept (Ermer, 2001) by equation (5).

$$S_e = s_s / S \times 100 \quad (4)$$

$$(b - s_b) < 0 < (b + s_b) \quad (5)$$

where S_e is the standard error of the slope, s_s – is the standard deviation of the slope, S is the slope of the calibration curve; b is the y-axis intercept of the calibration curve and s_b – the standard deviation of the y-axis intercept of the calibration curve.

The sensitivity of the analytical method was determined by S, considering that the greater its value, the more sensitive the method is (RELACRE, 2000).

Limits of detection and quantification

LOD and LOQ were calculated based on the standard deviation of the y-axis intercept of the calibration curve (S_b) and slope of the calibration curve (S) for each compound, expressed respectively by equations (2) and (3).

$$LOD = 3 \times S_b / S \quad (2)$$

$$LOQ = 10 \times S_b / S \quad (3)$$

Precision and Accuracy

Precision was evaluated through the repeatability of the method, using the concentrations in the linear range. Results were calculated by the coefficient of variation (CV) through the ratio of the standard deviation (σ_{lab}) to the mean of the laboratorial concentration (C_{lab}), expressed by the equation (6).

$$CV = \sigma_{lab} / C_{lab} \times 100 \quad (6)$$

Accuracy was determined by back calculation using the mean of the laboratorial and the expected concentrations (C_{exp}) in the linear range, expressed by equation (7).

$$Accuracy = C_{lab} / C_{exp} \times 100 \quad (7)$$

3.2.6. Quantification of phenolic compounds

For the quantification of PC, stock solutions of each standard compound were prepared at 1000 μg.mL^{-1} in MeOH. As working solutions, a concentration of 50 μg.mL^{-1} was prepared, using a gradual dilution of acidified water (acetic acid at 0.5% v/v), ACN and stock solution (90:5:5; v/v/v). For the analysis, a mixed standard solution of the compounds of interest was injected.

The injected samples were prepared with 1 mg of plant extract in 200 μL of mobile phase and filtered with VWR® syringe filter (nylon, 13 mm diameter) with a 0.22 μm pore diameter. The content was calculated based on the previously obtained and validated calibration equations of the standard solutions, and the concentration of the extract was expressed by equation (8) in μg.g^{-1} dry weight of extract.

$$Content = ((C_{re} \times V)/m) \times 1000 \quad (8)$$

where C_{re} is the concentration obtained using the peak area of each compound directly in the correspondent calibration equation, V is the volume of the extract solution in mL and m is the mass of the extract solution in mg.

3.3. Results and Discussion

3.3.1. Optimisation of the chromatographic conditions

The development and optimisation of the analytical method, briefly described below, was performed by a team of several investigators. Initially, for the identification and quantification of PC in the extracts under study, a HPLC-DAD method described by Dias *et al.* (1999) was selected, using acidified water (formic acid at 5% v/v) and MeOH as components of the mobile phase gradient. However, preliminary tests concluded that the method did not allow the correct separation of the interest compounds for the analysis of aqueous and methanolic extracts of *M. indicus* and *P. tridentatum*. Then, another mobile phase and elution gradient was tested according to the analytical method described be Irakli *et al.* (2012), using also MeOH and acidified water (acetic acid at 0.5% v/v), and an additional component, ACN. To optimise this method, standard solutions were prepared at three concentrations (1000, 50 and 5 μg.mL^{-1}) and injected with the flow rate 1.000 mL.min^{-1} and an injection volume of 20 μL. The chromatograms obtained allowed to observe a good separation of the compounds due to the gradient of the mobile phase. To improve this initial separation, an oven was coupled to the instrument to place the column and to maintain stationary phase temperature. Different temperatures were tested and the selected one was 25°C, allowing a drop of pressure in the column, compared to room temperature tests, and a better stability in the analysis. Additionally, the PC of interest were well separated. On elution with a

reverse mobile phase, polar compounds have a faster elution compared to more non-polar compounds (Hakkimane & Guru, 2017; Haminiuk *et al.*, 2012). Thus, phenolic acids have a retention time in the first 20 min, since at the beginning of the elution, the mobile phase is mostly acidified water. After 30 min, there is an increase in ACN that allows the detection and separation of the remaining flavonoids. The chromatographic analysis of any compound requires an ideal absorbance, and PC have maximum absorbance in the UV or visible regions (Haminiuk *et al.*, 2012). After an initial analysis of the chromatograms of each standard solution, the chosen wavelengths were 280 nm (HESP, NAR and GA), 320 nm (CA and CLA) and 350 nm (Q3G and RUT), as also described in the studies of Aires *et al.* (2016), Irakli *et al.* (2012) and Gonçalves *et al.* (2020).

3.3.2. Validation of the RP-HPLC-UV/DAD method

Method validation was performed for linearity, range, sensitivity, LOD, LOQ, precision and accuracy. CLA, CA and HESP were the compounds validated, while the remaining compounds of interest were treated by other team investigator and are not explored in the present study. Calibration curves for CLA, CA and HESP are presented in Figures 3.1, 3.2 and 3.3, respectively. For the first two compounds, it was necessary to perform two sequential linear ranges in order to cover all the analytical concentrations (Figures 3.1 and 3.2), while for HESP linearity was only achieved in higher concentrations in a single calibration curve (Figures 3.3).

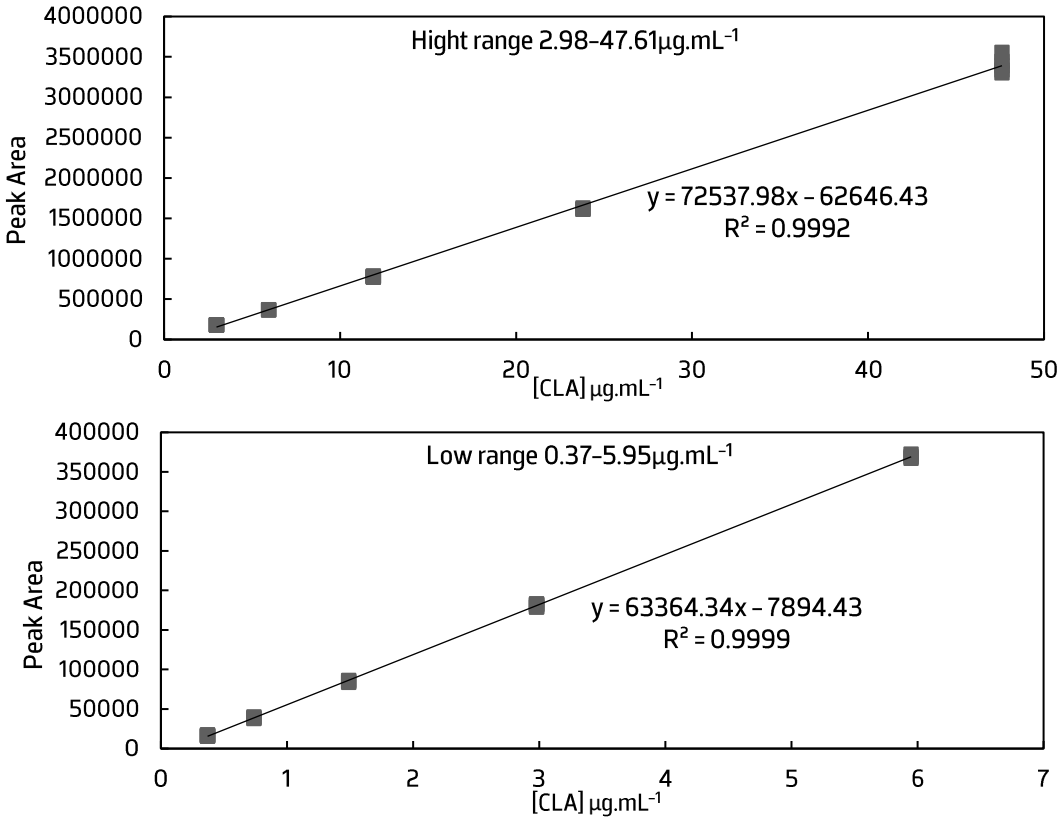


Figure 3.1 – Calibration plot of the chlorogenic acid standard at 320nm.

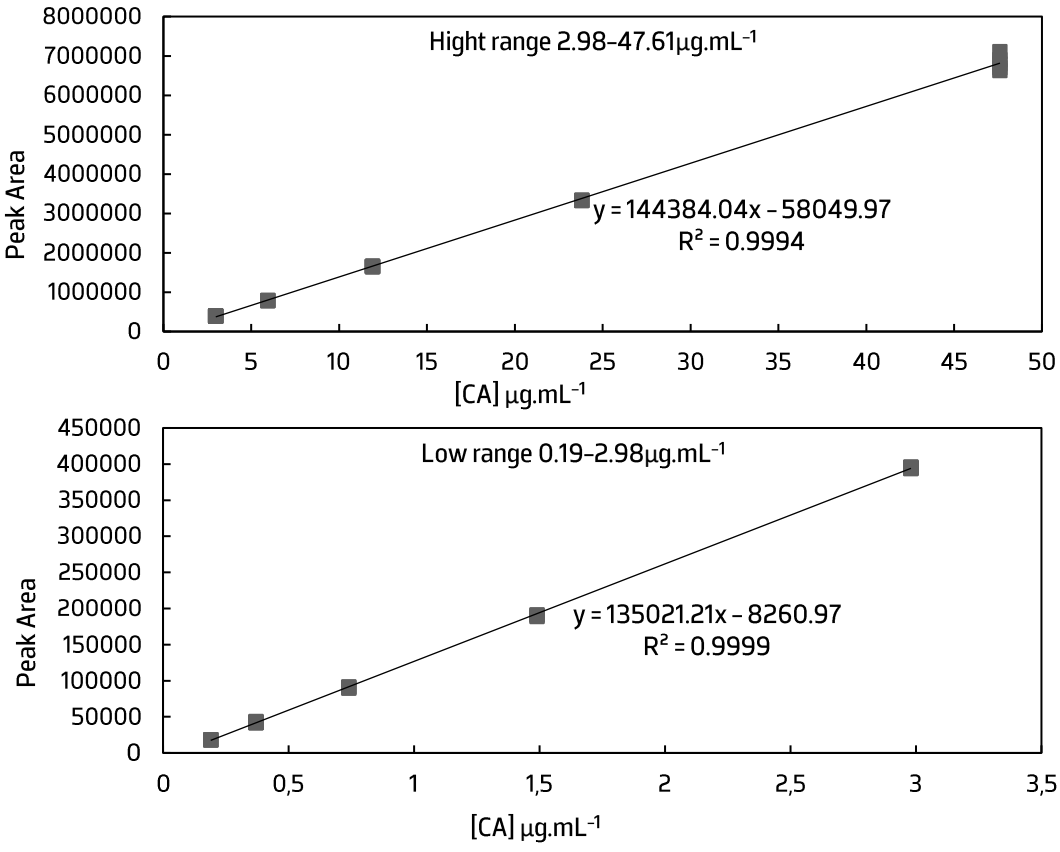


Figure 3.2 – Calibration plot of the caffeic acid standard at 320nm.

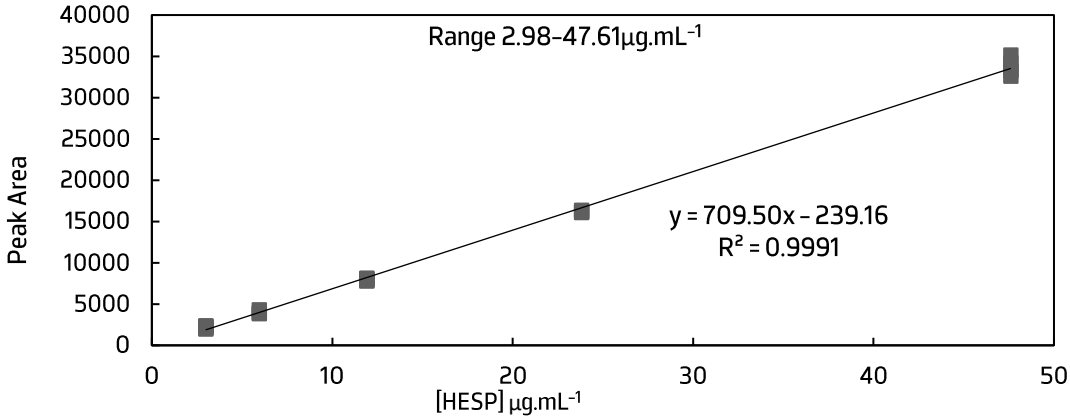


Figure 3.3 – Calibration plot of the hesperidin standard at 280nm.

Although eleven different standard concentrations were injected (47.61; 23.81; 11.90; 5.95; 2.98; 1.49; 0.74; 0.37; 0.19; 0.09 and 0.05 µg.ml^{-1}), the method showed good linearity for CLA in the range 0.37–47.61 µg.mL^{-1}, divided in two sub-ranges: 0.37–5.95 µg.mL^{-1} and 2.98–47.61 µg.mL^{-1} (Figure 3.1.). Linearity was also demonstrated in CA in a boarder range (0.19–47.61µg.mL^{-1}), once more divided in two sub-ranges: 0.19–2.98 µg.mL^{-1} and 2.98–47.61 µg.mL^{-1} (Figure 3.2). For HESP, linearity is only demonstrated in the range of 2.98–47.61 µg.mL^{-1} with a single calibration curve (Figure 3.3). All calibration data of the three interest compounds are resumed in Table 3.3.

Table 3.3 – Linearity validation parameters of the analytical method for quantification of chlorogenic and caffeic acids, and hesperidin.

Compound	Wavelength (nm)	Linear range (µg.mL^{-1})	S	s_s	S_e (%)	b	s_b	R
CLA	320	2.98–47.61	72537.98	627.87	0.87	-62646.43	15514.61	0.9992
		0.37–5.95	63364.34	188.84	0.30	-7894.43	535.11	0.9999
CA	320	2.98–47.61	144384.04	1054.93	0.73	-58049.97	26067.41	0.9994
		0.19–2.98	135021.21	271.47	0.20	-8260.97	468.38	0.9999
HESP	280	2.98–47.61	709.50	6.53	0.92	-239.16	161.27	0.9991

The values of R (Table 3.3) for the referred calibration curves of CLA (0.9992 and 0.9999) CA (0.9994 and 0.9999), and HESP (0.9991), were all above 0.995, the reference acceptable value (Kumar *et al.*, 2015; Ribani *et al.*, 2004). The standard error of the slope (Table 3.3) for CLA (between 0.30 and 0.87%), CA (0.20 and 0.73%) and HESP (0.92%), calculated by equation 4, was an analytical requirement considered satisfactory for being less than 5% (Ermer & Miller, 2005; RELACRE, 2000). Nevertheless, for the other linearity requirement, the inclusion of zero in confidence interval of the ordinate intercept of the calibration curve, no satisfactory values were obtained for any of the compounds analysed, because $b \pm s_b$

is always lower than the origin (Ermer, 2001; Ermer & Miller, 2005). The value of the calibration curve slope, *S* able also to evaluate sensitivity, because the greater the slope, the more sensitive the method is (RELACRE, 2000). Therefore, the developed analytical method is more sensitive for CA and less sensitive for HESP. In fact, that can be confirmed by the LOD values (Table 3.4) that were: 0.20 μg.mL^{-1}, for CLA; 0.09 μg.mL^{-1} for CA; 2.56 μg.mL^{-1}, for HESP. These results are considered significant, since they are below the lowest concentration of the linearity range and are in the range of the LOD found in literature: 0.00006 to 1.48 μg.mL^{-1} for CLA (Bataglion *et al.*, 2014; Bertin *et al.*, 2014; Canas *et al.*, 2015; Gökbulut *et al.*, 2013; Magwaza *et al.*, 2016; Mighri *et al.*, 2019; Skendi *et al.*, 2017; Vu *et al.*, 2018), 0.00008 to 1.35 μg.mL^{-1} for CA (Bataglion *et al.*, 2014; Bertin *et al.*, 2014; Bursal *et al.*, 2013; Canas *et al.*, 2015; Gökbulut *et al.*, 2013; Irakli *et al.*, 2012; Magwaza *et al.*, 2016; Mighri *et al.*, 2019; Sarikahya *et al.*, 2019; Skendi *et al.*, 2017; Vu *et al.*, 2018), and: 0.0008 to 5.02 μg.mL^{-1} for HESP (Irakli *et al.*, 2012; Magwaza *et al.*, 2016; Sarikahya *et al.*, 2019). Comparing the literature LOD values of CA, compound of interest present in all the extracts analysed, HESP and CLA, the methods with MS detectors show lower LOD values, demonstrating higher sensitivity than DAD and UV.

Table 3.4 – Limits of detection and quantification of the chlorogenic and caffeic acids, and hesperidin.

Compound	Linear range (μg.mL^{-1})	LOD (μg.mL^{-1})	LOQ (μg.mL^{-1})
CLA	0.37–47.61	0.20	0.38
CA	0.19–47.61	0.09	0.17
HESP	2.98–47.61	2.56	2.98

The results obtained for the LOQ (Table 3.4) of CLA (0.38 μg.mL^{-1}) and CA (0.17 μg.mL^{-1}) are very close to the lowest concentration of the linearity range (0.37 and 0.19 μg.mL^{-1}, respectively), which confirms the selected linearity ranges. Nevertheless, for HESP, the LOD value, also obtained by the application of equation 2, was 2.56 μg.mL^{-1}, and the LOQ value, obtained by the application of equation 3, was 7.75 μg.mL^{-1}. This LOQ value is above the lowest concentration in the linearity range (2.98 μg.mL^{-1}), enabling to calculate any concentration between 2.98 and 7.75 μg.mL^{-1}. Therefore, as presented in Table 3.4, the LOQ value was assumed to be equal to the lowest concentration in the linearity range (2.98 μg.mL^{-1}), since the calibration curve had a good fit. Nevertheless, it was convenient to perform a new calibration curve for HESP, using higher standard concentrations, in order to achieve a linear range with at least five points and adjust to the LOD and LOQ found. In fact, re-calibration at concentrations higher than 47.61 μg.mL^{-1}, may be viable for this compound. As described in the method of Magwaza *et al.* (2016), a linearity range of 5 to 150 μg.mL^{-1} was obtained for HESP. Also the LOQ values are in the range found in the literature: 0.0002 to 4.92 μg.mL^{-1} for CLA (Bataglion *et al.*, 2014; Bertin *et al.*, 2014; Canas *et al.*, 2015;

Gökbulut *et al.*, 2013; Magwaza *et al.*, 2016; Mighri *et al.*, 2019; Skendi *et al.*, 2017; Vu *et al.*, 2018), 0.0002 to 4.51 $\mu g.mL^{-1}$ for CA (Bataglion *et al.*, 2014; Bertin *et al.*, 2014; Bursal *et al.*, 2013; Canas *et al.*, 2015; Gökbulut *et al.*, 2013; Irakli *et al.*, 2012; Magwaza *et al.*, 2016; Mighri *et al.*, 2019; Sarikahya *et al.*, 2019; Skendi *et al.*, 2017; Vu *et al.*, 2018), and: 0.003 to 16.72 $\mu g.mL^{-1}$ for HESP (Irakli *et al.*, 2012; Magwaza *et al.*, 2016; Sarikahya *et al.*, 2019). These results follow the LOD values, with MS detectors showing the lowest values. HPLC-DAD of Irakli *et al.* (2012) demonstrates a good sensitivity of the method for the determination of PC.

The summary of results for precision and accuracy is described in Table 3.5. Annex 1 shows the values obtained for all concentrations tested. Precision in standards presented small CV values, ranging from a minimum of 0.18% (maximum precision) to 6.02% (minimum precision), in 2.98 $\mu g.mL^{-1}$ standards of CA and HESP, respectively. Nevertheless, precision was generally better in the middle standard concentrations than in the higher or lower concentrations. Relative to previous studies, in the method of Irakli *et al.* (2012), the CA showed similar values for precision, ranging from 0.8 to 6.9%, in the linearity range 0.0016–0.032 $\mu g.mL^{-1}$. The lowest precision value was also achieved at the middle concentrations (Irakli *et al.*, 2012). Skendi *et al.* (2017) presented lower values of CV, from 0.20 to 1.81, in the range 0.5–20 $\mu g.mL^{-1}$. The recommendations indicate that the CV values should not exceed 10% (RELACRE, 2000). Precision decreases in complex biological matrices, while in pharmaceutical quality control values below 1% are easily achievable (Chandran & Singh, 2007). Accuracy, expressed as recovery and achieved by back calculation, ranged from 97.27% to 114.07%, respectively in 11.90 and 2.98 $\mu g.mL^{-1}$ standards of HESP. The remaining overall accuracy results for CLA (97.66%–112.72%) and CA (99.68–105.23%) confirm the reliability of the method, within the application range of 80% to 120% (Ribani *et al.*, 2004). The results obtained for accuracy are similar to those in the literature, where Canas *et al.* (2015) obtained values from 87.7 to 91.3% in the linearity range from 0.25 to 50 $\mu g.mL^{-1}$, Irakli *et al.* (2012) from 84.3 to 98.8% in the range from 0.0016 to 0.032 $\mu g.mL^{-1}$ and Bataglion *et al.* (2014) from 82.88 to 112.42% in the range from 0.02 to 1 $\mu g.mL^{-1}$.

The remaining compounds of interest were validated by the research team, and the regression equations were: GA (for the hight range, y=53733x-31971, and low range, y=48961x-4464.9); RUT (for the hight range, y=13003x-8417.2, and low range y=11384x-1117.3); Q3G (y=703.72x-412.44); and NAR (for the hight range, y=17503x-6234.6, and low range, y=15938x-2088.1). Regarding LOD results were GA (0.07 $\mu g.mL^{-1}$) and RUT (0.15 $\mu g.mL^{-1}$). The LOQ values were GA (0.23 $\mu g.mL^{-1}$), Q3G (2.98 $\mu g.mL^{-1}$), RUT (0.44 $\mu g.mL^{-1}$) and NAR (0.29 $\mu g.mL^{-1}$). These results were used for the identification and quantification of PC in extracts of *M. indicus* and *P. tridentatum*.

Table 3.5 – Precision and accuracy in standards of chlorogenic and caffeic acids, and hesperidin, for concentrations of 47.61, 11.90, 2.98 and 0.37 µg.mL^{-1}.

Compound	Linear range (µg.mL^{-1})	Concentration (µg.mL^{-1})	Precision (%)	Accuracy (%)
CLA	2.98–47.61	47.61	2.86	100.49
		11.90	0.58	97.66
		2.98	0.72	112.72
	0.37–5.95	2,98	0.93	100.05
		0.37	2.99	103.23
CA	2.98–47.61	47.61	2.71	100.22
		11.90	0.23	99.68
		2.98	0.18	105.23
	0.19–2.98	2,98	0.20	100.16
		0.37	1.16	101.33
HESP	2.98–47.61	47.61	2.88	100.54
		11.90	1.53	97.27
		2.98	6.02	114.07

3.3.3. Quantification of phenolic compounds in extracts

Initially, samples were prepared at an extract concentration of 1.000 mg.mL^{-1}. However, the results were not within the range of linearity and only a screening was performed on aqueous and methanolic extracts of *M. indicus* and. The following compounds were not detected in *M. indicus*: GA, Q3G, NAR and HESP. For aqueous and methanolic extracts of *P. tridentatum* the same screening was performed and the compound that was not detected was only NAR. Thus, the solutions of the extracts were five times concentrated, using the same amount of lyophilized extract, 1.000 mg, in 0.2 mL of mobile phase (acidified water (acetic acid at 0.5% v/v), ACN and MeOH (90:5:5; v/v/v)). The chromatogram, obtained at 280 nm for the mixed standard solution injected in the same run as the extracts, is described in Figure 3.4. The remaining chromatograms, for other wavelengths, are described in Annex 2. The chromatogram results (Figures 3.4 and Annex 2) confirm the wavelengths described in the studies of Aires *et al.* (2016), Irakli *et al.* (2012) and Gonçalves *et al.* (2020) as optimal, and already used in the method validation: 280 nm for HESP, NAR and GA; 320 nm for CA and CLA; and 350 nm for Q3G and RUT.

The identification and quantification of PC was based on the comparison of the retention times obtained in the chromatograms of the extracts with those of the standard solution, at the wavelengths chosen for each compound. After validation of the HPLC-UV/DAD method, it was applied to aqueous and methanolic extracts of *M. indicus* and *P. tridentatum*. Figure 3.5 shows the compounds identified in the methanolic extract of *M. indicus* at 320 nm, and Figure 3.6 in the methanolic extract of *P. tridentatum* at 350 nm. The remaining chromatograms obtained are described in detail in Annex 3.

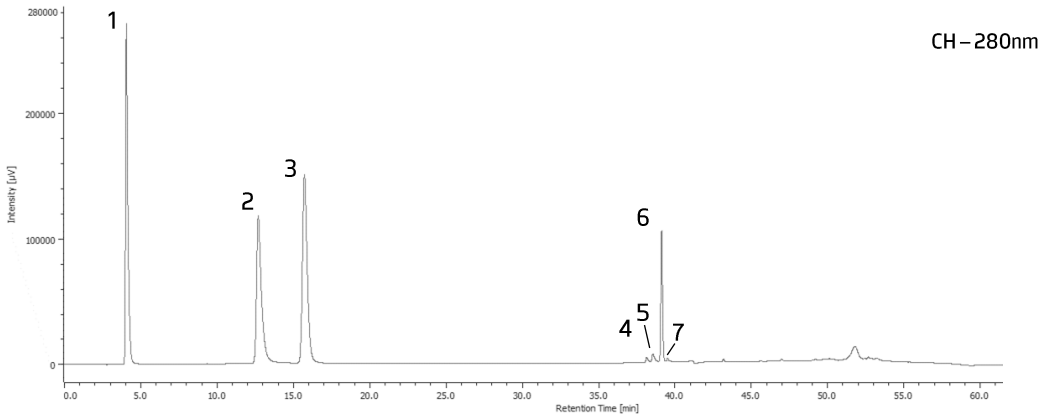


Figure 3.4 - Chromatogram of the mixed standard solution at 280nm.
1 - GA; 2 – CLA; 3 – CA; 4 – RUT; 5 – Q3G; 6 – NAR; 7 – HESP; CH – Chosen wavelength.

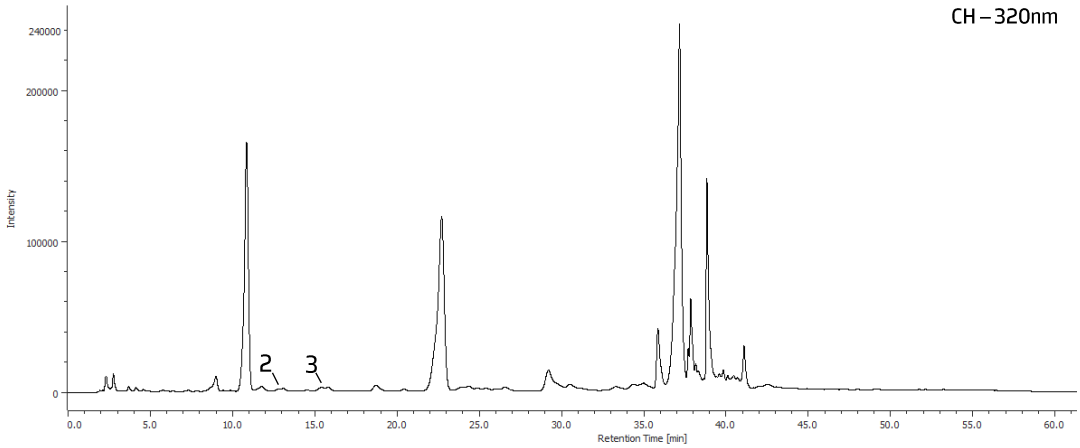


Figure 3.5 – Chromatograms of *Melilotus indicus* methanolic at 320nm.
2 – CLA; 3 – CA; CH – Chosen wavelength.

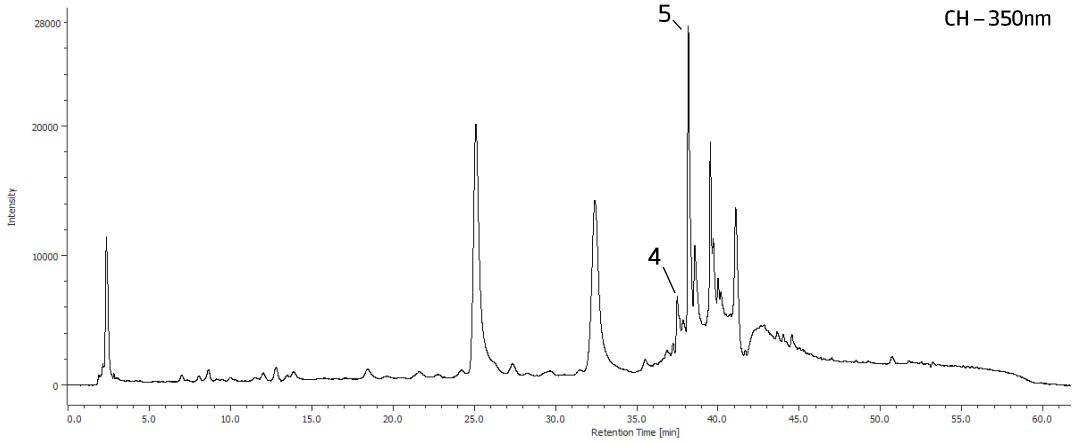


Figure 3.6 – Chromatograms of *Pterospartum tridentatum* methanolic at 350nm.
4 – RUT; 5 – Q3G; CH – Chosen wavelength.

The retention times of the compounds of interest found in the analysed extracts, as well as the respective contents in the different extracts are described in Table 3.6. These results are preliminary, so the contents should be confirmed with additional injections.

Table 3.6 – Retention times and phenolic compounds content in aqueous and methanolic extracts of *Melilotus indicus* and *Pterospartum tridentatum.*

Compound	Retention time (min.)	Contents (μg.g^{-1} dry weight)			
		M. indicus aqueous	*M. indicus* methanolic	*P. tridentatum* aqueous	*P. tridentatum* methanolic
GA	4.05	N/D	N/D	N/D	702.00
CLA	12.69	N/D	110.00	N/D	488.00
CA	15.71	106.00	96.00	D1	N/D
RUT	38.12	N/D	5678.00	N/D	848.00
Q3G	38.52	N/D	N/D	N/D	D2
NAR	39.10	N/D	N/D	N/D	N/D
HESP	39.50	N/D	N/D	D2	10370.00

N/D – Not detected (below LOD); D1 – Detected below linearity range and LOQ; D2 – Detected above linearity range.

In the aqueous *M. indicus* extract, it was only possible to quantify CA, with a concentration of 106.00 μg.g^{-1} dry weight, while in the methanolic extract, two phenolic acids were quantified, namely CLA and CA, with a content of 110.00 and 96.00 μg.g^{-1} dry weight, respectively, and the flavonol RUT with 5678.00 μg.g^{-1} dry weight. Regarding the aqueous extract of *P. tridentatum*, no compound could be quantified. Although CA and HESP could be detected (above LOD), additional injections with different sample dilutions are needed to confirm these results: CA was below the linearity range and LOQ, while HESP obtained results were above the linearity range (2.98–47.61 μg.mL^{-1}). Nevertheless, the methanolic extract of *P. tridentatum* showed the highest number of quantified compounds. The method quantified two phenolic acids (GA with 702.00 μg.g^{-1} dry weight and CLA with 488.00 μg.g^{-1} dry weight) and two flavonoids (RUT with 848.00 μg.g^{-1} dry weight and HESP with 10370.00 μg.g^{-1} dry weight). The obtained value HESP is outside the linearity range but very close to the upper limit, and was considered valid for a preliminary analisys. Q3G was detected but not quantified, since the result was above the linearity range (2.98–47.61 μg.mL^{-1}). Similar to HESP in the aqueous extract of *P. tridentatum*, also on this case the use of higher concentration ranges in calibration should demonstrate new results for Q3G. In comparison, between the quantified PC, HESP was present in highest amounts, with 10370.00 μg.g^{-1} dry weight, in the methanolic extract of *P. tridentatum*, while CA showed the lowest amount with 96.00 μg.g^{-1} dry weight, in the methanolic extract of *M. indicus*. In general, the results obtained are in agreement with the studies Gomma *et al.* (2015) where quantified in the methanolic extract of *M. indicus* 826.60 ± 21.10 μg.g^{-1} dry weight of RUT, being the most common compound. In the method of Luis *et al.* (2011) CLA was quantified (18200.0

μg.g^{-1} dry weight) in the methanolic extract of *P. tridentatum*, while Q3G and RUT was quantified in the methods of Roriz *et al.* (2014) with 963.2 and 125.7 μg.g^{-1} dry weight and Aires *et al.* (2016) with 1230.0 and 1580.0 μg.g^{-1} dry weight, respectively.The contents and compounds present variations due to several factors, including the stages of development and maturation, the harvest, the soil, storage after harvest, the extractive method and solvent, and the polarity of the compounds with the mobile and stationary phases (Arnoso *et al.*, 2019; Irakli *et al.*, 2012; Miguel *et al.*, 2014).

3.4. Conclusion

In the present study, RP-HPLC-DAD method adapted from Irakli *et al.* (2012) was validated for the compounds CLA, CA and HESP, for linearity, range, LOD, LOQ, precision and accuracy criteria. HPLC-DAD is a fast, sensitive and low-cost technique, when compared to other chromatographic techniques, which allows the identification and quantification of phenolic compounds in extracts. The use of a RP allows a better and faster separation of the compounds, as well as being more stable and less polluting than the normal mobile phase. The method optimisation obtained satisfactory values for the validation parameters. Other PC were similarly analysed by other team investigators (GA, RUT, Q3G and NAR) allowing the detection and quantification of a total of seven compounds. Concerning the identification and quantification of phenolic compounds, the method allowed the preliminary detection and quantification of three compounds in *M. indicus* extracts (CLA, CA and RUT). In *P. tridentatum* extracts, the method identified six compounds (GA, CLA, CA, RUT, Q3G and HESP) and quantified four (GA, CLA, RUT and HESP). From this preliminary results, it seems like methanolic extracts of *M. indicus* and *P. tridentatum* presented higher content of PC when compared with the aqueous extracts, concluding that the PC analysed are better extracted by MeOH than by water. The most concentrated analysed compounds were RUT, in *M. indicus* extracts, and HESP, in *P. tridentatum* extracts.

These results will need to be repeated and confirmed, allowing the determination of precision in samples. The determination of accuracy in samples, using the standard addition method is also needed, and a new validation for different concentrations may be considered as well.

4. Conclusion

In conclusion, there are several methods described for the determination of bioactive compounds in *M. indicus* and *P. tridentatum* extracts, of which HPLC is one of the most widely used due to its great power and resolution, ease of use and ability to identify and quantify compounds in herbal preparations. However, studies for the quantification of PC, especially for *M. indicus* extracts, are still understudied. Published methods have identified different classes of phenolic acid and flavonoids, namely HCA and HBA, flavonols, flavones, isoflavones and flavanones in *M. indicus*, and HCA and HBA, flavonols, flavones and isoflavones in *P. tridentatum*. In *M. indicus* extracts there was quantification in the range from 0.16 ± 0.02 to 826.60 ± 21.10 $\mu g.g^{-1}$ dry weight (HESP and RUT, respectively) while in *P. tridentatum* extracts in the range from 53.2 ± 0.2 to 32200.0 ± 2600.0 $\mu g.g^{-1}$ dry weight (sissotrin and vanillic acid, respectively).

Regardless of the selected method, all require previous validation to prove the quality of the assay. Among the validation parameters are linearity, range, sensitivity, LOD, LOQ, precision and accuracy. The adapted, optimised, and validated RP-HPLC-DAD method of the present study showed linearity in the range of 0.19 to 47.61 $\mu g.mL^{-1}$, R higher than 0.999, LOD and LOQ from 0.09 to 2.56 $\mu g.mL^{-1}$ and from 0.17 to 2.98 $\mu g.mL^{-1}$, respectively. Precision ranged from 0.18 to 6.02%, while accuracy ranged from 97.27 to 114.07%. In addition to the good sensitivity for the determination of CA, CLA and HESP, the method was considered satisfactory for the identification and quantification of PC. This method allowed the identification of phenolic compounds (GA, CLA, CA, RUT, Q3G and HESP) in aqueous and methanolic extracts of *M. indicus* and *P. tridentatum* and the quantification in *M. indicus* extracts, in a range between 96.00 and 5678.00 $\mu g.g^{-1}$ dry weight (CA and RUT, respectively) and *P. tridentatum* extracts, between 488.00 and 10370.00 $\mu g.g^{-1}$ dry weight (CLA and HESP, respectively).

The non-repetition of the injections and confirmation of the quantification values were limitations of the present study, as well as the non-determination of the precision and accuracy of the method in extracts (e.g., standard addition method). The methanolic extracts showed higher content of phenolic compounds, which suggests that the polarity of the extracts is changed with the extraction method and the solvent used. The use of new extractive solvents and/or extraction methods could be a suggestion for future investigation because it could allow the detection and quantification of other compounds what would allow a more profound knowledge about *M. indicus* and *P. tridentatum*. In addition, a new validation for different concentrations could be considered. As the HPLC-DAD technique, the use of a reversed phase and a gradient elution demonstrated satisfactory values in the detection and quantification of CA, CLA, GA, HESP, Q3G and NAR, this method could be extended to new compounds.

References

Abd El-Hafeez, A. A., Khalifa, H. O., Elgawish, R. A., Shouman, S. A., Abd El-Twab, M. H., & Kawamoto, S. (2018). *Melilotus indicus* extract induces apoptosis in hepatocellular carcinoma cells via a mechanism involving mitochondria-mediated pathways. *Cytotechnology*, *70*(2), 831–842. https://doi.org/10.1007/s10616-018-0195-7

Ahangarpour, A., Sayahi, M., & Sayahi, M. (2019). The antidiabetic and antioxidant properties of some phenolic phytochemicals: A review study. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, *13*(1), 854–857. https://doi.org/10.1016/j.dsx.2018.11.051

Ahmed, D., Baig, H., & Zara, S. (2012). Seasonal variation of phenolics, flavonoids, antioxidant and lipid peroxidation inhibitory activity of methanolic extract of *Melilotus indicus* and its sub-fractions in different solvents. *International Journal of Phytomedicine*, *4*(3), 326. http://www.arjournals.org/index.php/ijpm/index

Ahmed, S. A. K., & Al-Refai, M. (2014). Chemical constituents and cytotoxic activities of the extracts of *Melilotus indicus*. *European Journal of Chemistry*, *5*(3), 503–506. https://doi.org/10.5155/eurjchem.5.3.503-506.1070

Aires, A., Marrinhas, E., Carvalho, R., Dias, C., & Saavedra, M. J. (2016). Phytochemical Composition and Antibacterial Activity of Hydroalcoholic Extracts of *Pterospartum tridentatum* and *Mentha pulegium* against *Staphylococcus aureus* Isolates. *BioMed Research International*, *2016*, 1–11. https://doi.org/10.1155/2016/5201879

Al-Ishaq, R. K., Overy, A. J., & Büsselberg, D. (2020). Phytochemicals and Gastrointestinal Cancer: Cellular Mechanisms and Effects to Change Cancer Progression. *Biomolecules*, *10*(1), 105. https://doi.org/10.3390/biom10010105

Albuquerque, B. R., Heleno, S. A., Oliveira, M. B. P. P., Barros, L., & Ferreira, I. C. F. R. (2021). Phenolic compounds: current industrial applications, limitations and future challenges. *Food & Function*, *12*(1), 14–29. https://doi.org/10.1039/d0fo02324h

Alkhalidy, H., Moore, W., Wang, Y., Luo, J., Mcmillan, R., Zhen, W., Zhou, K., & Liu, D. (2018). The Flavonoid Kaempferol Ameliorates Streptozotocin-Induced Diabetes by Suppressing Hepatic Glucose Production. *Molecules*, *23*(9), 2338. https://doi.org/10.3390/molecules23092338

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D., & Lightfoot, D. (2017). Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. *Plants*, *6*(4), 42. https://doi.org/10.3390/plants6040042

Araujo, P. (2009). Key aspects of analytical method validation and linearity evaluation. *Journal of Chromatography B*, *877*(23), 2224–2234. https://doi.org/10.1016/j.jchromb.2008.09.030

Araújo, P. V., Clamote, F., Carapeto, A., Almeida, J. D., Porto, M., Holyoak, D. T., Lourenço, J., & Aguiar, C. (2021). *Pterospartum tridentatum (L.) Willk* [Mapa de distribuição]. Sociedade Portuguesa de Botânica, Flora-On: Flora de Portugal Interactiva. http://www.flora-on.pt/#w*Pterospartum+tridentatum*

Arnoso, B. J. D. M., Costa, G. F. d., & Schmidt, B. (2019). Biodisponibilidade e classificação de compostos fenólicos. *Nutrição Brasil*, *18*(1), 39. https://doi.org/10.33233/nb.v18i1.1432

Bacanli, M., Dilsiz, S. A., Başaran, N., & Başaran, A. A. (2019). Chapter Five - Effects of phytochemicals against diabetes. In F. Toldrá (Ed.), *Advances in Food and Nutrition Research* (Vol. 89, pp. 209–238). Academic Press. https://doi.org/10.1016/bs.afnr.2019.02.006

Bagetta, G., Cosentino, M., Corasaniti, M. T., & Sakurada, S. (2012). *Herbal Medicines: Development and Validation of Plant-Derived for Human Health* (Vol. XXI). CRC Press.

Balanč, B., Kalušević, A., Drvenica, I., Coelho, M. T., Djordjević, V., Alves, V. D., Sousa, I., Moldão-Martins, M., Rakić, V., Nedović, V., & Bugarski, B. (2016). Calcium-Alginate-Inulin Microbeads as Carriers for Aqueous Carqueja Extract. *Journal of Food Science*, *81*(1), E65–E75. https://doi.org/10.1111/1750-3841.13167

Barbosa, S. (2021). *Determinação do Potencial de Proteção Solar de Plantas da Flora Portuguesa* [Instituto Politécnico do Porto - Escola Superior de Saúde]. Porto. http://hdl.handle.net/10400.22/17543

Bashir, A., Asif, M., Saadullah, M., Saleem, M., Khalid, S. H., Hussain, L., Ullah Khan, I., Sidra Yaseen, H., Zubair, H. M., Shamas, M. U., Al Zarzour, R., & Chohan, T. A. (2022). Therapeutic Potential of Standardized Extract of *Melilotus indicus* (L.) All. and Its Phytochemicals against Skin Cancer in Animal Model: *In Vitro*, *In Vivo*, and *In Silico* Studies. *ACS Omega*, *7*(29), 25772–25782. https://doi.org/10.1021/acsomega.2c03053

Bataglion, G. A., da Silva, F. M. A., Eberlin, M. N., & Koolen, H. H. F. (2014). Simultaneous quantification of phenolic compounds in buriti fruit (*Mauritia flexuosa* L.f.) by ultra-high performance liquid chromatography coupled to tandem mass spectrometry. *Food Research International*, *66*, 396–400. https://doi.org/10.1016/j.foodres.2014.09.035

Batanony, N. H. E., Castellano-Hinojosa, A., Mamdouh, A., Ashraf, N., & Bedmar, E. J. (2020). Agronomical parameters of host and non-host legumes inoculated with *Melilotus indicus*-isolated rhizobial strains in desert unreclaimed soil. *Archives of Microbiology*, *202*(7), 1929–1938. https://doi.org/10.1007/s00203-020-01907-x

Bento-Silva, A., Koistinen, V. M., Mena, P., Bronze, M. R., Hanhineva, K., Sahlstrøm, S., Kitrytė, V., Moco, S., & Aura, A.-M. (2020). Factors affecting intake, metabolism and health benefits of phenolic acids: do we understand individual variability? *European Journal of Nutrition*, *59*(4), 1275–1293. https://doi.org/10.1007/s00394-019-01987-6

Bertin, R. L., Gonzaga, L. V., Borges, G. d. S. C., Azevedo, M. S., Maltez, H. F., Heller, M., Micke, G. A., Tavares, L. B. B., & Fett, R. (2014). Nutrient composition and, identification/quantification of major phenolic compounds in *Sarcocornia ambigua* (Amaranthaceae) using HPLC–ESI-MS/MS. *Food Research International*, *55*, 404–411. https://doi.org/10.1016/j.foodres.2013.11.036

Borges, A., Abreu, A., Dias, C., Saavedra, M., Borges, F., & Simões, M. (2016). New Perspectives on the Use of Phytochemicals as an Emergent Strategy to Control Bacterial Infections Including Biofilms. *Molecules*, *21*(7), 877. https://doi.org/10.3390/molecules21070877

Bursal, E., Koksal, E., Gülçin, I., Bilsel, G., & Gören, A. (2013). Antioxidant activity and polyphenol content of cherry stem (*Cerasus avium* L.) determined by LC–MS/MS. *Food Research International*, *51*, 66–74. https://doi.org/10.1016/j.foodres.2012.11.022

Byun, M. R., Jeong, H., Bae, S. J., Kim, A. R., Hwang, E. S., & Hong, J.-H. (2012). TAZ is required for the osteogenic and anti-adipogenic activities of kaempferol. *Bone*, *50*(1), 364–372. https://doi.org/10.1016/j.bone.2011.10.035

Canas, S., Assunção, M., Brazão, J., Zanol, G., & Eiras-Dias, J. E. (2015). Phenolic Compounds Involved in Grafting Incompatibility of *Vitis* spp: Development and Validation of an Analytical Method for their Quantification. *Phytochemical Analysis*, *26*(1), 1–7. https://doi.org/10.1002/pca.2526

Chandran, S., & Singh, R. (2007). Comparison of various international guidelines for analytical method validation. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *62*(1), 4–14. https://doi.org/10.1691/ph.2007.1.5064

Chaves, D. F. S. (2015). *Compostos Bioativos dos Alimentos* (1 ed.). VP Editora.

Chen, R., Qi, Q.-L., Wang, M.-T., & Li, Q.-Y. (2016). Therapeutic potential of naringin: an overview. *Pharmaceutical Biology*, *54*(12), 3203–3210. https://doi.org/10.1080/13880209.2016.1216131

Cheng, T. O. (2001). Hippocrates and cardiology. *American Heart Journal*, *141*(2), 173–183. https://doi.org/10.1067/mhj.2001.112490

Choi, H. G., Tran, P. T., Lee, J.-H., Min, B. S., & Kim, J. A. (2018). Anti-inflammatory activity of caffeic acid derivatives isolated from the roots of Salvia miltiorrhiza Bunge. *Archives of Pharmacal Research*, *41*(1), 64–70. https://doi.org/10.1007/s12272-017-0983-1

Deshetty, U. M., Tamatam, A., Bhattacharjee, M., Perumal, E., Natarajan, G., & Khanum, F. (2020). Ameliorative Effect of Hesperidin Against Motion Sickness by Modulating Histamine and Histamine H1 Receptor Expression. *Neurochemical Research*, *45*(2), 371–384. https://doi.org/10.1007/s11064-019-02923-0

Dias, A. C. P., Seabra, R. M., Andrade, P. B., & Fernandes-Ferreira, M. (1999). The development and evaluation of an HPLC–DAD method for the analysis of the phenolic fractions from *in vivo* and *in vitro* biomass of *Hypericum* species. *Journal of Liquid Chromatography & Related Technologies*, *22*(2), 215–227. https://doi.org/10.1081/JLC-100101655

Dobrikova, A. G., & Apostolova, E. L. (2015). Damage and protection of the photosynthetic apparatus from UV-B radiation. II. Effect of quercetin at different pH. *Journal of Plant Physiology*, *184*, 98–105. https://doi.org/10.1016/j.jplph.2015.06.008

EMA. (1995). ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf

Ermer, J. (2001). Validation in pharmaceutical analysis. Part I: An integrated approach. *Journal of Pharmaceutical and Biomedical Analysis*, *24*(5), 755–767. https://doi.org/10.1016/S0731-7085(00)00530-6

Ermer, J., & Miller, J. H. M. (2005). *Method validation in pharmaceutical analysis*. Wiley - VCH.

FDA. (2015). Analytical Procedures and Methods Validation for Drugs and Biologics. https://www.fda.gov/files/drugs/published/Analytical-Procedures-and-Methods-Validation-for-Drugs-and-Biologics.pdf

Fernandes, F. H. A., & Salgado, H. R. N. (2016). Gallic Acid: Review of the Methods of Determination and Quantification. *Critical Reviews in Analytical Chemistry*, *46*(3), 257–265. https://doi.org/10.1080/10408347.2015.1095064

Ferreira, F. M., Dinis, L. T., Azedo, P., Galhano, C. I. C., Simões, A., Cardoso, S. M., Rosário, M., Domingues, M., Pereira, O. R., Palmeira, C. M., & Peixoto, F. P. (2012). Antioxidant capacity and toxicological evaluation of *Pterospartum tridentatum* flower extracts. *CyTA - Journal of Food*, *10*(2), 92–102. https://doi.org/10.1080/19476337.2011.590233

Ferreira, I. C. F. R., & Abreu, R. M. V. (2007). Stress Oxidativo, Antioxidantes e Fitoquímicos. *Bioanálise*, *IV:2*, 32–39. http://hdl.handle.net/10198/2711

Fischer, U. A., Carle, R., & Kammerer, D. R. (2011). Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MSn. *Food Chemistry*, *127*(2), 807–821. https://doi.org/10.1016/j.foodchem.2010.12.156

Gil, M., & Wianowska, D. (2017). Chlorogenic acids – their properties, occurrence and analysis. *Annales Universitatis Mariae Curie-Sklodowska, sectio AA – Chemia*, *72*(1), 61. https://doi.org/10.17951/aa.2017.72.1.61

Gökbulut, A., Özhana, O., Satılmiş, B., Batçioğlu, K., Günal, S., & Şarer, E. (2013). Antioxidant and Antimicrobial Activities, and Phenolic Compounds of Selected *Inula* species from Turkey. *Natural Product Communications*, *8*(4), 475–478. https://doi.org/10.1177/1934578x1300800417

Gomaa, N. H., Hassan, M. O., Fahmy, G. M., González, L., Hammouda, O., & Atteya, A. M. (2015). Flavonoid profiling and nodulation of some legumes in response to the allelopathic stress of *Sonchus oleraceus* L. *Acta Botanica Brasilica*, *29*(4), 553–560. https://doi.org/10.1590/0102-33062015abb0153

Gonçalves, A. C., Bento, C., Nunes, A. R., Simões, M., Alves, G., & Silva, L. R. (2020). Multitarget protection of *Pterospartum tridentatum* phenolic-rich extracts against a wide range of free radical species, antidiabetic activity and effects on human colon carcinoma (Caco-2) cells. *Journal of Food Science*, *85*(12), 4377–4388. https://doi.org/10.1111/1750-3841.15511

Griffin, J. P., & D'Arcy, P. F. (1996). Synergistic Drug Interactions. In *Mechanisms of Drug Interactions* (pp. 235–248). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-61015-8_8

Hakkimane, S. S., & Guru, B. R. (2017). Nano formulation analysis: analytical method development of isoniazid and simultaneous estimation of anti-tubercular drugs isoniazid and rifampicin by RP-HPLC. *Asian Journal of Pharmaceutical and Clinical Research*, 330–335.

Haminiuk, C. W. I., Maciel, G. M., Plata-Oviedo, M. S. V., & Peralta, R. M. (2012). Phenolic compounds in fruits – an overview. *International Journal of Food Science & Technology*, *47*(10), 2023–2044. https://doi.org/10.1111/j.1365-2621.2012.03067.x

Hosseinzadeh, E., Hassanzadeh, A., Marofi, F., Alivand, M. R., & Solali, S. (2020). Flavonoid-Based Cancer Therapy: An Updated Review. *Anticancer Agents Med Chem*, *20*(12), 1398–1414. https://doi.org/10.2174/1871520620666200423071759

ICH. (s.d.). *ICH Harmonisation for Better Health*. Retrieved 20/06/2021 from https://www.ich.org/page/quality-guidelines

Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*(4), 1821–1835. https://doi.org/10.1016/j.foodchem.2010.12.026

Irakli, M. N., Samanidou, V. F., Biliaderis, C. G., & Papadoyannis, I. N. (2012). Simultaneous determination of phenolic acids and flavonoids in rice using solid-phase extraction and RP-HPLC with photodiode array detection. *Journal Separation Science 35*(13), 1603–1611. https://doi.org/10.1002/jssc.201200140

Kamel, R., & Mostafa, D. M. (2015). Rutin nanostructured lipid cosmeceutical preparation with sun protective potential. *Journal of Photochemistry and Photobiology B: Biology*, *153*, 59–66. https://doi.org/10.1016/j.jphotobiol.2015.09.002

Kanzana, G., Musaza, J., Wu, F., Ouyang, Z., Wang, Y., Ma, T., Akoy, B. I. R., & Zhang, J. (2021). Genome-wide development and application of miRNA-SSR markers in *Melilotus* genus. *Physiology and Molecular Biology of Plants*, *27*(10), 2269–2282. https://doi.org/10.1007/s12298-021-01086-z

Khan, H. (2014). Medicinal Plants in Light of History: Recognized Therapeutic Modality. *Journal of Evidence-Based Complementary & Alternative Medicine*, *19*(3), 216–219. https://doi.org/10.1177/2156587214533346

Kumar, G. P., & Khanum, F. (2012). Neuroprotective potential of phytochemicals. *Pharmacognosy reviews*, *6*(12), 81–90. https://doi.org/10.4103/0973-7847.99898

Kumar, N., & Goel, N. (2019). Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnology Reports*, *24*, e00370. https://doi.org/10.1016/j.btre.2019.e00370

Kumar, V. S., Dhiman, V., Giri, K. K., Sharma, K., Zainuddin, M., & Mullangi, R. (2015). Development and validation of a RP-HPLC method for the quantitation of tofacitinib in rat plasma and its application to a pharmacokinetic study. *Biomedical Chromatography*, *29*(9), 1325–1329. https://doi.org/10.1002/bmc.3426

Lattanzio, V. (2013). Phenolic Compounds: Introduction 50. *Natural Products*, 1543–1580. https://doi.org/10.1007/978-3-642-22144-6_57

Lee, H., Kim, B. G., Kim, M., & Ahn, J.-H. (2015). Biosynthesis of Two Flavones, Apigenin and Genkwanin, in Escherichia coli. *Journal of Microbiology and Biotechnology*, *25*(9), 1442–1448. https://doi.org/10.4014/jmb.1503.03011

Li, C., & Schluesener, H. (2017). Health-promoting effects of the citrus flavanone hesperidin. *Critical Reviews in Food Science and Nutrition*, *57*(3), 613–631. https://doi.org/10.1080/10408398.2014.906382

Li, Z.-J., Liu, M., Dawuti, G., Dou, Q., Ma, Y., Liu, H.-G., & Aibai, S. (2017). Antifungal Activity of Gallic Acid *In Vitro* and *In Vivo*. *Phytotherapy Research*, *31*(7), 1039–1045. https://doi.org/10.1002/ptr.5823

Liao, W., Chen, L., Ma, X., Jiao, R., Li, X., & Wang, Y. (2016). Protective effects of kaempferol against reactive oxygen species-induced hemolysis and its antiproliferative activity on human cancer cells. *European Journal of Medicinal Chemistry*, *114*, 24–32. https://doi.org/10.1016/j.ejmech.2016.02.045

Liu, W.-N., Shi, J., Fu, Y., & Zhao, X.-H. (2019). The Stability and Activity Changes of Apigenin and Luteolin in Human Cervical Cancer Hela Cells in Response to Heat Treatment and Fe2+/Cu2+ Addition. *Foods*, *8*(8), 346. https://doi.org/10.3390/foods8080346

Lourenço, J., Araújo, P. V., Holyoak, D. T., Carapeto, A., Clamote, F., Gomes, C. T., Aguiar, C., & Almeida, J. D. (2021). *Melilotus indicus (L.) All.* [Mapa de distribuição]. Sociedade Portuguesa de Botânica, Flora-On: Flora de Portugal Interactiva. http://www.flora-on.pt/#w*Melilotus+indicus*

Luís, Â., Domingues, F., & Duarte, A. P. (2011). Bioactive Compounds, RP-HPLC Analysis of Phenolics, and Antioxidant Activity of Some Portuguese Shrub Species Extracts. *Natural Product Communications*, *6*(12), 1934578X1100601. https://doi.org/10.1177/1934578x1100601219

Lyu, J. I., Ryu, J., Jin, C. H., Kim, D.-G., Kim, J. M., Seo, K.-S., Kim, J.-B., Kim, S. H., Ahn, J.-W., Kang, S.-Y., & Kwon, S.-J. (2020). Phenolic Compounds in Extracts of *Hibiscus acetosella* (Cranberry Hibiscus)

and Their Antioxidant and Antibacterial Properties. *Molecules*, *25*(18), 4190. https://doi.org/10.3390/molecules25184190

Magwaza, L. S., Opara, U. L., Cronje, P. J. R., Landahl, S., Ortiz, J. O., & Terry, L. A. (2016). Rapid methods for extracting and quantifying phenolic compounds in citrus rinds. *Food Science & Nutrition*, *4*(1), 4–10. https://doi.org/10.1002/fsn3.210

Mahdi-Pour, B., Jothy, S. L., Latha, L. Y., Chen, Y., & Sasidharan, S. (2012). Antioxidant activity of methanol extracts of different parts of Lantana camara. *Asian Pacific Journal of Tropical Biomedicine*, *2*(12), 960–965. https://doi.org/10.1016/s2221-1691(13)60007-6

Manzoor, M. F., Ahmad, N., Ahmed, Z., Siddique, R., Zeng, X. A., Rahaman, A., Muhammad Aadil, R., & Wahab, A. (2019). Novel extraction techniques and pharmaceutical activities of luteolin and its derivatives. *Journal of Food Biochemistry*, *43*(9), e12974. https://doi.org/10.1111/jfbc.12974

Mattonai, M., Vinci, A., Degano, I., Ribechini, E., Franceschi, M., & Modugno, F. (2019). Olive mill wastewaters: quantitation of the phenolic content and profiling of elenolic acid derivatives using HPLC-DAD and HPLC/MS2 with an embedded polar group stationary phase. *Natural Product Research*, *33*(21), 3171–3175. https://doi.org/10.1080/14786419.2018.1522632

Mighri, H., Akrout, A., Bennour, N., Eljeni, H., Zammouri, T., & Neffati, M. (2019). LC/MS method development for the determination of the phenolic compounds of Tunisian *Ephedra alata* hydro-methanolic extract and its fractions and evaluation of their antioxidant activities. *South African Journal of Botany*, *124*, 102–110. https://doi.org/10.1016/j.sajb.2019.04.029

Miguel, M. G., Nunes, S., Dandlen, S. A., Cavaco, A. M., & Antunes, M. D. (2014). Phenols, flavonoids and antioxidant activity of aqueous and methanolic extracts of propolis (*Apis mellifera* L.) from Algarve, South Portugal. *Food Science and Technology (Campinas)*, *34*(1), 16–23. https://doi.org/10.1590/s0101-20612014000100002

Nabavi, S. F., Braidy, N., Gortzi, O., Sobarzo-Sanchez, E., Daglia, M., Skalicka-Woźniak, K., & Nabavi, S. M. (2015). Luteolin as an anti-inflammatory and neuroprotective agent: A brief review. *Brain Research Bulletin*, *119*, 1–11. https://doi.org/10.1016/j.brainresbull.2015.09.002

Naveed, M., Hejazi, V., Abbas, M., Kamboh, A. A., Khan, G. J., Shumzaid, M., Ahmad, F., Babazadeh, D., FangFang, X., Modarresi-Ghazani, F., WenHua, L., & XiaoHui, Z. (2018). Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomedicine & Pharmacotherapy*, *97*, 67–74. https://doi.org/10.1016/j.biopha.2017.10.064

Nollet, L. M., & Gutierrez-Uribe, J. A. (2017). *Phenolic Compounds in Food: Characterization and Analysis* (1 ed.). CRC Press. https://doi.org/10.1201/9781315120157

Paulo, A., Martins, S., Branco, P., Dias, T., Borges, C., Rodrigues, A. I., do Céu Costa, M., Teixeira, A., & Mota-Filipe, H. (2008). The opposing effects of the flavonoids isoquercitrin and Sissotrin, isolated from *Pterospartum tridentatum*, on oral glucose tolerance in rats. *Phytotherapy Research*, *22*(4), 539–543. https://doi.org/10.1002/ptr.2403

Paz, R. A. D. (2018). *Determinação simultânea, por cromatografia líquida de alta resolução, das vitaminas A, D e E em amostras compostas por diferentes matrizes alimentares* Instituto Superior de Engenharia de Lisboa]. Lisboa. http://hdl.handle.net/10400.21/8611

Pedro, L. F., Catarina, F., Ana, M. R., Pedro, C., Paulo, J. A. M., Maria, H. F., ncio, Fatima, N. F., & Maria, L. S. (2013). Antioxidant and anti-acetylcholinesterase activity of commercially available medicinal infusions after *in vitro* gastrointestinal digestion. *Journal of Medicinal Plants Research*, *7*(20), 1370–1378.

Peraman, R., Mallikarjuna, S., Ammineni, P., & Kondreddy, V. K. (2014). RP-HPLC Method Development and Validation for Simultaneous Estimation of Atorvastatin Calcium and Pioglitazone Hydrochloride in Pharmaceutical Dosage Form. *Journal of Chromatographic Science*, *52*(9), 1038–1042. https://doi.org/10.1093/chromsci/bmt156

Pereira, A. J. (s.d.). *Pterospartum tridentatum* [Imagem]. [Licença de Creative Commons Atribuição—NãoComercial 4.0 Internacional.]. Sociedade Portuguesa de Botânica, Flora-On: Flora de Portugal Interactiva. https://flora-on.pt/#/hoDzF

Phan, M. A. T., Paterson, J., Bucknall, M., & Arcot, J. (2018). Interactions between phytochemicals from fruits and vegetables: Effects on bioactivities and bioavailability. *Critical Reviews in Food Science and Nutrition*, *58*(8), 1310–1329. https://doi.org/10.1080/10408398.2016.1254595

Porto, M. (s.d.-a). *Melilotus indicus* [Imagem]. [Licença de Creative Commons Atribuição—NãoComercial 4.0 Internacional.]. Sociedade Portuguesa de Botânica, Flora-On: Flora de Portugal Interactiva. https://flora-on.pt/index.php#/hsh3w

Porto, M. (s.d.-b). *Melilotus indicus* [Imagem]. [Licença de Creative Commons Atribuição—NãoComercial 4.0 Internacional.]. Sociedade Portuguesa de Botânica, Flora-On: Flora de Portugal Interactiva. https://flora-on.pt/index.php#/ho3jH

Porto, M. (s.d.-c). *Pterospartum tridentatum* [Imagem]. [Licença de Creative Commons Atribuição—NãoComercial 4.0 Internacional.]. Sociedade Portuguesa de Botânica, Flora-On: Flora de Portugal Interactiva. https://flora-on.pt/#/h_ZIz

Prince, P. S. M., Rajakumar, S., & Dhanasekar, K. (2011). Protective effects of vanillic acid on electrocardiogram, lipid peroxidation, antioxidants, proinflammatory markers and histopathology in isoproterenol induced cardiotoxic rats. *European Journal of Pharmacology*, *668*(1), 233–240. https://doi.org/10.1016/j.ejphar.2011.06.053

Ramzan, I. (2015). *Phytotherapies: Efficacy, Safety, and Regulation* (XXII ed.). John Wiley & Sons.

RELACRE. (2000). Guia RELACRE 13 - Validação de Métodos Internos de Ensaio em Análise Química. In R.-A. d. L. A. d. Portugal (Ed.). RELACRE.

Ribani, M., Bottoli, C. B. G., Collins, C. H., Jardim, I. C. S. F., & Melo, L. F. C. (2004). Validação em métodos cromatográficos e eletroforéticos. *Química Nova*, *27*(5), 771–780. https://doi.org/10.1590/s0100-40422004000500017

Roriz, C. L., Barros, L., Carvalho, A. M., Santos-Buelga, C., & Ferreira, I. C. F. R. (2014). *Pterospartum tridentatum*, *Gomphrena globosa* and *Cymbopogon citratus*: A phytochemical study focused on antioxidant compounds. *Food Research International*, *62*, 684–693. https://doi.org/10.1016/j.foodres.2014.04.036

Rosa, L. A. d. l., Moreno-Escamilla, J. O., Rodrigo-García, J., & Alvarez-Parrilla, E. (2019). Chapter 12 - Phenolic Compounds. In E. M. Yahia (Ed.), *Postharvest Physiology and Biochemistry of Fruits and Vegetables* (pp. 253–271). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-813278-4.00012-9

Roy, M., & Datta, A. (2019). Fundamentals of Phytochemicals. In *Cancer Genetics and Therapeutics* (pp. 49–81). Springer Singapore. https://doi.org/10.1007/978-981-13-9471-3_3

Saleem, M., Asif, M., Parveen, A., Yaseen, H. S., Saadullah, M., Bashir, A., Asif, J., Arif, M., Khan, I. U., & Khan, R. U. (2021). Investigation of *in vivo* anti-inflammatory and anti-angiogenic attributes of coumarin-rich ethanolic extract of *Melilotus indicus*. *Inflammopharmacology*, *29*(1), 281–293. https://doi.org/10.1007/s10787-020-00703-9

Salehi, B., Venditti, A., Sharifi-Rad, M., Kręgiel, D., Sharifi-Rad, J., Durazzo, A., Lucarini, M., Santini, A., Souto, E. B., Novellino, E., Antolak, H., Azzini, E., Setzer, W. N., & Martins, N. (2019). The Therapeutic Potential of Apigenin. *International Journal of Molecular Sciences*, *20*(6), 1305. https://doi.org/10.3390/ijms20061305

Santana-Gálvez, J., & Jacobo-Velázquez, D. A. (2018). Classification of phenolic compounds. In *Phenolic Compounds in Food* (pp. 3–20). CRC Press.

Šantić, Ž., Pravdić, N., Bevanda, M., & Galić, K. (2017). The historical use of medicinal plants in traditional and scientific medicine. *Psychiatria Danubina*, *29*(Suppl 4), 787–792.

Santos, W. N. L. D., & Magalhães, B. E. A. D. (2020). Phenolic content and antioxidant capacity of infusions herbs: Optimization of phenolic extraction and HPLC-DAD method. *Anais da Academia Brasileira de Ciências*, *92*(3). https://doi.org/10.1590/0001-3765202020190646

Sarikahya, B. N., Goren, A. C., & Kirmizigul, S. (2019). Simultaneous determination of several flavonoids and phenolic compounds in nineteen different *Cephalaria* species by HPLC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, *173*, 120–125. https://doi.org/10.1016/j.jpba.2019.05.019

Seelinger, G., Merfort, I., & Schempp, C. (2008). Anti-Oxidant, Anti-Inflammatory and Anti-Allergic Activities of Luteolin. *Planta Medica*, *74*(14), 1667–1677. https://doi.org/10.1055/s-0028-1088314

Sen, T., & Samanta, S. K. (2014). Medicinal Plants, Human Health and Biodiversity: A Broad Review. In *Biotechnological Applications of Biodiversity* (pp. 59–110). Springer Berlin Heidelberg. https://doi.org/10.1007/10_2014_273

Shabir, G. A., John Lough, W., Arain, S. A., & Bradshaw, T. K. (2007). Evaluation and Application of Best Practice in Analytical Method Validation. *Journal of Liquid Chromatography & Related Technologies*, *30*(3), 311–333. https://doi.org/10.1080/10826070601084753

Singh, R. (2013). HPLC method development and validation-an overview [Article]. *Journal of Pharmaceutical Education & Research*, *4*(1), 26–33. https://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=88925378&lang=pt-pt&site=ehost-live&scope=site

Skarupova, D., Vostalova, J., & Rajnochova Svobodova, A. (2020). Ultraviolet A protective potential of plant extracts and phytochemicals. *Biomedical Papers*, *164*(1), 1–22. https://doi.org/10.5507/bp.2020.010

Skendi, A., Irakli, M., & Chatzopoulou, P. (2017). Analysis of phenolic compounds in Greek plants of Lamiaceae family by HPLC. *Journal of Applied Research on Medicinal and Aromatic Plants*, *6*, 62–69. https://doi.org/10.1016/j.jarmap.2017.02.001

Sociedade Portuguesa de Botânica. (s.d.-a). *Melilotus indicus*. Retrieved 20/06/2021 from https://flora-on.pt/index.php#/1*melilotus+indicus*

Sociedade Portuguesa de Botânica. (s.d.-b). *Pterospartum tridentatum*. Retrieved 20/06/2021 from https://flora-on.pt/?q=Pterospartum

Stevens, Y., Rymenant, E. V., Grootaert, C., Camp, J. V., Possemiers, S., Masclee, A., & Jonkers, D. (2019). The Intestinal Fate of Citrus Flavanones and Their Effects on Gastrointestinal Health. *Nutrients*, *11*(7), 1464. https://doi.org/10.3390/nu11071464

Takó, M., Kerekes, E. B., Zambrano, C., Kotogán, A., Papp, T., Krisch, J., & Vágvölgyi, C. (2020). Plant Phenolics and Phenolic-Enriched Extracts as Antimicrobial Agents against Food-Contaminating Microorganisms. *Antioxidants*, *9*(2), 165. https://doi.org/10.3390/antiox9020165

Talavera, S., Aedo, C., Castroviejo, S., Herrero, A., Zarco, C. R., Salgueiro, F. J., & Velayos, M. (2000). *Flora iberica* (Vol. VII (II) Leguminosae (partim)). Real Jardin Botanico, C.S.I.C.

Tatsimo, S. J. N., Tamokou, J. D. D., Havyarimana, L., Csupor, D., Forgo, P., Hohmann, J., Kuiate, J.-R., & Tane, P. (2012). Antimicrobial and antioxidant activity of kaempferol rhamnoside derivatives from Bryophyllum pinnatum. *BMC Research Notes*, *5*(1), 158. https://doi.org/10.1186/1756-0500-5-158

Thangavel, P., Puga-Olguín, A., Rodríguez-Landa, J. F., & Zepeda, R. C. (2019). Genistein as Potential Therapeutic Candidate for Menopausal Symptoms and Other Related Diseases. *Molecules*, *24*(21), 3892. https://doi.org/10.3390/molecules24213892

Tilburt, J. (2008). Herbal medicine research and global health: an ethical analysis. *Bulletin of the World Health Organization*, *86*(8), 594–599. https://doi.org/10.2471/blt.07.042820

Tuenter, E., Creylman, J., Verheyen, G., Pieters, L., & Van Miert, S. (2020). Development of a classification model for the antigenotoxic activity of flavonoids. *Bioorganic Chemistry*, *98*, 103705. https://doi.org/10.1016/j.bioorg.2020.103705

Ulusoy, H. G., & Sanlier, N. (2020). A minireview of quercetin: from its metabolism to possible mechanisms of its biological activities. *Critical Reviews in Food Science and Nutrition*, *60*(19), 3290–3303. https://doi.org/10.1080/10408398.2019.1683810

Vitor, R. F., Mota-Filipe, H., Teixeira, G., Borges, C., Rodrigues, A. I., Teixeira, A., & Paulo, A. (2004). Flavonoids of an extract of *Pterospartum tridentatum* showing endothelial protection against oxidative injury. *Journal of Ethnopharmacology*, *93*(2), 363–370. https://doi.org/10.1016/j.jep.2004.04.003

Vu, D. C., Vo, P. H., Coggeshall, M. V., & Lin, C.-H. (2018). Identification and Characterization of Phenolic Compounds in Black Walnut Kernels. *Journal of Agricultural and Food Chemistry*, *66*(17), 4503–4511. https://doi.org/10.1021/acs.jafc.8b01181

Waters Corporation. (s.d.). *How Does High Performance Liquid Chromatography Work?* Retrieved 28/11/2021 from https://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_US

WHO. (2021). Key technical issues of herbal medicines with reference to interaction with other medicines. 70. file:///C:/Users/josep/Downloads/9789240019140-eng.pdf

Yan, Z., Wu, F., Luo, K., Zhao, Y., Yan, Q., Zhang, Y., Wang, Y., & Zhang, J. (2017). Cross-species transferability of EST-SSR markers developed from the transcriptome of *Melilotus* and their application to population genetics research. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-18049-8

Zhu, F., Du, B., & Xu, B. (2018). Anti-inflammatory effects of phytochemicals from fruits, vegetables, and food legumes: A review. *Critical Reviews in Food Science and Nutrition*, *58*(8), 1260–1270. https://doi.org/10.1080/10408398.2016.1251390

Annexes

Annex 1 – Accuracy and precision for all concentrations of chlorogenic and caffeic acids and hesperidin.

Table 4.1 – Precision and accuracy of the analytical method for chlorogenic acid, caffeic acid and hesperidin, for all concentration.

Compound	Linear range (µg.mL^{-1})	Concentration (µg.mL^{-1})	Precision (%)	Accuracy (%)
CLA	2.98–47.61	47.61	2.86	100.49
		23.81	0.59	97.50
		11.90	0.58	97.66
		5.95	0.60	100.11
		2.98	0.72	112.72
	0.37–5.95	5.95	0.68	100.08
		2,98	0.93	100.05
		1.49	1.33	98.09
		0.74	2.16	99.61
		0.37	2.99	103.23
CA	2.98–47.61	47.61	2.71	100.22
		23.81	0.12	98.59
		11.90	0.23	99.68
		5.95	0.57	98.71
		2.98	0.18	105.23
	0.19–2.98	2,98	0.20	100.16
		1.49	0.56	98.76
		0.74	0.93	99.08
		0.37	1.16	101.33
		0.19	0.85	103.25
HESP	2.98–47.61	47.61	2.88	100.54
		23.81	0.87	97.30
		11.90	1.53	97.27
		5.95	5.87	100.41
		2.98	6.02	114.07

Annex 2 – Chromatograms of the mixed standard solution at 320 and 360 nm.

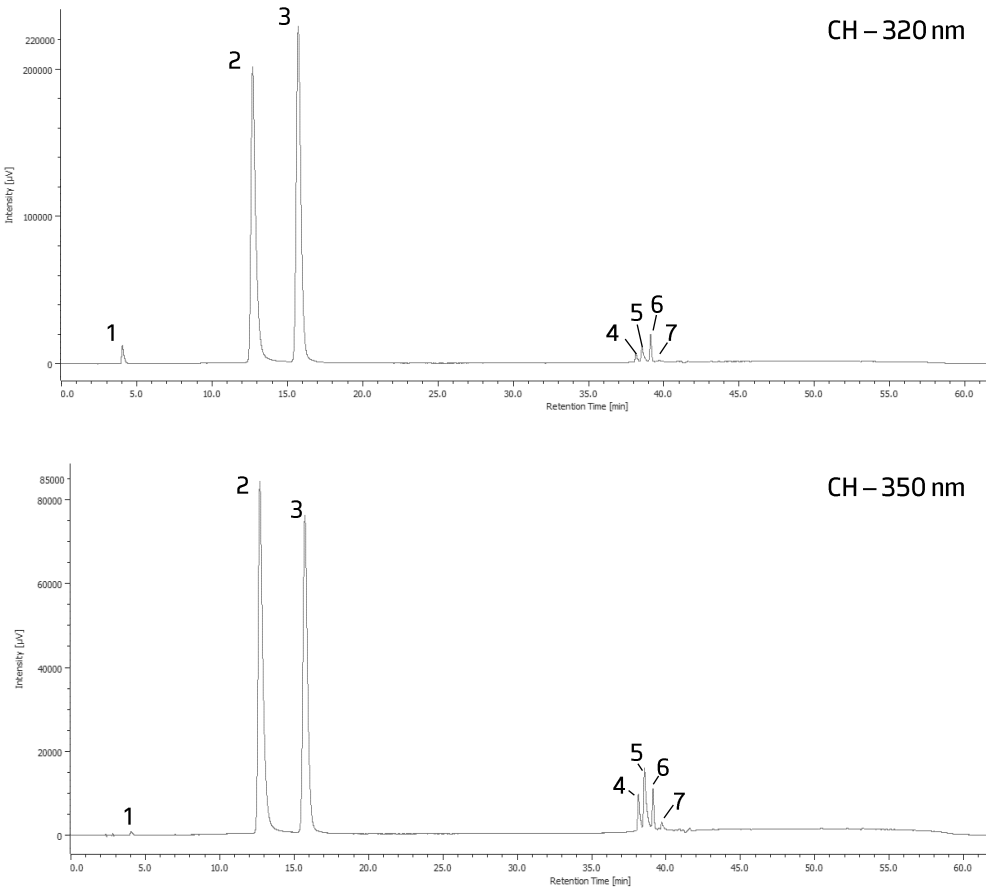


Figure 4.1 – Chromatograms of the mixed standard solution at 320 and 360 nm.
1 – GA; 2 – CLA; 3 – CA; 4 – RUT; 5 – Q3G; 6 – NAR; 7 – HESP; CH – Wavelength.

Annex 3 – Chromatograms of *Melilotus indicus* and *Pterospartum tridentatum* in different wavelengths.

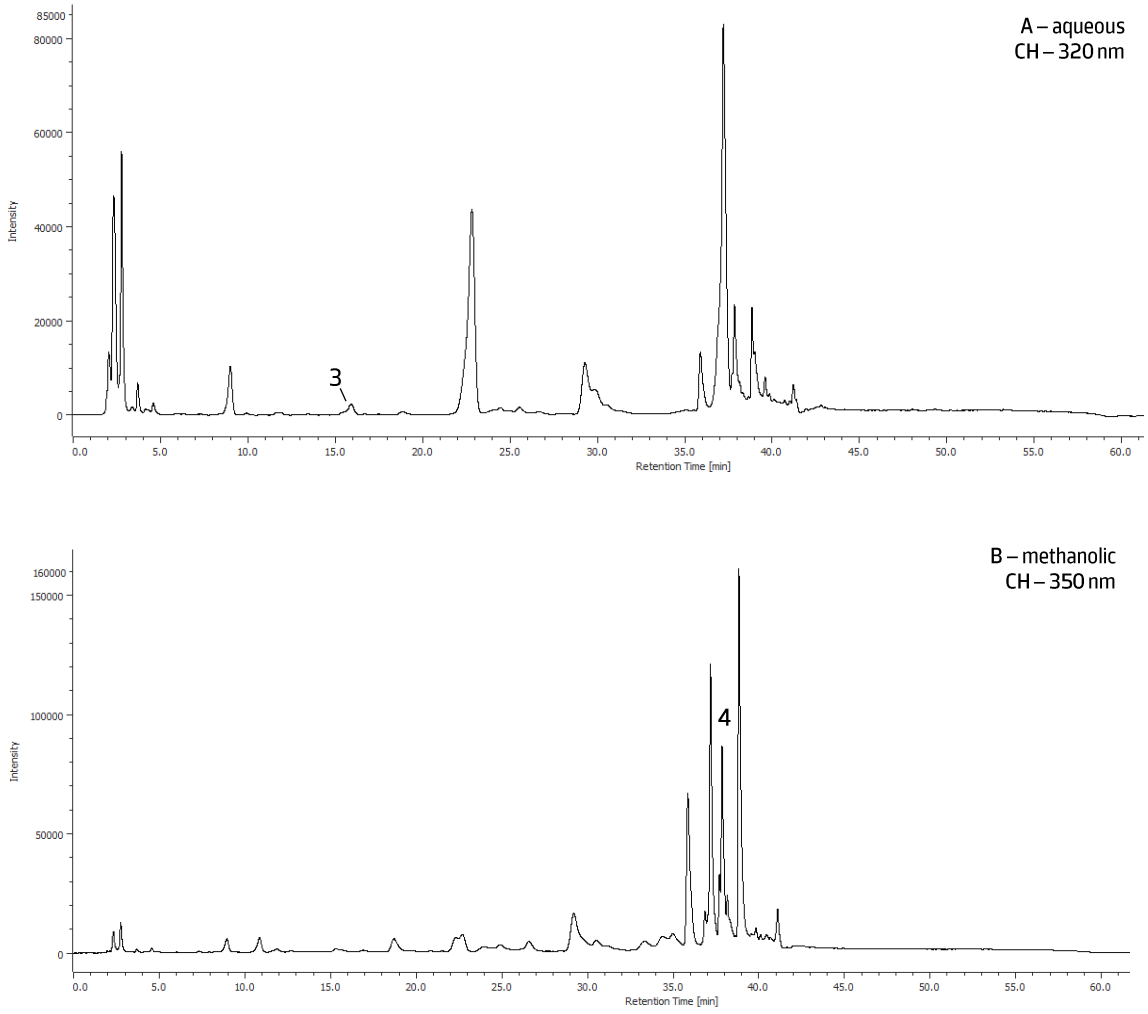
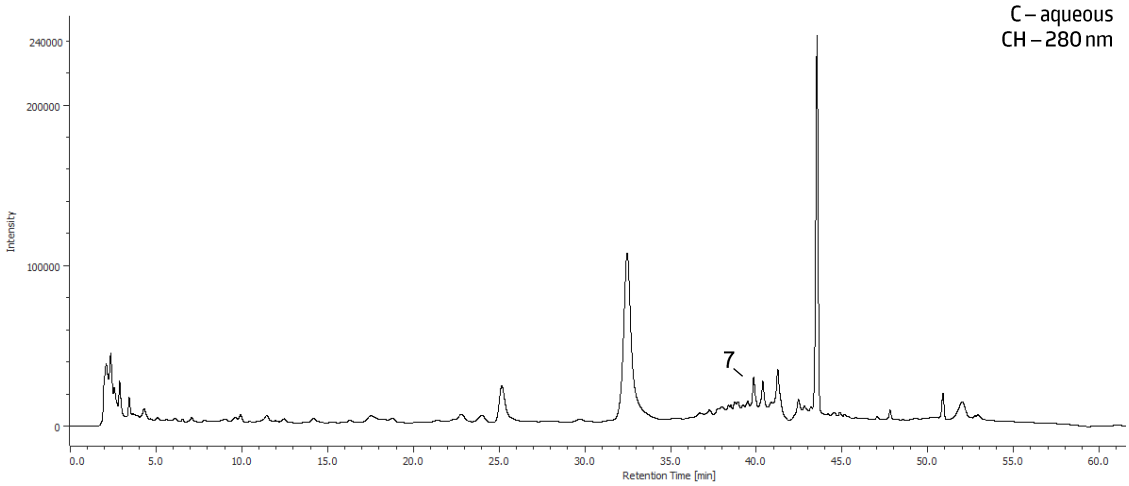


Figure ~ 4.2 – Chromatograms of *Melilotus indicus* (A) aqueous at 320 nm and (B) methanolic at 350 nm.
2 – CLA; 3 – CA; 4 – RUT; CH – Wavelength.



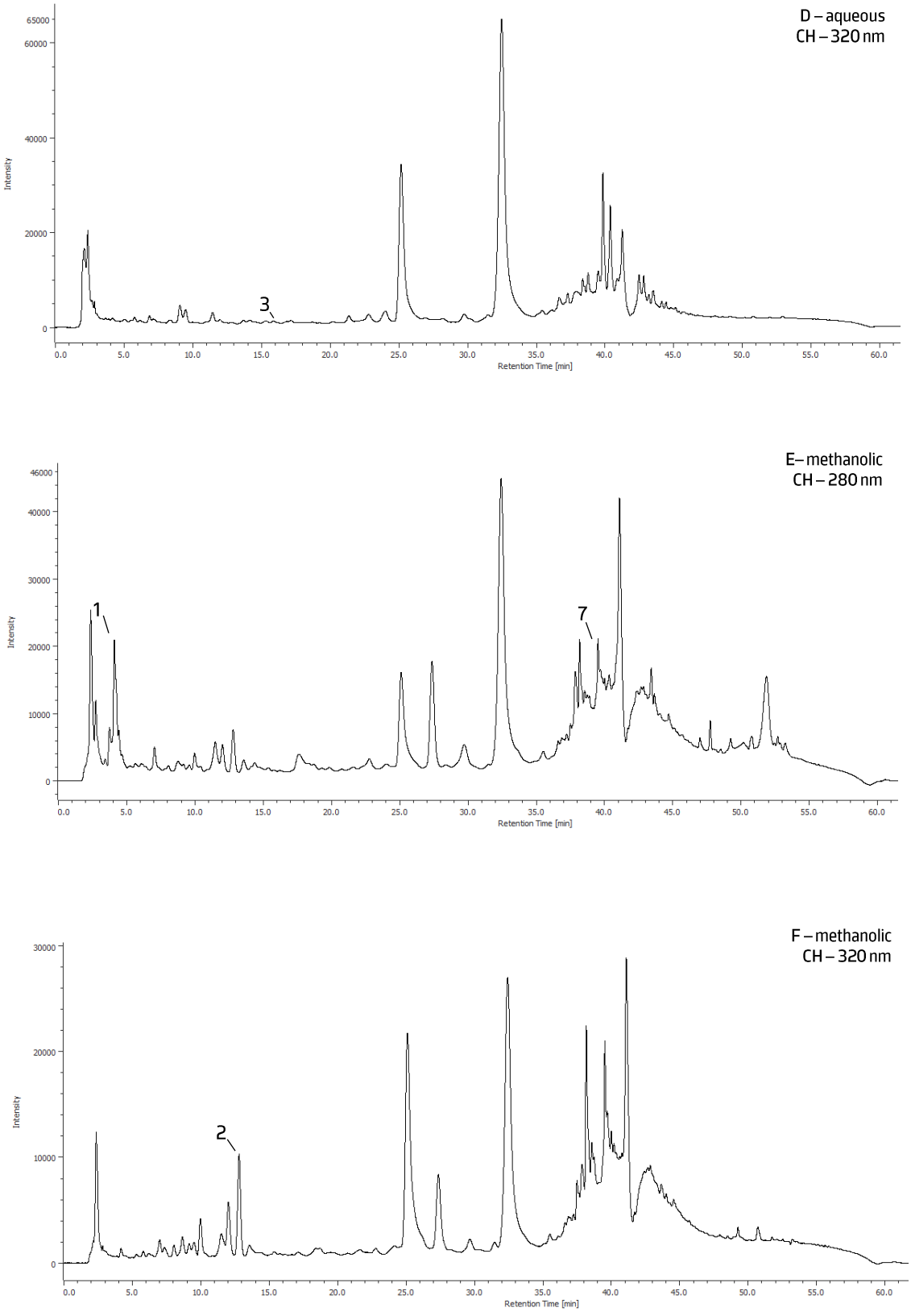


Figure 4.3 - Chromatograms of *Pterospartum tridentatum* (C) aqueous at 280 nm, (D) aqueous at 320 nm, (E) methanolic at 280 nm and (F) methanolic at 320 nm.
1 – GA; 2 – CLA; 3 – CA; 7 – HESP; CH – Wavelength.