UNIVERSITY OF TRÁS-OS-MONTES AND ALTO DOURO

Chemical Characterization and Bioactivity of Aqueous and Organic Extracts of *Echinacea purpurea* (L.) Moench

THESIS OF MASTER OF SCIENCE IN BIOCHEMISTRY

JOANA FILIPA PEREIRA COELHO

Supervisor: *Prof. Doutor Paulo Fernando da Conceição Santos*

Co-Supervisor: *Prof. Doutora Isabel Cristina Fernandes Rodrigues Ferreira*

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Vila Real, 2019

Declaration

I, Joana Filipa Pereira Coelho, declare, on my honour, that this work is original and that all non-original contributions were duly referenced with source identification.

Vila Real, 18 de March de 2019

(Joana Filipa Pereira Coelho)

"Failure is so important. We speak about success all the time. It is the ability to resist failure or use failure that often leads to greater success. I've met people who don't want to try for fear of failing."

J.K. Rowling

Agradecimentos

Chegar ao fim desta etapa é uma grande recompensa por todo o esforço, trabalho e dedicação durante estes dois anos de mestrado. Mas se consegui chegar aqui foi graças a muitas pessoas importantes que direta ou indiretamente me ajudaram a atingir os meus objetivos, sendo com trabalho académico, sendo com apoio emocional, devo agradecer-lhes por tudo.

Começo desde já a agradecer ao meu orientador, Paulo Santos, pela oportunidade de trabalhar neste projeto, por toda a paciência que tinha para comigo sempre que surgiam as dúvidas e os obstáculos. Nada disto seria possível sem a sua ajuda, generosidade e os seus conselhos ao longo deste ano.

Agradeço também à minha coorientadora e diretora do CIMO (Centro de Investigação de Montanha), Isabel Ferreira, pela oportunidade trabalhar consigo e o seu grupo de investigação pelo qual eu tenho uma grande admiração. Agradeço também à Lilian Barros, Inês Dias, ao Ricardo Calhelha e à Tânia Pires por toda a ajuda e orientação no meu percurso do projeto no CIMO.

Expresso também a minha gratidão ao Cantinho das Aromáticas pelo fornecimento do material de estudo desta dissertação.

Não posso deixar de agradecer aos meus colegas de laboratório, especialmente à Céu Sousa e Fátima Correia, que me ajudaram sempre em momentos de aflição tentando sempre me ajudarem com dúvidas de última hora.

Agradeço também, com muito carinho, aos meus amigos Raquel Vieira, Pedro Barbosa e Sílvia Lemos antes de mais pela ajuda na integração neste mestrado, sem vocês eu ainda andaria à deriva, por todo o apoio e por me aturarem em tempos de desespero e ansiedade. A nossa jornada começou no início do mestrado e neste tempo passamos vários momentos de diversão, de estudo intensivo, desespero e troca de sabedoria. Obrigada por me terem acolhido.

À Joana Gonçalves e Diana Teixeira pela amizade ao longo de quase já uma década, todo o vosso carinho e compreensão foi essencial para mim, mesmo longe conseguem estar bastante perto.

A família também foi uma grande ajuda por me apoiarem, deram a oportunidade de expandir os meus horizontes e pelos melhores conselhos possíveis. Obrigada do fundo do coração.

Por fim, mas não menos importante, agradeço ao meu namorado, Miguel Costa. Obrigada por todo o teu carinho e dedicação, por nunca me deixares desistir de nada em tempos de

desespero, por me ajudares a ultrapassar todos os meus momentos de angustia e ansiedade. Obrigada por estares lá para ouvir todos os meus desabafos e por me aconselhares sobre as minhas dúvidas e inquietações.

A todos um grande e sincero OBRIGADA!

Abstract

Echinacea purpurea (L.) Moench is known for its medicinal properties such as anti-inflammatory, antioxidant, antimicrobial, antiviral and cytotoxic, and it is the most known and used medicinal plan for its stimulating properties. *E. purpurea* can be used in infusions, tinctures or capsule forms and it is available on the market. The information about the cytotoxic activity of *E. purpurea* is scarce and this study was designed essentially to assesses the anticancer and antimicrobial properties of the plant.

Five organic extracts were obtained by sequential extraction with different solvents and two aqueous extracts (decoction and infusion) of *E. purpurea* and it was determined and quantified their phenolic composition. The antimicrobial activity of the extracts was assessed against *Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis, Listeria monocytogenes*, MRSA, MSSA and *Candida albicans*. The cytotoxicity activity was tested against several human cancer cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cells lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Additionally, the hepatotoxicity was tested against a non-tumor porcine liver primary cell line (PLP2).

Concerning the phenolic composition, the methanol extract was the only organic extract that was rich in phenolic acids and flavonoids, both aqueous extracts possessing a much bigger content of phenolic acids than flavonoids.

In what respects antimicrobial activity, the dichloromethane, ethyl acetate and acetone extracts showed moderate activity towards *Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis, Listeria monocytogenes*, MRSA, MSSA and *Candida albicans*.

The dichloromethane and *n*-hexane extracts were the ones that stood out with great cytotoxicity against the tumor cell lines, but these extracts also showed some toxicity towards the PLP2 cell line.

As the cytotoxic results were promising for the *n*-hexane and dichloromethane extracts, both extracts were fractionated by gradient elution column chromatography and their cytotoxicity as well as their hepatotoxicity were evaluated. With the fractionation of both extracts, fourteen fractions of the *n*-hexane extract and fifteen fractions of dichloromethane extract were obtained and their cytotoxicity as well as their hepatotoxicity was evaluated. All *n*-hexane fractions showed cytotoxic properties against HepG2 and only the last fraction of *n*-

hexane extract didn't show cytotoxicity towards NCI H460, HeLa and MCF-7. Five dichloromethane fractions didn't show cytotoxicity against all tumour cell lines and only two fractions showed toxicity towards primary cell line of pork liver.

In general, the cytotoxicity of the extracts was superior to that of the obtained fractions.

Moreover, the phenolic compounds do not seem to contribute to the cytotoxic properties of *n*-hexane and dichloromethane extracts since these compounds were not detected.

Keywords: *Echinacea purpurea* (L.) Moench; antimicrobial activity; cytotoxicity; phenolic compounds;

Resumo

Echinacea purpurea (L.) Moench é conhecida pelas suas propriedades medicinais, tais como, atividade anti-inflamatória, antioxidante, antimicrobiana, antiviral e citotóxica, e é a planta medicinal mais conhecida e usada para a estimulação do sistema imunitário. Ela está disponivel no mercado e pode ser usada sob a forma de infusões, tinturas ou capsulas. É escassa a informação sobre a atividade citotóxica da *E. purpurea* e este estudo foi projetado essencialmente para avaliar as propriedades anticancerígenas e antimicrobianas da planta.

Cinco extractos organicos foram obtidos por extração sequencial com diferentes solventes e dois extractos aquosos (decocção e infusão) de *E. purpurea* e foi determinada e quantificada a sua composição fenólica. A atividade antimicrobiana dos extractos foi avaliada em *Escherichia coli; Klebsiella pneumoniae; Morganella morganii; Proteus mirabilis; Pseudomonas aeruginosa; Enterococcus faecalis; Listeria monocytogenes*; MRSA; MSSA e *Candida albicans*. A atividade citotóxica foi testada em várias células tumorais humanas: MCF-7 (adenocarcinoma da mama), NCI-H460 (cancro do pulmão), HeLa (carcinoma do cérvix) e HepG2 (carcinoma hepatocelular). Adicionalmente, a hepatotoxicidade foi testada numa linha celular primária não tumoral de fígado de porco (PLP2).

No que diz respeito à composição fenólica, o extrato de metanol foi o único extrato rico em ácido fenólicos e flavonoides, os extratos aquosos possuem um maior conteúdo de ácidos fenólicos do que de flavonoides.

A respeito da atividade antimicrobiana, os extratos de diclorometano, acetato de etilo e acetona mostraram uma atividade moderada perante *Escherichia coli; Klebsiella pneumoniae; Morganella morganii; Proteus mirabilis; Pseudomonas aeruginosa; Enterococcus faecalis; Listeria monocytogenes*; MRSA; MSSA and *Candida albicans*.

Os extratos de diclorometano e *n*-hexano foram os únicos que se destacaram com uma ótima citotoxicidade nas linhas celulares tumorais, mas estes extratos também mostraram alguma toxicidade perante a linha celular PLP2.

Como os resultados da citotoxicidade foram promissores para os extratos de *n*-hexano e diclorometano, ambos foram fracionados por cromatografia em coluna de eluição em gradiente e a sua citotoxicidade assim como a sua hepatotoxicidade foi avaliada. Com o fracionamento de ambos os extratos, foram obtidas catorze frações do extrato de *n*-hexano e quinze do extrato de diclorometano e foi avaliada a sua citotoxicidade assim como a sua hepatotoxicidade. Todas as frações do extrato de *n*-hexano mostraram propriedades citotóxicas na linha celular HepG2

e só a última fração não mostrou possuir atividade citotóxica perante as linhas celulares NCI H460, HeLa e MCF-7. Cinco frações do extrato de diclorometano não mostraram possuir citotoxicidade e só duas frações mostraram toxicidade perante a linha primária.

No geral, a citotoxicidade dos extratos foi superior do que a citotoxicidade obtida nas frações.

Além disso, os compostos fenólicos parecem não contribuir para as propriedades citotóxicas dos extratos de *n*-hexano e diclorometano uma vez que não foram detetados compostos.

Palavras-chave: *Echinacea purpurea* (L.) Moench; atividade antimicrobiana; atividade citotóxica; composição fenólica;

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List of abbreviations

AA – Ascorbic acid

AAE – Ascorbic acid equivalents

CA – Columbia agar

CFU – Colony forming unit

DAD – Diode array detector

DCM - Dichloromethane

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

dw – Dry weight

EC_{50} – Concentration at which 50% of radical scavenging occurred

EtOAc – Ethyl acetate

FBS – Fetal Bovine Serum

GI_{50} – Half maximal inhibitory growth

HBSS – Hank's Balanced Salt Solution

HeLa – Cervical carcinoma

HepG2 – Hepatocellular carcinoma

HPLC – High performance liquid chromatography

IC_{50} – Half maximal inhibitory concentration

INT – *p*-iodonitrotetrazolium chloride

MBC – Minimum bactericidal concentration

MCF-7 – Breast adenocarcinoma

MeOH – Methanol

MIC – Minimum inhibitory concentration

MRSA – Methicillin-resistant *Staphylococcus aureus*

MS – Mass spectrometer

MSSA – Methicillin-sensitive *Staphylococcus aureus*

NCI-H460 – Non-small cell lung cancer

NK – Natural killer

rpm – Revolutions per minute

RPMI – Roswell Park Memorial Institute

R_t – Retention time

SRB – Sulforhodamine B

TCA – Trichloroacetic acid

TLC – Thin layer chromatography

u - Unified atomic mass unit

UV-Vis – Ultraviolet–visible

WCB – Wilkins-Chalgren broth

CHAPTER I

Introduction

In this chapter it is made an introduction about medicinal plants and their importance in medicine, a brief botanical description of *Echinacea purpurea* (L.) Moench and its traditional uses, biological properties and phytochemicals found in the plant.

1.1. Medicinal plants

Herbal medicines are becoming increasingly more atractive due to the side effects of certain drugs, which encouraged people to look for more natural forms of treatment, and because the efficacy of convencional medicines is decaying once infectious organisms had developed resistance to synthesized drugs over the years (Thomford et al., 2015). Since ever, humans have trusted on plants to treat all kinds of illnesses from coughs or colds to tuberculosis or malaria (Chevallier, 1996).

A diversity of plants can be used as a tool for disease prevention and, traditionally, they have been used for ethnomedicinal applications such as insect and animal bites, skin eruptions (Miller, 2012) , coughs, sore throat, chest conditions and thrush (Hudson *et al*., 2005). Plants can be used fresh, dried, whole or chopped and they can be used by being added to meals and as herbal infusions (Pereira *et al.*, 2015). The World Health Organization estimated that 80% of people in the world trust in herbal medicines for the treatment of various diseases (Palhares et al., 2015). Close to 30% of the pharmaceutical market and 11% of basic drugs (considered drugs intended to primary cares) derive from plants (Martins, 2013).

Plants produce thousands of bioactive compounds like vitamins, antioxidants and dietary fibers and these have shown a variety of health benefits (Sultan *et al.*, 2014). A diet rich in fruits and vegetables can protect us against cardiovascular diseases and cancer (Aune et al., 2017).

1.2. Botanical description and traditional uses of *Echinacea purpurea* (L.) Moench

Echinacea purpurea (L.) Moench (**Figure 1**) is a perennial plant native from eastern North American and it belongs to the Asteraceae family (Chicca *et al*., 2007). *E. purpurea* is cultivated in the United States, Canada and Europe, not only for its beauty but also for its reported medicinal properties (Barrett, 2003). It is the most known and used medicinal plant and it has been used for a variety of treatments such as snake bites, syphilis, wound infections (Barnes *et al*., 2005), common colds (Goel *et al*., 2005), sore throat (Hudson *et al*., 2005),

toothache, bowel pain, rabies, tonsillitis, seizures, septic conditions and cancer (Grimm and Müller, 1999).

Figure 1 – *Echinacea purpurea* (L.) Moench (Source: https://jb.utad.pt/especie/Echinacea_purpurea).

E. purpurea can be used in infusions, tinctures or capsule forms and it is available on the market (standardized preparations) as water or ethanol solutions (fluid forms) or capsules (containing dried *E. purpurea*). These products are used to stimulate the immune system and can contain only parts of the plant such as roots, stems and floral parts or the whole plant (Miller, 2012; Tsai *et al*., 2012). These standardized preparations have antiviral, immunomodulatory and antimicrobial activities (Hudson, 2012). Extracts and products from the whole plant embrace the largest sector of herbal medicine market in Europe (Barrett, 2003). It is very common for patients in phase I of chemotherapy trials to ingest *E. purpurea* preparations due to its immunostimulatory effect (Chicca *et al*., 2007).

E. purpurea is a safe herbal medicine (Barrett, 2003) and its extracts are used mostly in a self-medication manner (Goel *et al*., 2005) and the effects can depend on the host conditions (Zhai *et al*., 2007). There are reports about allergic reactions to *E. purpurea*, but no deaths were verified (Barrett, 2003). Echinacin® (*E. purpurea* juice), a standardized preparation of the plant, is well tolerated when administrated by oral or parenteral via (Parnham, 1996). *E. purpurea* is contraindicated in patients with tuberculosis, leukaemia, leukaemia-like diseases, collagen disorders, multiple sclerosis and other autoimmune diseases because of its potential for stimulating autoimmune processes (Barnes *et al.*, 2005).

There are several studies about the pharmacological activities of *E. purpurea* and these are diversified based on which part of the plant is used, like aerial parts or roots. The combination of various activities of this plant has can led to an improvement of the symptoms of infections (Hudson, 2012). According to Sultan *et al* (2014)*., E. purpurea* "could lead to life

span prolongation greater than immunization alone". These pharmacological activities such as anti-inflammatory, antioxidant, antiviral, cytotoxic (Yildiz *et al*., 2014) and antimicrobial (Hudson, 2012) are originated by its phytochemical compounds (Pleschka *et al*., 2009).

1.3. Biological activities of *E. purpurea*

1.3.1. Anti-inflammatory activity

E. purpurea can stimulate the immune system raising body resistance to bacterial and viral infections, and its extracts are mostly used for prevention and treatment of infections of the in upper respiratory tract.

The most frequently reported pharmacological activity of *E. purpurea* is the activation of macrophages. *E. purpurea* stimulates macrophages and neutrophils to produce anti-inflammatory cytokines and free radicals and it also stimulates natural killer cells (these also are important in the defence of cancer immunosurveillance) (Chevallier, 1996; Chicca *et al.*, 2007; Miller, 2005; Wen *et al.*, 2012; Zhai *et al.*, 2007).

Root extracts of *E. purpurea* showed potential anti-inflammatory activity inhibiting 5-lipoxygenases and displaying an IC_{50} value of 0.642 μg/mL (Barnes *et al.*, 2005; Merali *et al.*, 2003).

Ethanol extracts of the roots and aerial parts of *E. purpurea* inhibited fibroblast-induced collagen contraction and have potential anti-inflammatory activity, but this was most evident *in vitro* than *in vivo* (Barnes *et al*., 2005). Administration of oral doses of *E. purpurea* inhibited carrageenan-paw oedema induced in mice (Zhai *et al*., 2007). Vimalanathan *et al*. (2009) studied several organic and aqueous *E. purpurea* extracts derived from roots, leaves, stems and flowers and observed that they reduced the levels of pro-inflammatory cytokines, the extract with best results being the aqueous extract.

1.3.2. Antimicrobial activity

Some *E. purpurea* preparations or extracts possess selective antibacterial activity against candidiasis, pathogenic respiratory bacteria and *staphylococcus* and also possess antifungal activity against some fungi causing dermatophytosis (Hudson, 2012; Pleschka *et al*., 2009).

Table 1 describes some of the microbial species that are susceptible to *E. purpurea*. In some of the studies evaluating the antimicrobial activity, *E. purpurea* showed activity but for *Legionella pneumophila*, *Candida parapsilosis* (strain: ATCC 22019 – reference) and *Staphylococcus aureus E. purpurea* showed almost no activity and for: *Candida albicans* (strains: ATCC 90028 - reference; 575541 - urinary; 557834 - vaginal and 558234 - vaginal), *Candida glabrata* (strains: ATCC 2001 - reference; D1 - oral and 513100 - urinary), *Candida parapsilosis* (strains: AM2 - oral; AD - oral; 491861 - vaginal and 513143 - vaginal) and . *Candida tropicalis* (strains: ATCC 750 - reference; AG1 - oral; 75 - vaginal; 12 - vaginal; 544123 - urinary; 519468 - urinary and T2.2 - oral) *E. purpurea* showed an absence of activity.

Table 1 - Antimicrobial activity of *E. purpurea* against several microbial species.

Plant material	Extract	Microbial species	Activity	References
Roots	EtOH (40%)	*Candida albicans*	X	Sharma *et al.*, 2008
Aerial parts	MeOH/H2O (80:20 v/v)	*Candida albicans* (strains: ATCC 90028; 575541; 557834 and 558234)	X	Martins *et al.*, 2015
Aerial parts	MeOH/H2O (80:20 v/v)	*Candida glabrata* (strains: ATCC 2001; D1 and 513100)	X	Martins *et al.*, 2015
Aerial parts	MeOH/H2O (80:20 v/v)	*Candida tropicalis* (strains: ATCC 750; AG1; 75; 12; 544123; 519468 and T2.2)	X	Martins *et al.*, 2015
Roots	*n*-hexane	*Saccharomyces cerevisiae*	+	Barnes *et al.*, 2005
Roots	EtOH (40%)	*Haemophilus influenzae*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Legionella pneumophila*	+/-	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Klebsiella pneumuniae*	++	Sharma *et al.*, 2008
Roots/Aerial parts	EtOH (40/65%)	*Propionibacterium acne*	++	Sharma *et al.*, 2008, Sharma *et al.*, 2011
Roots	EtOH (40%)	*Mycobacterium smegmatis*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	C*lostridium difficile*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Acinetobacter baumanii*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Bacillus cereus*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Bacillus subtilis*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Enterococcus faecalis*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Escherichia coli*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Pseudomonas aeruginosa*	++	Sharma *et al.*, 2008
Aerial parts	MeOH/H2O (80:20 v/v)	*Candida parapsilosis* (strain: ATCC 22019)	+/-	Martins *et al.*, 2015
Aerial parts	MeOH/H2O (80:20 v/v)	*Candida parapsilosis* (strains: AM2; AD; 491861 and 513143)	X	Martins *et al.*, 2015
Roots	EtOH (40%)	*Typanosoma brucei*	++	Sharma *et al.*, 2008
Roots	EtOH (40%)	*Trichoderma viride*	++	Sharma *et al.*, 2008

Notes: ++: intense activity; +: normal activity; +/-: negligible activity; X: absence of activity

1.3.3. Antiviral activity

A variety of virus are susceptible to *E. purpurea* extracts and their antiviral effect is manifested in an early stage of infection.

Standardized preparations of *E. purpurea* were shown to possess antiviral activity against influenza virus type IV (H3N2) and other membrane containing viruses (Hudson, 2012; Pleschka *et al.*, 2009). These preparations also showed reverse of pro-inflammatory virus-induced responses in epithelial cells.

Ethanol and EtOAc extracts of *E. purpurea* have antiviral activity against herpes simplex virus (HSV) and influenza virus (Hudson *et al.*, 2005; Pleschka *et al.*, 2009).

Echinaforce® (standardized *E. purpurea* extract) has antiviral activity against influenza A virus subtypes H3N2, H1N1 (PR8), H5N1, H7N7 and H1N1 S-OIV (swine origin) only with direct contact and against respiratory syncytial virus (Hudson, 2012).

A water-soluble antiviral compound present in the roots of *E. purpurea* was active against HSV and influenza virus but it wasn't active against rhinovirus of common cold (Hudson *et al.*, 2005).

Orally administered *E. purpurea* extracts to mice infected with influenza A virus were shown to significantly increase the survival rate (Bodinet *et al.*, 2002).

Sharma *et al*. (2009) studied a standardized preparation of *E. purpurea* which showed an impressive antiviral activity against HSV and influenza virus with MIC_{100} (concentration leading to complete prevention of cytopathic effect) values of 0.39 and 0.58 µg/mL, respectively. Vimalanathan *et al*., 2005 reported for HSV and influenza virus a MIC_{100} value of 1.5 µg/mL from a *E. purpurea* EtOAc extract and a value of 5.8 µg/mL with a 70% ethanol extract.

1.3.4. Antioxidant activity

The antioxidant system plays a very important role in our body's defence by protecting it from oxidative stress with elimination of free radicals (Baek *et al.*, 2016). Supplementation with *E. purpurea* can restore the normal redox status helping in infections. Usually, *E. purpurea* extracts showed lower activity compared to other medicinal plants.

Alcoholic extracts from *E. purpurea* roots and leaves have free radical scavenging activity (Barnes *et al.*, 2005; Hudson, 2012; Stanisavljević *et al.*, 2009). Pellati *et al*. (2004) evaluated the antioxidant activity of *E. purpurea*'s capsules and tablets as well as the methanolic extract of the plant (described in Table 2).

Pires *et al*. (2016) also studied the antioxidant activity of *E. purpurea* and Echinacea tablets and syrup. For the evaluation of the antioxidant activity in this study were used a hydroethanolic extract, an infusion and a decoction from fresh and dried *E. purpurea*.

Table 2 describes some of the methods used to evaluate the antioxidant activity of *E. purpurea* and their results.

Table 2 - Antioxidant activity of *E. purpurea* extracts.

Plant material	Method	Extract	Results	References
Aerial parts and inflorescences	DPPH	Infusion	2.41 ± 0.22 (mg/mL)	Pires *et al.*, 2016
		Decoction	1.43 ± 0.03 (mg/mL)	
		Hydroethanolic (80/20)	0.76 ± 0.02 (mg/mL)	
Roots		Methanolic	134 ± 0.7 (µg/mL)	Pellati *et al.*, 2004
Aerial parts		Ultrasound (Hydroethanolic 70/30)	34.16 ± 0.65 (mg/mL)	Stanisavljević *et al.*, 2009
		Hydroethanolic (70/30)	65.48 ± 1.12 (mg/mL)	
Roots	ABTS	$CHCl_3$	3.2 ± 0.2 (mg/mL)	Hu and Kitts, 2000
		CH_3OH	11.0 ± 0.1 (mg/mL)	

Results for DPPH method are shown as EC_{50} and for the ABTS method as AAE. AA was used as reference compound (3.48 ± 0.07 mg/mL).

1.3.5. Cytotoxicity

Until 2007 there was no available data on the effect of *E. purpurea* on human cancer cells. Chicca *et al*. (2007) were the first to study the activity of *E. purpurea* in human cancer cells: MIA PaCa-2 (human pancreatic cancer) and COLO320 (colon cancer) cell lines. *E. purpurea* showed a better activity against COLO320.

Aqueous ethanol extracts of flowers of *E. purpurea* also showed some cell growth inhibition in CaCo-2 (human colon adenocarcinoma) and HCT-116 (human colon cancer) cell cultures (Tsai *et al.*, 2012).

Several extracts have been assessed against different cancer cell lines. **Table 3** describes the cancer cell lines that were susceptible to *E. purpurea* and the results of the cytotoxic activity of its extracts expressed as IC_{50}.

Yildiz *et al*. (2014) studied the cytotoxicity of *E. purpurea* supercritical CO2, supercritical H2O, EtOH and MeOH extracts on CaCo-2, MCF-7, HeLa and A549 (human alveolar adenocarcinoma). *E. purpurea* showed no inhibition of cell proliferation in all these cell lines.

Table 3 – Cytotoxic activity of *E. purpurea* in studies currently available.

Plant material	Tumour cell lines	*E. purpurea* extracts	IC_{50} (µg/mL)	References
Commercial sample	MCF-7	Hydroalcoholic	Not active	Aarland *et al.*, 2016
Aerial		Supercritical CO_2, supercritical H_2O, EtOH and MeOH	Not active	Yildiz *et al.*, 2014
Commercial sample	HeLa	Hydroalcoholic	Not active	Aarland *et al.*, 2016
Aerial		Supercritical CO_2, supercritical H_2O, EtOH and MeOH	Not active	Yildiz *et al.*, 2014
Commercial sample	HCT-15	Hydroalcoholic	Not active	Aarland *et al.*, 2016
Aerial	Caco-2	Supercritical CO_2, supercritical H_2O, EtOH and MeOH	Not active	Yildiz *et al.*, 2014
Aerial	A549	Supercritical CO_2, supercritical H_2O, EtOH and MeOH	Not active	Yildiz *et al.*, 2014
Roots	MIA PaCa-2	*n*-Hexane	62.92	Chicca *et al.*, 2007
Roots	COLO320	*n*-Hexane	25.36	Chicca *et al.*, 2007

1.3.6. Clinical studies

The majority of clinical trials performed with *E. purpurea* were carried out to test the effects in the treatment and prevention of common cold and upper respiratory tract infections (Barnes *et al.*, 2005). Barrett *et al*. (2010) performed a randomized trial for the treatment of common cold with *E. purpurea* counting with 719 participants within four groups (no pills, placebo pills, *Echinacea* pills (blinded) or *Echinacea* pills (unblinded)). The trial showed that the duration of the common cold and the severity of this illness were not statistically significant

to compare *E. purpurea* with the placebo because the dose regiment of *E. purpurea* didn't have a large effect compared with placebo.

Rauš *et al*. (2015) carried out a clinical trial to examine the effects of a hot drink based on *E. purpurea* in influenza treatment. Counting with 473 participants it was concluded that the *E. purpurea*-based hot drink was effective in the early treatment with a reduced risk of complications and adverse events.

Sperber *et al*. (2004) conducted a randomized, double-blind, placebo-controlled clinical trial using *E. purpurea* to prevent infection with rhinovirus type 39 (common cold virus) and it counted with 48 participants. All participants received *E. purpurea* juice or placebo for 7 days before and 7 days after intranasal inoculation with rhinovirus type 39. It was observed that *E. purpurea* didn't decrease the rate of infection with rhinovirus.

There are a few clinical studies evaluating the cytotoxic effects of *E. purpurea* using its immunostimulant properties against liver cancer and colorectal cancer with intramuscular *E. purpurea* juice (Barrett, 2003).

1.4. Phytochemistry

E. purpurea is a plant rich in sugars and organic acids. Acording to Pires *et al*. (2016), fresh *E. purpurea* is much richier in those components (sugars > tocopherols > organic acids) than the dried plant. They also succed to identify and quantify four different sugars: arabinose, fructose, glucose and sucrose, being sucrose the constituint in higher concentrations in the fresh plant (4.39 ± 0.17 g/100g) and glucose the one in higher concentrations in the dried plant (1.15 ± 0.02 g/100g). Six organic acids – oxalic, quinic, malic, shikimic, citric and succinic acid were identified, being citric acid the one organic acid in higher concentration in the fresh plant (5.19 ± 0.18 g/100g) and succinic acid the organic acid in higher concentrations in the dried plant (2.15 ± 0.07 g/100g).

The protective effects of fruits and vegtables have been atributted to plant phenolics, flavonoids and phenylpropanoids that act as antioxidants or contribute to anticarcinogenic or cardioprotective action (Pellati *et al*., 2004).

E. purpurea is the richest of the Echinacea species in total phenolics and their extracts were the most effective concerning antioxidant activity (Pellati *et al.*, 2004). The most common phytochemicals of *E. purpurea* are alkamides, caffeic acid (**7**) derivatives, polysaccharides and

lipoproteins but it possesses also betain (**1**), sesquiterpenes, rosmarinic acid (**2**), glycosides, echinacoside (**3**), chicoric acid (**4**) and polyacetylene (**5**) (Sultan *et al.*, 2014; Wen *et al.*, 2012). *E. purpurea* is also chemically characterized by containing hypoxanthine (**6**), chlorogenic acid, caffeic acid (**7**), chicoric acid (**4**), quercetin (**8**) and kaempferol (**9**) derivatives such as quercetin-3-*O*-rhamnosyl-(1-6)-galactoside, kaempferol-3-*O*-rhamnosyl-(1-6)-galactoside and rutin (**10**) (**Figure 2**) (Miller, 2012; Wen *et al*., 2012).

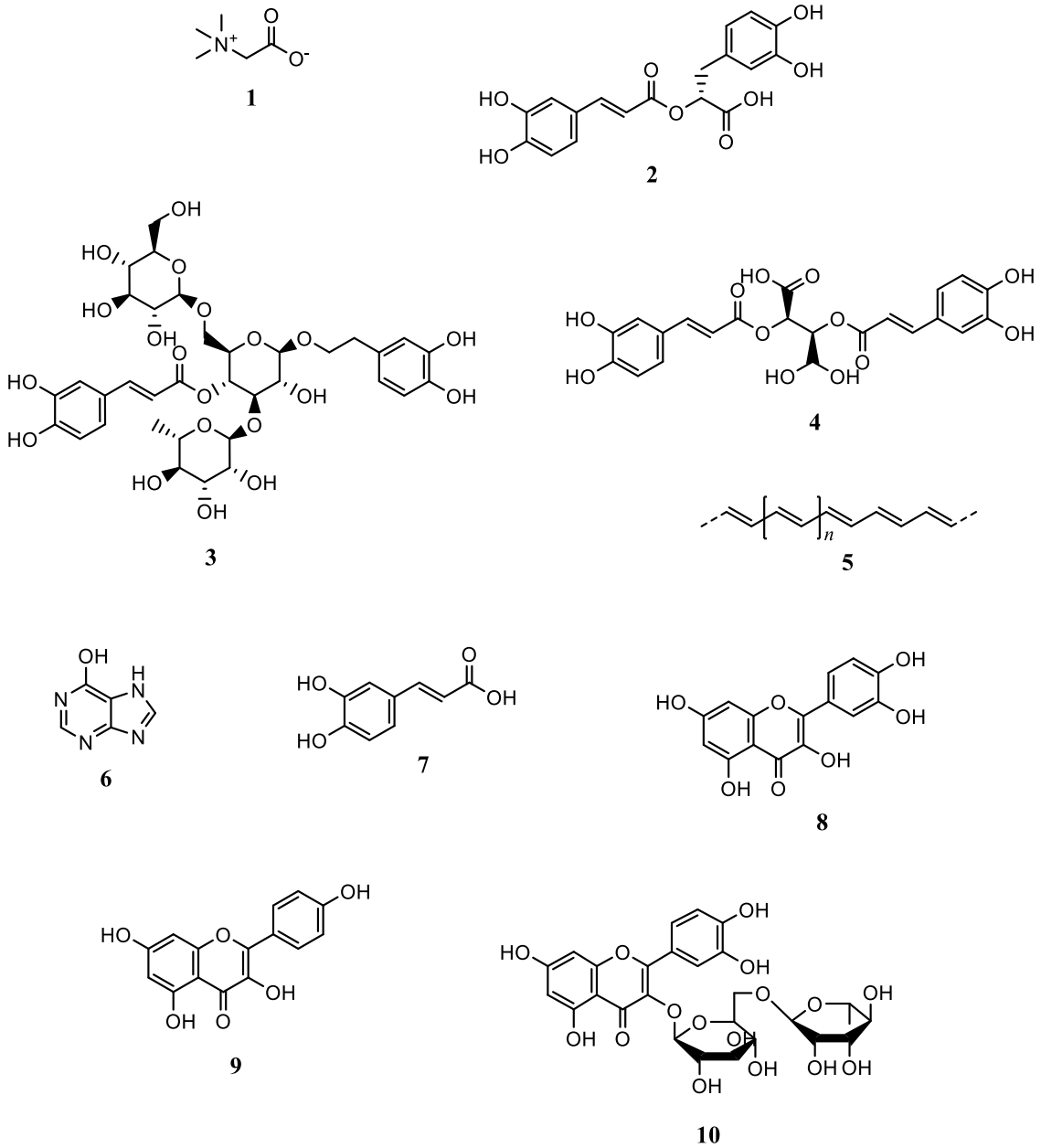

Figure 2 - Some of the phytochemicals present in *E. purpurea*.

Polysaccharides present in herbal medicines confer them various pharmacological activities, such as antitumoral effects and cancer chemopreventive effects. Cafeic acid derivatives, alkylamides and polysaccharides are responsible for the medicinal properties of *E. purpurea* extracts (Pleschka *et al.*, 2009; Wen *et al.*, 2012).

There are several studies about the medicinal properties of some of the chemical constituints of *E. purpurea*. It is know that alkamides present in *E. purpurea* inhibit 5-lipoxygenase and cyclooxygenase who are essential to the production of prostaglandins.

Caffeic acid derivatives protect collagen from free radical induced degradation and has antiviral activity (Miller, 2012; Tsai *et al*., 2012).

It has been shown that polisaccharides, caffeic acid derivatives and alkamides from Echinacea possess antibiotic, antiviral and immune-modultaing activities (Vimalanathan *et al.*, 2009).

CHAPTER II

Objectives

The objectives of the present study were made the chemical characterization of a commercial sample of *Echinacea purpurea* L. Moench of biological production in Portugal the determination of its nutritional value. Organic (*n*-hexane, DCM, EtOAc, acetone and MeOH) and aqueous (infusion and decoction) extracts were obtained and their bioactive properties evaluated. The antimicrobial activity was assessed in vitro against *Escherichia coli*; *Klebsiella pneumoniae*; *Morganella morganii*; *Proteus mirabilis*; *Pseudomonas aeruginosa*; *Enterococcus faecalis*; *Listeria monocytogenes*; MRSA and MSSA and its antitumoral activity was evaluated in several human cancer cell lines: MCF-7, NCI-H460, HeLa and HepG2. Its hepatotoxicity was evaluated against a primary cell line obtained from fresh pork liver. The phenolic profile of the organic and aqueous extracts of E. purpurea was determined by HPLC.

CHAPTER III

Experimental Part

In this chapter it is described the equipment and methods used in each stage of the work. In the first stage of the work were obtained seven extracts from *E. purpurea*, five organic and two aqueous extracts and it was evaluated their antimicrobial and cytotoxicity properties and it was identified the phenolic profile of these extracts. It was also evaluated the macronutrient composition of *E. purpurea.* In the second stage of the experimental part it was carried out the fractionation of the two extracts with better cytotoxicity against the tumour cell lines. It was obtained a total of twenty-nine fractions and their cytotoxicity was evaluated.

3.1. Standards and reagents

Acetonitrile was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Caffeic acid and quercetin-3-*O*-rutinoside were from Extrasynthese (Genay, France). Formic acid, acetic acid, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Fetal Bovine Serum (FBS), solutions of penicillin (100 U/mL) and streptomycin (100 mg/mL), RPMI-1640 medium, trypsin-EDTA (ethylenediaminetetraacetic acid), L-glutamine and Hank's Balanced Salt Solution (HBSS) were purchased from Hyclone (Logan, Utah, USA). Silica gel 0.060–0.200 mm, 60 Å was obtained from Acros Organics (Geel, Belgium).

3.2. Plant material

Echinacea purpurea (L.) Moench (aerial parts) was acquire from Cantinho das Aromáticas, Vila Nova de Gaia, Portugal in September 2017. According to the supplier the *E. purpurea* was from biological production, harvested when flowering and dried at 40-45ºC for three days in a dryer with controlled ventilation. The sample was reduced to a fine powder, mixed to obtain a homogeneous sample and stored at room temperature protected from light.

3.3. Preparation of aqueous and organic extracts

The organic (*n*-hexane, DCM, EtOAc, acetone and MeOH) and aqueous (decoction and infusion) extracts were prepared from the powdered plant as described in Graça *et al*. (2016).

For the preparation of the organic extracts a sample (150 g) was extracted with 500 mL of *n*-hexane by stirring vigorously (500 rpm) at room temperature for approximately 48 hours. The mixture was then filtered under reduced pressure through a Fioroni 0601A00010 filter paper using a Büchner funnel, and the filtrate was further filtered under reduced pressure through a sintered glass funnel. The solid residue was extracted with an additional 500 mL of *n*-hexane under the same conditions. The combined organic extracts were evaporated to dryness at 40ºC under reduced pressure and then stored at room temperature protected from light until

further analysis. The resulting residue was further extracted sequentially with DCM, EtOAc, acetone and methanol, in this order, according to the procedure described above.

The infusion was prepared by adding the sample (1 g) to 100 mL of boiling distilled water, left to stand at room temperature for 5 min and then filtered under reduced pressure through a sintered glass funnel. The decoction was prepared by adding the sample (1 g) to 100 mL of distilled water and boiling the mixture for 5 minutes. This mixture was left to stand for 5 min at room temperature and then filtered under reduced pressure through a sintered glass funnel. The decoctions and the infusions obtained were frozen and lyophilized and then stored at room temperature protected from light until further analysis.

3.4. Chemical characterization

3.4.1. Nutritional value of *E. purpurea*

E. purpurea was analysed in terms of macronutrients (proteins, fat, carbohydrates and ash) as described in Petropoulos *et al*. (2018). For the crude protein content (Nx6.25) was used the macro-Kjeldahl method. To determine the fat content a Soxhlet extraction of a sample of dried powder of *E. purpurea* (3 g) with petroleum ether was carried out for 7-8h. For the determination of the ash content a sample of dried powder of *E. purpurea* (250 mg) was incinerated at a 550±15 ºC for 12h and then the ashes were weight. Total carbohydrates were calculated by the difference: 100 - (g protein + g fat + g ash) and the energetic content was calculated by the following equation: Kcal = 4 x (g protein + g carbohydrate) + 9 x (g fat).

3.4.2. Analysis of phenolic compounds in *E. purpurea* extracts

The phenolic compounds in *E. purpurea* extracts were analysed as described in Bessada *et al*. (2016) with some modifications. The *n*-hexane, DCM, EtOAc and acetone extracts were dissolved in methanol, the MeOH extract was dissolved in methanol/water (1:4 v/v) and the infusion and the decoction extracts were dissolved in distilled water at a final concentration of 5 mg/mL and filtered through a 0.22-µm disposable LC filter disk. The extracts analysed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to an electrospray ionization mass detector (LC-DAD-ESI/MSn),

a quaternary pump, an auto-sampler (kept at 5 ºC), a degasser and an automated thermostatted column compartment.

Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column thermostatted at 35 °C.

The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B (5 min), 15% B to 20% B (5 min), 20-25% B (10 min), 25-35% B (10 min), 35-50% B (10 min), and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280, 330 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 5 kV, a source temperature of 325 °C, a capillary voltage of -20 V. The tube lens offset was kept at a voltage of -66 V. The full scan covered the mass range from *m*/*z* 100 to 1500. The collision energy used was 35 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

The phenolic compounds were identified by comparing their retention times, UV-vis and mass spectra with those obtained from standard compounds, when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve (2 – 200 μg/mL) for each available phenolic standard was constructed based on the UV signal caffeic acid (y = 388345x + 406369, R^2=0.999) and quercetin-3-*O*-rutinoside (y = 13343x + 76751, R^2=0.999). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The results were expressed as mg per g of extract.

3.5. Evaluation of the bioactive properties of the extracts

3.5.1. Antimicrobial activity

The antimicrobial evaluation of the extracts was performed using the same methodology performed by Alves *et al*. (2012) with some modifications. The microorganisms used to assess

the antimicrobial activity of the extracts of *E. purpurea* were clinical isolates from patients hospitalized in various departments of the Centro Hospitalar de Trás-os-Montes e Alto Douro (Vila Real and Bragança). Five Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis* and *Pseudomonas aeruginosa*), four Gram-positive bacteria (*Enterococcus faecalis*, *Listeria monocytogenes*, MRSA and MSSA) and a yeast *Candida albicans* were used. All these microorganisms were incubated at 37 ºC in appropriate fresh medium for 24 h before analysis to maintain the exponential growth phase.

The MIC minimum inhibitory concentration (lowest concentration of the *E. purpurea* extracts able to inhibit microbial growth) determinations on all bacteria were conducted using colorimetric assay. The samples were first of all dissolved in 5% (v/v) Dimethyl sulfoxide (DMSO)/Mueller-Hinton Broth (MHB)/Tryptic Soy Broth (TSB) to give a final concentration of 20 mg/mL for the stock solution. 190 µL of this concentration was added in the first well (96-well microplate) in duplicate. In the remaining wells it was placed 90 µL of medium MHB or TSB, then the samples were serially diluted, obtaining the concentration ranges from 20 at 0.15 mg/mL. To finish 10 µL of inoculum (standardized at 1.5×10^8 Colony Forming Unit (CFU) /ml) was added at all well. Three negative controls were prepared (one with (MHB)/(TSB), another one with the extract, and the third with medium, antibiotic, and bacteria). One positive control was prepared with MHB/TSB and each inoculum. Ampicillin and imipenem were used for all Gram-negative bacteria tested and *Listeria monocytogenes*. Ampicillin and vancomycin were selected for *Enterococcus faecalis*, MSSA, and MRSA. The microplates were covered and incubated at 37°C for 24 h. The MIC of samples was detected following addition (40 µl) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37°C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determined by the change of coloration from yellow to pink if the microorganisms are viable. For the determination of MBC, 10 µL of each well that showed no change in colour was plated on a solid medium, blood agar (7% sheep blood) and incubated at 37°C for 24 h. The lowest concentration that yielded no growth determine the MBC. MBC was defined as the lowest concentration required to kill bacteria.

3.5.2. Cytotoxicity in human tumour cell lines

The cytotoxic evaluation of the extracts was performed using the same methodology performed by Graça *et al*. (2016) with some modifications. The *E. purpurea* extract were

dissolved in 1 mL of aqueous DMSO (50%) in order to obtain a concentration of 8 mg/mL. The final solutions were previously diluted to different concentrations (400 to 6.25 μg/mL).

Four human tumour cell lines were used: NCI-H460 (non-small cell lung cancer); MCF-7 (breast adenocarcinoma); HepG2 (hepatocellular carcinoma) and HeLa (cervical carcinoma) acquired from DSMZ – *German Collection of Microorganisms and Cell Cultures*. The cells were routinely maintained as adherent cell cultures in RPMI-1640 medium at 37°C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density of $5.0x10^4$ cells/mL. The cells were then treated for 48 h with the different diluted sample solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% TCA (100 μL) and incubated for 60 min at 4°C. Plates were then washed with deionized water and dried; SRB solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate-well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, the bound SRB was solubilised with 10 mM Tris (200 μL, pH 7.4) and the absorbance was measured at 540 nm in the microplate reader (Biotek, ELX800). Ellipticine was used as a positive control and results were expressed in GI_{50} values corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines.

3.5.3. Hepatotoxicity in non-tumour cells

For hepatotoxicity assay, a cell culture was prepared from a freshly harvested porcine liver (obtained from a local slaughter house) and designated as PLP2 (Abreu *et al*., 2011). Briefly, the liver tissue was rinsed in Hank's balanced salt solution containing 100 U/mL penicillin + 100 μg/mL streptomycin and was divided into 1 × 1 mm^3 explants. Some of them were placed into 25 cm^2 tissue flasks containing DMEM medium (supplemented with 10% fetal bovine serum, 2 mM non-essential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin) and incubated at 37 °C under a humidified atmosphere with 5% CO_2. Phase contrast microscope was used for direct monitoring of the cell cultivation every 2 to 3 days. Before reaching the confluence, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well and cultivated in commercial DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

The evaluation of the samples was carried out in the same way as the cytotoxicity of the tumour cell lines. Ellipticine was used as a positive control and results were expressed in GI_{50}

values corresponding to the sample concentration achieving 50% of growth inhibition in liver primary culture PLP2.

3.6. Fractionation of extracts

The fractionation of the extracts were conducted based on methodology described in Graça *et al*. (2017) with some modifications. The DCM extract was diluted in the minimum amount of DCM and it was added a small amount of silica gel. The mixture was evaporated to dryness at 40ºC under reduced pressure and, afterwards, placed on the top of a silica gel column. The dry-loaded extract was fractionated by gradient elution column chromatography using: DCM; DCM/EtOAc − (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9); EtOAc, EtOAc/acetone − (9:1), (8:2), (7:3), (6:4), (5:5), (6:4), (7:3), (8:2), (9:1); acetone; acetone/MeOH − (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), (1:9); MeOH; MeOH/formic acid (99:1), (97:3), (95:5). Seven hundred and twenty-four samples (~23 mL each) were collected and grouped in fifteen fractions according to the similarity of their TLC profiles. The solvent of these final fractions was removed under reduced pressure until complete dryness.

The *n*-hexane extract was diluted in the minimum amount of DCM as possible and it was added a small amount of silica gel. The mixture was evaporated to dryness at 40ºC under reduced pressure and, afterwards, placed on the top of a silica gel column. The dry-loaded extract was fractionated in the same way as the DCM extract. Eight hundred and thirty-six samples (~23 mL each) were collected and grouped in fourteen fractions according the similarity of their TLC profiles. The solvent of these final fractions was removed under reduced pressure until complete dryness.

3.7. Evaluation of the bioactive properties of the fractions

3.7.1. Cytotoxicity in human tumour cell lines

For the cytotoxic evaluation of the fractions, 1 mL of DMSO was added to 8 mg of each fraction. Four human tumour cell lines: NCI-H460 (non-small cells lung cancer); MCF-7 (breast adenocarcinoma); HepG2 (hepatocellular carcinoma) and HeLa (cervical carcinoma) were used to accessed this methodology as described above in **CHAPTER II**, **section 2.5.2.**.

3.7.2. Hepatotoxicity in non-tumour cell line

For the hepatotoxicity evaluation of the fractions, 1 mL of DMSO was added to 8 mg of each fraction. The hepatotoxicity assessment was performed in a non-tumour primary cell line (PLP). The evaluation of the samples was carried out in the same way as mentioned in **CHAPTER II**, **section 2.5.2.**.

3.8. Statistical analysis

For all the experiments, three samples were analysed, and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation. The differences between the different samples were analysed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. This treatment was carried out using the SPSS v. 23.0 program (SPSS v. 23.0; IBM Corp., Armonk, NY, USA).

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CHAPTER III

Results and Discussion

In this chapter are presented and discussed the results of the chemical characterization of the *E. purpurea*. The results of the antimicrobial and cytotoxicity of all seven extracts of the whole plant are also presented and discussed. The cytotoxicity of the fractions from the most promising extracts - *n*-hexane and DCM - are also presented and discussed.

4.1. Chemical characterization

4.1.1. Nutritional value of *E. purpurea*

The results of the chemical characterizations of *E. purpurea* in terms of macronutrients are presented in **Table 4**. The contents evaluated for the nutritional value of the plant were fat, proteins, ash and carbohydrates and the energetic contribution.

Table 4 - Chemical characterization of *E. purpurea* in terms of macronutrients.

Fat	Protein	Ash	Carbohydrates	kcal	kj
1.73 ± 0.06	13.1 ± 0.6	12.62 ± 0.05	72.6 ± 0.6	358.2 ± 0.5	1500 ± 2

The results of fat, protein, ash and carbohydrates are expressed as g per 100g dw. Energy is expressed as kcal per 100 g dw or kj per 100 g dw.

Fat was the macronutrient that less contributed to the nutritional value, representing only 1.73 g per 100 g of the dried plant while carbohydrates were the macronutrient which contributes most for the nutritional value of this plant, presenting values of 72.5 g per 100 g of dried plant. It can also be assumed that this plant is rich in proteins since it has values of 13.1 g per 100 g of dried plant. The plant showed an energetic contribution of 358.2 kcal and 1500 kj per 100 g of dry weigh.

4.1.2. Phenolic compounds of *E. purpurea* extracts

Table 5 presents the data obtained from the HPLC-DAD-ESI/MS analysis (retention time, λ_{max}, pseudomolecular ions, main fragment ions in MS^2, tentative identification and quantification) present in the EtOH, the acetone, MeOH, infusion and decoction extracts of *E. purpurea*. An exemplificatives chromatograms of the phenolic profile recorded at 280, and 370 nm of the methanolic extract is shown in **Figure 3** (sample that presented most of the phenolic compounds tentatively identified). The *n*-hexane and DCM extracts were not considered for this analysis, since there were no phenolic compounds detected in these extracts. Fifteen phenolic compounds were tentatively identified, seven phenolic acids (mainly caffeic, chicoric and caftaric acid derivatives, peaks **1**, **2**, **3**, **4**, **5**, **8** and **14**) and eight flavonoids (mainly quercetin, kaempferol, and diosmetin glycosylated derivatives, peaks **6**, **7**, **9**, **10**, **11**, **12**, **13** and

15). Peaks **2**, **3** and **7** were identified as 5-*O*-caffeoylquinic acid, caffeic acid and quercetin-3-*O*-rutinoside, respectively, according to their retention time, mass and UV-Vis characteristics in comparison with commercial standards. All the remaining compounds were tentatively identified according to their mass and UV-Vis characteristics in comparison with information found in literature.

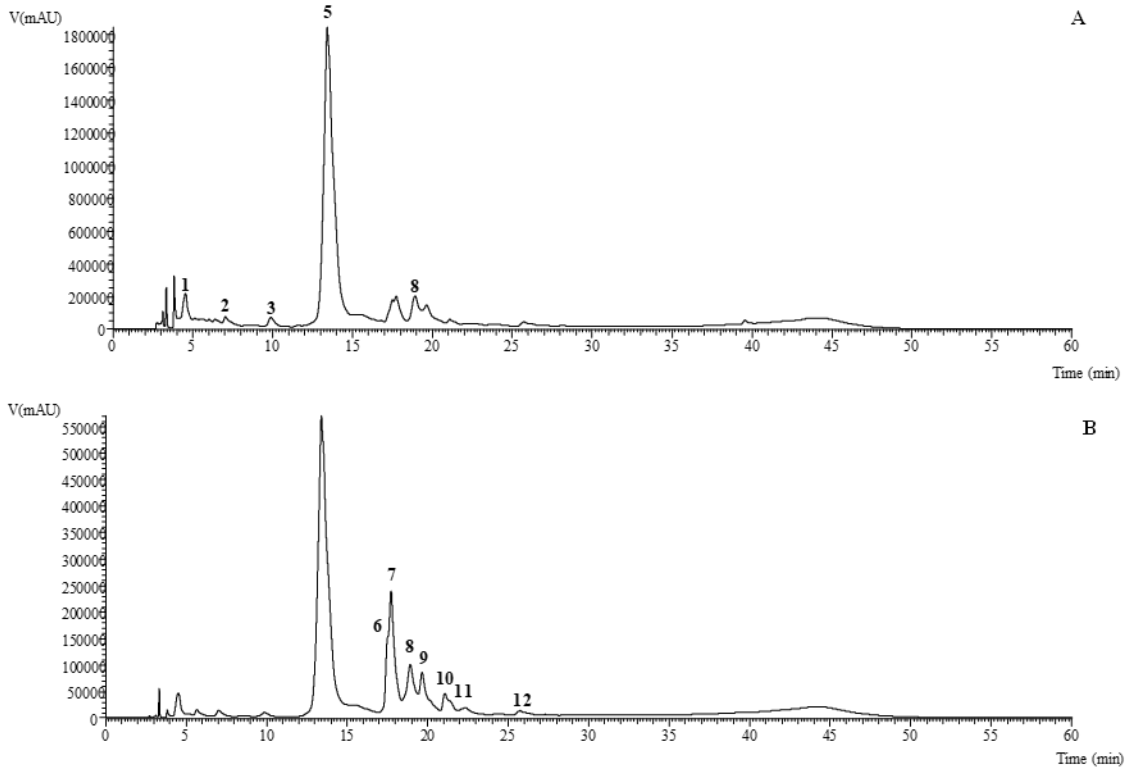

Figure 3 - Phenolic profile of the MeOH extract of *E. purpurea* recorded at 280 nm (A) and 370 nm (B).

Peaks **1** ([M - H]$^-$ at *m/z* 311) and **4/5** ([M - H]$^-$ at *m/z* 473) were tentatively identified as caftaric acid and *cis* and *trans* chicoric acid, respectively, according to their pseudomolecular ion, MS^2 fragmentation and UV-Vis spectra. *trans*-Chicoric acid was the main compound found in methanolic extract; however, in the infusion and the decoction the *cis* form was found in higher amounts. Peaks **8** ([M - H]$^-$ at *m/z* 487) and **14** ([M - H]$^-$ at *m/z* 501) were identified as caffeic acid derivatives, presenting a characteristic MS^2 fragment at *m/z* 179 (corresponding to the caffeic acid moiety) and also characteristic UV-vis spectra at 328-327 nm. Peak **8**, besides the 179 MS^2 fragment, also presented another one at *m/z* 325 which corresponded to the successive loss of an hexosyl and glucuronyl moieties, being tentatively identified as caffeoyl hexosyl-glucuronide. Peak **14**, presented an additional MS^2 fragment at *m/z* 307 which

corresponded to the loss of a deoxyhexosyl moiety followed by a glucuronyl moiety, being tentatively identified as caffeoyl deoxyhexosyl-glucuronide.

Regarding the flavonoids identified in *E. purpurea* samples, kaempferol derivatives were the main compounds found in these samples. Peaks **9**, **10**, **11**, and **13** ($[M-H]^-$ at *m/z* 607) presented a unique MS^2 fragment at *m/z* 285, corresponding to kaempferol aglycone, as also its characteristic UV-vis spectrum. Peaks **9**, **10** and **11** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 593, being all tentatively identified as kaempferol-*O*-deoxyhexosyl-hexoside, thus compound **10** was identified as kaempferol-3-*O*-rutinoside, presenting a similar retention time to the commercial standard. Peak **13** present a pseudomolecular ion $[M-H]^-$ at *m/z* 607, being tentatively identified as kaempferol-*O*-deoxyhexosyl-glucuronide.

Regarding the quercetin derivatives, peak **6** presented the same chromatographic characteristics as peak **7**, except for its retention time, being tentatively identified as quercetin-*O*-deoxyhexosyl-hexoside. Peak **12** ($[M-H]^-$ at *m/z* 549) revealed MS^2 fragments at *m/z* 505 (44 u), 463 (42 u), and 301 (162 u, quercetin aglycone), being tentatively identified as quercetin-*O*-malonylhexoside. Finally, peak **15** ($[M-H]^-$ at *m/z* 577) showed an MS^2 fragment at *m/z* 299 (diosmetin moiety). Given its late retention time the -146 u should correspond to the loss of a *p*-coumaroyl moiety and the -132 u to a pentosyl moiety, being tentatively identified as diosmetin-*O*-*p*-coumaroyl-pentoside.

As previously stated the methanolic extract was the one that presented the highest number of phenolic compounds identified, being also the richest extract in phenolic acids (45.2 ± 0.4 mg per g of extract, mainly due to *trans* chicoric acic) and flavonoids (8.6 ± 0.2 mg per g of extract, mainly due to quercetin-3-*O*-rutinoside).

Table 5 - Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g extract) of the phenolic compounds present in five different extracts of *E. purpurea*.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Organic			Aqueous	
						EtOAc	Acetone	MeOH	Infusion	Decoction
1	4.54	327	311	179(6),149(100),135(5),113(3)	Caftaric acid	nd	nd	1.19 ± 0.02^{a}	0.16 ± 0.01^{c}	0.22 ± 0.01^{b}
2	7.04	325	353	191(100),179(8),173(3),135(3)	5-*O*-Caffeyolquinic acid	nd	nd	0.286 ± 0.003^{a}	0.024 ± 0.001^{b}	nd
3	9.87	324	179	135(100)	Caffeic acid	0.20 ± 0.01^{b}	0.066 ± 0.001^{c}	0.63 ± 0.02^{a}	nd	nd
4	11.84	328	473	311(100),293(87),179(5),149(5)	*cis*-Chicoric acid	nd	nd	nd	$12.0 \pm 0.1^{*}$	$10.5 \pm 0.1^{*}$
5	13.40	328	473	311(100),293(87),179(5),149(5)	*trans*-Chicoric acid	nd	nd	41.0 ± 0.3^{a}	4.05 ± 0.08^{b}	4.47 ± 0.08^{b}
6	17.50	342	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside	nd	tr	1.8836 ± 0.0005	nd	nd
7	17.73	342	609	301(100)	Quercetin-3-*O*-rutinoside	nd	tr	5.6 ± 0.2^{a}	1.40 ± 0.07^{b}	0.26 ± 0.01^{c}
8	18.93	328	487	325(95),179(9)	Caffeoyl hexosyl-glucuronide	nd	tr	2.14 ± 0.07	nd	tr
9	19.66	329	593	285(100)	Kampferol-*O*-deoxyhexosyl-hexoside	nd	tr	0.60 ± 0.01	nd	tr
10	21.10	334	593	285(100)	Kampferol-3-*O*-rutinoside	nd	tr	0.536 ± 0.001	nd	tr
11	21.42	334	593	285(100)	Kampferol-*O*-deoxyhexosyl-hexoside	nd	tr	0.058 ± 0.003	nd	nd
12	22.35	327	549	505(5),463(13),301(30)	Quercetin-*O*-malonylhexoside	nd	nd	tr	nd	nd
13	24.10	340	607	285(100)	Kampferol-O-deoxyhexosyl-glucuronide	nd	tr	nd	nd	nd
14	25.73	327	501	307(100),179(5)	Caffeoyl deoxyhexosyl-glucuronide	nd	nd	tr	tr	tr
15	39.56	308	577	299(100)	Diosmetin-*O*-*p*-coumaroyl-pentoside	nd	5.29 ± 0.04	nd	nd	nd
					Total phenolic acids	**0.20 ± 0.01^{b}**	**0.066 ± 0.001^{a}**	**45.2 ± 0.4^{b}**	**16.2 ± 0.2^{b}**	**15.1 ± 0.2^{b}**
					Total flavonoids	**nd**	**5.29 ± 0.04^{b}**	**8.6 ± 0.2^{a}**	**1.40 ± 0.07^{c}**	**0.26 ± 0.01^{d}**
					Total phenolic compounds	**0.20 ± 0.01^{e}**	**5.36 ± 0.04^{d}**	**53.9 ± 0.6^{a}**	**17.7 ± 0.1^{b}**	**15.4 ± 0.2^{c}**

nd - not detected. tr – traces. ***** Samples differ significantly ($p < 0.05$), obtained by Student's *t*-test. Results expressed in mean values ± standard deviation (SD). Different letters represent significant differences ($p < 0.05$).

4.2. Antimicrobial properties of *E. purpurea* extracts

Presently, it is quite common to find bacteria not affected by treatment with antibiotics; so, it is important to find some alternatives to this problem. We assessed the antimicrobial activity of *E. purpurea* extracts against clinical isolates of several bacteria (gram-positive and gram-negative) and yeast.

Concerning the antimicrobial activity were evaluated the ability of all seven extracts of *E. purpurea* to inhibit the growth of five gram-negative bacteria, four gram-positive bacteria and one yeast. Was also investigated the bactericidal concentration for each of the gram-negative and gram-positive bacteria and the yeast. Results are presented in **Table 6**.

In general, the DCM, EtOAc and acetone extracts were the ones with the best antimicrobial activity. All gram-positive bacteria showed some susceptibility for these three extracts, being *Enterococcus faecalis* and *Listeria monocytogenes* the most susceptible for the DCM extract with a MIC value of 2.5 mg/mL and MSSA the most susceptible for the acetone extract with a MIC value of 2.5 mg/mL. The DCM, EtOAc and acetone extracts showed similar activity against MRSA being their MIC values of 5 mg/mL. The activity of the *n*-hexane extract against these bacteria was not significant because all gram-positive bacteria showed MIC values of 10 mg/mL, except *Listeria monocytogenes*, that presented MIC values greater than 20 mg/mL. For the MeOH extract only MRSA and MSSA were susceptible with MIC values of 20 mg/mL; the remaining gram-positive bacteria presented MIC values greater than 20 mg/mL. For the infusion and the decoction all gram-positive bacteria had MIC values superior to 20 mg/mL.

Gram-negative bacteria showed some susceptibility against the DCM, AtOAc and acetone extracts, being *Morganella morganii* the most susceptible to the DCM extract with a MIC value of 5 mg/mL, for the EtOAc extract the most susceptible bacteria were *Escherichia coli* and *Morganella morganii* with MIC values identical to those of the DCM extract. For the acetone extract, all gram-negative bacteria showed MIC values of 10 mg/mL. For the *n*-hexane extract, all gram-negative bacteria presented MIC values of 20 mg/mL. The MeOH extract, the infusion and the decoction didn't show any effect against the bacteria or the yeast being most of the MIC values >20 mg/mL.

Table 6 - Results of antimicrobial activity of all seven *E. purpurea* extracts against clinical isolates of five Gram-negative bacteria, four Gram-positive bacteria and one yeast.

	Organic extracts										**Aqueous extracts**			
	***n*-Hexane**		**DCM**		**EtOAc**		**Acetone**		**MeOH**		**Infusion**		**Decoction**	
Antimicrobial activity	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria														
Escherichia coli	20	>20	10	>20	5	>20	10	>20	>20	>20	>20	>20	>20	>20
Klebsiella pneumoniae	20	>20	20	>20	10	>20	10	>20	>20	>20	>20	>20	20	>20
Morganella morganii	20	>20	5	>20	5	>20	10	>20	>20	>20	>20	>20	>20	>20
Proteus mirabilis	20	>20	20	>20	10	>20	10	>20	>20	>20	>20	>20	>20	>20
Pseudomonas aeruginosa	20	>20	10	>20	10	>20	10	>20	>20	>20	>20	>20	>20	>20
Gram-positive bacteria														
Enterococcus faecalis	10	>20	2.5	>20	10	>20	5	>20	>20	>20	>20	>20	>20	>20
Listeria monocytogenes	>20	>20	2.5	>20	20	>20	20	>20	>20	>20	>20	>20	>20	>20
MRSA	10	>20	5	>20	5	>20	5	>20	20	>20	>20	>20	>20	>20
MSSA	10	>20	5	>20	5	>20	2.5	>20	20	>20	>20	>20	>20	>20
Yeasts														
Candida albicans	10	>20	5	>20	10	>20	5	>20	>20	>20	>20	>20	>20	>20

MIC and MBC are expressed in mg/mL.

Table 7 – Controls for the microorganism used in antimicrobial activity.

	Ampicillin (20 mg/mL)		**Imipenem (1 mg/mL)**		**Vancomycin (1 mg/mL)**		**Fluconazol (1 mg/mL)**	
Antibacterial activity)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria								
Escherichia coli	<0.15	< 0.15	<0.0078	<0.0078	nt	nt	nt	nt
Klebsiella pneumoniae	10	20	<0.0078	<0.0078	nt	nt	nt	nt
Morganella morganii	20	>20	<0.0078	<0.0078	nt	nt	nt	nt
Proteus Mirabilis	<0.15	< 0.15	<0.0078	<0.0078	nt	nt	nt	nt
Pseudomonas aeruginosa	>20	>20	0.5	1	nt	nt	nt	nt
Gram-positive bacteria								
Enterococcus faecalis	<0.15	<0.15	nt	nt	<0.0078	<0.0078	nt	nt
Listeria monocytogenes	<0.15	<0.15	<0.0078	<0.0078	nt	nt	nt	nt
MRSA	<0.15	<0.15	nt	nt	<0.0078	<0.0078	nt	nt
MSSA	<0.15	<0.15	nt	nt	0.25	0.5	nt	nt
Antifungal activity								
Candida albicans	nt	nt	nt	nt	nt	nt	0.06	0.06

nt – not tested

Comparing the results obtained in this study with the controls that are presented in **Table 7** it is evident that most of the values of MIC and MBC for the bacteria and the yeast obtained were much higher than those obtained from controls. But in the case of Klebsiella pneumoniae, *Morganella morganii* and *Pseudomonas aeruginosa* the results obtained in this study for DCM and EtOAc were lower than those obtained from ampicillin control.

Candida albicans was most susceptible to the DCM and acetone extracts showing a MIC of 5 mg/mL. This yeast also showed some susceptibility against *n*-hexane and EtOAc extracts with a MIC values of 10 mg/mL. The remaining extracts didn't show any activity against *Candida albicans* being their MIC values superior to 20 mg/mL. Relatively to MBC, any of the extracts show bactericidal activity, being the MBC values superior to 20 mg/mL against all bacteria and the yeast for all extracts.

In Sharma *et al*. (2008) study, they tested the antimicrobial activity of *E. purpurea* commercial extracts (roots + aerial parts with 40% of EtOH content and only roots) in the dark and in the light and they concluded that *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* showed some resistance against these extracts.

4.3. Cytotoxicity of all *E. purpurea* extracts against tumour cell lines

The cytotoxic activity of all seven extracts of E. purpurea was evaluated and the results for this assay are showed in **Table 8**. Almost all extracts showed activity against the four human tumour cell lines. The extract that showed a better cytotoxicity against all four tumour cell lines was DCM extract [GI_{50} = 48 ± 4 µg/mL (NCI H460), GI_{50} = 36.7 ± 0.6 µg/mL (HepG2), GI_{50} = 51 ± 4 µg/mL (HeLa) and GI_{50} = 21 ± 2 µg/mL (MCF-7)]. Although this extract was the most effective against tumour cell lines. The tumour cell line that was more affected by the DCM extract was MCF-7, this extract also displayed higher toxicity against the PLP2 cell line (GI_{50} = 100 ± 8 µg/mL), showing only a difference of ~80 µg/mL from the better cytotoxicity value (DCM extract against MCF-7 tumour cell line) which is very low and give a very small therapeutic window. The *n*-hexane extract showed also very good results against all four cell lines [GI_{50} = 70 ± 2 µg/mL (NCI H460), GI_{50} = 47 ± 3 µg/mL (HepG2), GI_{50} = 58 ± 5 µg/mL (HeLa) and GI_{50} = 29 ± 2 µg/mL (MCF-7)], but it also showed toxicity against PLP2 cell line (GI_{50} = 104 ± 7 µg/mL).

The EtOAc and acetone extracts showed some good results against all tumour cell lines, but also presented some hepatotoxicity. The MeOH extract was only effective against the HeLa and MCF-7 tumour cell lines and it was ineffective against the NCI H460 and HepG2 tumour cell lines. This extract didn't show toxicity against the PLP2 primary cell line at the maximum concentration assayed (400 µg/mL). Both aqueous extracts showed some growth inhibition against HeLa cells, but the results were already very close to the maximum concentration. They also didn't revealed toxicity towards PLP2 cells. The infusion, in addition to influence HeLa cell line, also showed some effects against the MCF-7 cell line which was, in general, the most affected by almost all extracts, being the decoction the only one that wasn't effective even at the maximum concentration used.

Ellipticine was used as the positive control in the cytotoxic activity assay showing excellent results, thus these cannot be compared to the results obtained for the extracts, because ellipticine is an individual compound being tested in a much higher concentration the compounds present in the extract.

Table 8 – Cytotoxicity of all seven extracts of *E. purpurea* against four human tumour cell lines and one non-tumor cell line that it is a primary cell line from pork liver.

	Cytotoxicity in human tumour cell lines (GI_{50}, µg/mL)						
	Organic extracts					**Aqueous extracts**	
	***n*-Hexane**	**DCM**	**EtOAc**	**Acetone**	**MeOH**	**Infusion**	**Decoction**
NCI H460	70 ± 2c	48 ± 4d	192 ± 4a	142 ± 10b	<400	<400	<400
HepG2	47 ± 3c	36.7 ± 0.6c	226 ± 15a	82 ± 4b	<400	<400	<400
HeLa	58 ± 5e	51 ± 4e	85 ± 6d	98 ± 5cd	111 ± 9c	305 ± 23b	319 ± 12a
MCF-7	29 ± 2d	21 ± 2e	51 ± 5c	50 ± 4c	76 ± 5b	247 ± 5a	<400
	Hepatoxicity in non-tumour cells (GI_{50}, µg/mL)						
PLP2	104 ± 7c	100 ± 8c	166 ± 9b	195 ± 15a	<400	<400	<400

GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. Results expressed in mean values ± standard deviation (SD). Different letters represent significant differences ($p < 0.05$).

The study of the cytotoxic properties of *E. purpurea* are virtually scarce. Aarland *et al*. (2016) were the only ones to investigate cytotoxicity of a hydroalcoholic extract of the roots, leaves and flowers and only roots of *E. purpurea* against MCF-7, HeLa and HCT-15 but both extracts showed to be ineffective towards all three tumour cell lines. There are other studies that were referred in **CHAPTE R I**, **section 1.3.5** but their *E. purpurea* extracts investigated didn't show cytotoxic properties towards the tumour cell lines evaluated.

Several authors associate *E. purpurea* biological properties to their phytochemical composition. In this study, the cytotoxic properties of *n*-hexane and DCM extracts can't be associated with their phenolic composition, because no flavonoids or phenolic acids were detected in both extracts.

4.4. Cytotoxic properties of *E. purpurea* fractions

Since the cytotoxic activity of *E. purpurea* extracts was promising it was considerated interesting to investigate the cytotoxicity of the fractions obtained from both extracts with better cytotoxic activity. Were obtained fourteen fractions from the n-hexane extract all fifteen fractions from the DCM and the results of their cytotoxicity are showed in **Table 9**.

Almost all fractions from the of *n*-hexane extract showed activity against the four human tumour cell lines. The fraction that showed better results was the FH11 fraction [GI_{50} = 100 ± 7 µg/mL (NCI H460), GI_{50} = 93 ± 4 µg/mL(HepG2), GI_{50} = 105 ± 9 µg/mL (HeLa) and GI_{50} = 106 ± 10 µg/mL (MCF-7)] but it also showed some toxicity against PLP2 primary cell line (GI_{50} = 269 ± 6 µg/mL) being the GI_{50} values towards PLP2 only approximately the double of the higher concentration needed of this fraction for obtained 50% of growth inhibition of tumour cell lines. Only the activity of the FH14 against the NCI H460, HeLa and MCF-7 human tumour cell lines exceeded the maximum concentration assayed (400 µg/mL). All other fractions from the *n*-hexane extract showed some activity against all four human tumour cell lines. FH1 and fractions FH7 to FH11 showed toxicity against the non-tumour cell line.

Fractions FD1, FD3, FD4, FD14 and FD15 from the DCM extract exhibited cytotoxicity results that exceeded the maximum concentration assayed for all tumour cell lines. Only fractions FD5 and FD7 fractions showed toxicity towards the PLP2 cell line. The fractions that showed the better results were fraction FD5 [GI_{50} = 128 ± 4 µg/mL (NCI H460), GI_{50} = 146 ±

4 µg/mL(HepG2), GI_{50} = 116 ± 4 µg/mL (HeLa) and GI_{50} = 89 ± 8 µg/mL (MCF-7)] and fraction FD6 [GI_{50} = 135 ± 4 µg/mL (NCI H460), GI_{50} = 141 ± 3 µg/mL (HepG2), GI_{50} = 113 ± 4 µg/mL (HeLa) and GI_{50} = 81 ± 3 µg/mL (MCF-7)]. All other DCM fractions showed some cytotoxicity to all four tumour cell lines.

Ellipticine was used as positive controls in the cytotoxic activity assays but as ellipticine is individual compound it should not be considered as standard and the comparison with the results obtained for the fractions should be avoided since an eventual synergistic effect of the mixtures cannot be excluded.

There are no virtual data about cytotoxic activity of fractionated extracted of *E. purpurea*. With these fractions results it is possible to assume that both extracts have a synergetic effect and that's why in the fractions results the values weren't so good.

Table 9 - Results for the cytotoxic activity of fractions from *n*-hexane and DCM extracts against four human tumour cell lines and one non-tumour primary cell line from pork liver.

	Cytotoxicity in human tumour cell lines and hepatoxicity in non-tumor cells				
Fractions	**NCI H46**	**HepG2**	**HeLa**	**MCF-7**	**PLP2**
FH1	105 ± 7h	206 ± 12e	150 ± 5d	110 ± 3fg	326 ± 17b
FH2	142 ± 5g	344 ± 10ab	180 ± 7c	140 ± 7e	>400
FH3	184 ± 15ef	308 ± 19c	212 ± 6b	182 ± 9c	>400
FH4	243 ± 16a	233 ± 3d	240 ± 3a	222 ± 5b	>400
FH5	238 ± 4ab	359 ± 7a	232 ± 5a	239 ± 4a	>400
FH6	250 ± 1a	327 ± 16bc	235 ± 5a	237 ± 8a	>400
FH7	197 ± 12de	159 ± 6gh	213 ± 8b	179 ± 9c	343 ± 22ab
FH8	222 ± 5bc	168 ± 5fg	241 ± 2a	121 ± 10f	356 ± 11a
FH9	206 ± 11cd	159 ± 3gh	181 ± 8c	171 ± 4cd	342 ± 10ab
FH10	211 ± 5cd	141 ± 4hi	170 ± 9c	181 ± 5c	331 ± 6ab
FH11	100 ± 7h	93 ± 4j	105 ± 9f	106 ± 10g	269 ± 6c
FH12	128 ± 5g	131 ± 6i	127 ± 9e	111 ± 3fg	>400
FH13	168 ± 12f	187 ± 12ef	176 ± 12c	160 ± 7d	>400
FH14	>400	338 ± 13b	>400	>400	>400
FD1	>400	>400	>400	>400	>400
FD2	236 ± 11c	278 ± 9ab	178 ± 12c	146 ± 11c	>400
FD3	>400	>400	>400	>400	>400
FD4	>400	>400	>400	>400	>400
FD5	128 ± 4e	146 ± 4f	116 ± 4e	89 ± 8d	267 ± 13*
FD6	135 ± 4e	141 ± 3f	113 ± 4e	81 ± 3d	>400
FD7	170 ± 2d	187 ± 16e	150 ± 3d	139 ± 5c	302 ± 18*
FD8	225 ± 9c	212 ± 3d	174 ± 9c	151 ± 4bc	>400
FD9	238 ± 4c	205 ± 7de	178 ± 9c	144 ± 9c	>400
FD10	341 ± 9a	298 ± 19a	241 ± 5a	181 ± 9a	>400
FD11	291 ± 9b	253 ± 8c	220 ± 5b	149 ± 3bc	>400
FD12	292 ± 17b	256 ± 11c	223 ± 14b	144 ± 3c	>400
FD13	336 ± 10a	271 ± 4bc	254 ± 6a	161 ± 4b	>400
FD14	>400	>400	>400	>400	>400
FD15	>400	>400	>400	>400	>400

FH (fractions from the *n*-hexane extract) FD (fractions from the DCM extract). Results expressed in mean values ± standard deviation (SD). Different letters correspond to significant differences ($p<0.05$). *Samples differ significantly ($p < 0.05$), obtained by Student's *t*-test. Ellipticine GI_{50} values: 1.21 μg/mL (MCF-7), 1.03 μg/mL (NCI-H460), 0.91 μg/mL (HeLa), 1.10 μg/mL (HepG2) and 2.29 μg/mL (PLP2).

CHAPTER IV

Conclusions

The present study aimed at testing the cytotoxic and antimicrobial properties of different organic and aqueous extracts of *E. purpurea* and determining their phenolic composition.

Once the *n*-hexane and DCM extracts showed considerable cytotoxic activity they were also fractionated by gradient elution column chromatography and the cytotoxicity of the fractions obtained from both extracts was assessed.

In a global perspective, this study allowed to conclude that:

1. Only the MeOH and the aqueous extracts were rich in phenolic acids and flavonoids, as expected, once they are polar solvents and these compounds have more affinity for them than for *n*-hexane or DCM whose extracts showed negligible phenolic compounds content.
2. The *n*-hexane and the DCM extracts displayed the best cytotoxic activities, thereby indicating that presumably the cytotoxic properties of *E. purpurea* weren't conferred by phenolic compounds. The pharmacological activity should thus be conferred by other compounds that weren't analysed in this work.
3. In general, the cytotoxicity of the extracts was superior to that of the obtained fractions, but their hepatotoxicity wasn't so high compared with that of the extracts. This points out to a possible synergistic effect of the mixture of compounds present in the extracts.
4. In general, the DCM, EtOAc and acetone extracts showed moderate antimicrobial activity. The DCM and EtOAc extracts showed the higher antimicrobial activity against *Klebsiella pneumoniae*, *Morganella morganii* and *Pseudomonas aeruginosa* compared with ampicillin. It can't be forgotten that these were clinical isolates and, therefore, they had multiple resistances that ATCC strains didn't have. So, if this study was performed with those strains instead of clinical isolates the results could be more promising than those obtained.

CHAPTER V

References

Aarland, R.C., Bañuelos-Hernández, A.E., Fragoso-Serrano, M., Sierra-Palacios, E.D.C., Díaz de León-Sánchez, F., Pérez-Flores, L.J., Rivera-Cabrera, F., Mendoza-Espinoza, J.A., 2016. Studies on phytochemical, antioxidant, anti-inflammatory, hypoglycaemic and antiproliferative activities of Echinacea purpurea and Echinacea angustifolia extracts. Pharm. Biol. 55, 649–656.

Abreu, R.M. V, Ferreira, I.C.F.R., Calhelha, R.C., Lima, R.T., Helena Vasconcelos, M., Adega, F., Chaves, R., Queiroz, M.-J.R.P., 2011. Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno 3,2-b pyridine-2-carboxylate derivatives: In vitro evaluation, cell cycle analysis and QSAR studies. Eur. J. Med. Chem. 46, 5800–5806.

Alves, M.J., Ferreira, I.C.F.R., Martins, A., Pintado, M., 2012. Antimicrobial activity of wild mushroom extracts against clinical isolates resistant to different antibiotics. J. Appl. Microbiol. 113, 466–475.

Aune, D., Giovannucci, E., Boffetta, P., Fadnes, L.T., Keum, N., Norat, T., Greenwood, D.C., Riboli, E., Vatten, L.J., Tonstad, S., 2017. Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality—a systematic review and dose-response meta-analysis of prospective studies. Int. J. Epidemiol. 46, 1029–1056.

Baek, M.K., Kim, K.O., Kwon, H.J., Kim, Y.W., Woo, J.H., Kim, D.Y., 2016. Age-related changes in antioxidative enzyme capacity in tongue of fischer 344 rats. Clin. Exp. Otorhinolaryngol. 9, 352–357.

Barnes, J., Anderson, L.A., Gibbons, S., Phillipson, J.D., 2005. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J. Pharm. Pharmacol. 57, 929–954.

Barrett, B., 2003. Medicinal properties of *Echinacea*: A critical review. Phytomedicine 10, 66–86.

Barrett, B., Brown, R., Rakel, D., Mindt, M., Bone, K., Barlow, S., 2010. *Echinacea* for Treating the Common Cold. Ann. Intern. Med. 12/21/2010 153, 771–777.

Bessada, S.M.F., Barreira, J.C.M., Barros, L., Ferreira, I.C.F.R., Oliveira, M.B.P.P., 2016. Phenolic profile and antioxidant activity of Coleostephus myconis (L.) Rchb.f.: An underexploited and highly disseminated species. Ind. Crops Prod. 89, 45–51.

Bodinet, C., Mentel, R., Wegner, U., Lindequist, U., Teuscher, E., Freudenstein, J., 2002. Effect of oral application of an immunomodulating plant extract on influenza virus type A infection in mice. Planta Med. 68, 896–900.

Chevallier, A., 1996. Encyclopedia of Medicinal Plants, First Amer. ed. DK Publisher Inc.

Chicca, A., Adinolfi, B., Martinotti, E., Fogli, S., Breschi, M.C., Pellati, F., Benvenuti, S., Nieri, P., 2007. Cytotoxic effects of *Echinacea* root hexanic extracts on human cancer cell lines. J. Ethnopharmacol. 110, 148–153.

Goel, V., Lovlin, R., Chang, C., Slama, J. V., Barton, R., Gahler, R., Bauer, R., Goonewardene, L., Basu, T.K., 2005. A proprietary extract from the echinacea plant (*Echinacea purpurea*) enhances systemic immune response during a common cold. Phyther. Res. 19, 689–694.

Graça, V.C., Barros, L., Calhelha, R.C., Dias, M.I., Carvalho, A.M., Santos-Buelga, C., Santos, P.F., Ferreira, I.C.F.R., 2016. Chemical characterization and bioactive properties of aqueous and organic extracts of Geranium robertianum L. Food Funct. 7, 3807–3814.

Graça, V.C., Barros, L., Calhelha, R.C., Dias, M.I., Ferreira, I.C.F.R., Santos, P.F., 2017. Bio-guided fractionation of extracts of Geranium robertianum L.: Relationship between phenolic profile and biological activity. Ind. Crops Prod. 108, 543–552.

Grimm, W., Müller, H.H., 1999. A randomized controlled trial of the effect of fluid extract of *Echinacea purpurea* on the incidence and severity of colds and respiratory infections. Am.

J. Med. 106, 138–143.
Hu, C., Kitts, D.D., 2000. Studies on the antioxidant activity of *Echinacea* root extract. J. Agric. Food Chem. 48, 1466–1472.
Hudson, J., Vimalanathan, S., Kang, L., Amiguet, V.T., Livesey, J., Arnason, J.T., 2005. Characterization of antiviral activities in *Echinacea* root preparations. Pharm. Biol. 43, 790–796.
Hudson, J.B., 2012. Applications of the phytomedicine *Echinacea purpurea* (purple coneflower) in infectious diseases. J. Biomed. Biotechnol. 1–16.
Martins, J.C., 2013. Medicamentos à base de plantas: contributo para o aproveitamento dos recursos naturais nacionais. Rev. Port. Farmacoter. 5, 22–38.
Martins, N., Ferreira, I.C.F.R., Barros, L., Carvalho, A.M., Henriques, M., Silva, S., 2015. Plants used in folk medicine: The potential of their hydromethanolic extracts against *Candida* species. Ind. Crops Prod. 66, 62–67.
Merali, S., Binns, S., Paulin-Levasseur, M., Ficker, C., Smith, M., Baum, B., Brovelli, E., Arnason, J.T., 2003. Antifungal and Anti-inflammatory Activity of the Genus *Echinacea*. Pharm. Biol. 41, 412–420.
Miller, S.C., 2005. *Echinacea*: A miracle herb against aging and cancer? Evidence *in vivo* in mice. Evidence-based Complement. Altern. Med. 2, 309–314.
Miller, S.C., 2012. Can herbs be useful in cancer therapy? A review of studies on the influence of *Echinacea* on cells of the immune system and on tumor amelioration. Biomed. Res. 23, 9–16.
Palhares, R.M., Drummond, M.G., Brasil, B. dos S.A.F., Cosenza, G.P., Brandão, M. das G.L., Oliveira, G., 2015. Medicinal Plants Recommended by the World Health Organization : DNA Barcode Identification Associated with Chemical Analyses Guarantees Their Quality. PLoS One 1–29.
Parnham, M.J., 1996. Benefit-risk assessment of the squeezed sap of the purple coneflower (*Echinacea purpurea*) for long-term oral immunostimulation. Phytomedicine 3, 95–102.
Pellati, F., Benvenuti, S., Magro, L., Melegari, M., Soragni, F., 2004. Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. J. Pharm. Biomed. Anal. 35, 289–301.
Pereira, C., Barros, L., Ferreira, I.C.F.R., 2015. A comparison of the nutritional contribution of thirty-nine aromatic plants used as condiments and/or herbal infusions. Plant Foods Hum. Nutr. 70, 176–183.
Petropoulos, S., Fernandes, Â., Barros, L., Ferreira, I.C.F.R., 2018. Chemical composition, nutritional value and antioxidant properties of Mediterranean okra genotypes in relation to harvest stage. Food Chem. 242, 466–474.
Pires, C., Martins, N., Carvalho, A.M., Barros, L., Ferreira, I.C.F.R., 2016. Phytopharmacologic preparations as predictors of plant bioactivity: A particular approach to *Echinacea purpurea* (L.) Moench antioxidant properties. Nutrition 32, 834–839.
Pleschka, S., Stein, M., Schoop, R., Hudson, J.B., 2009. Anti-viral properties and mode of action of standardized *Echinacea purpurea* extract against highly pathogenic avian Influenza virus (H5N1, H7N7) and swine-origin H1N1 (S-OIV). Virol. J. 6, 1–9.
Rauš, K., Pleschka, S., Klein, P., Schoop, R., Fisher, P., 2015. Effect of an Echinacea-Based Hot Drink Versus Oseltamivir in Influenza Treatment: A Randomized, Double-Blind, Double-Dummy, Multicenter, Noninferiority Clinical Trial. Curr. Ther. Res. - Clin. Exp. 77, 66–72.
Sharma, M., Anderson, S.A., Schoop, R., Hudson, J.B., 2009. Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized *Echinacea*, a potent antiviral herbal extract. Antiviral Res. 83, 165–170.

Sharma, M., Schoop, R., Suter, A., Hudson, J.B., 2011. The potential use of *Echinacea* in acne: Control of *Propionibacterium acnes* growth and inflammation. Phyther. Res. 25, 517–521.

Sharma, M., Vohra, S., Arnason, J.T., Hudson, J.B., 2008. *Echinacea* extracts contain significant and selective activities against human pathogenic bacteria. Pharm. Biol. 46, 111–116.

Sperber, S.J., Shah, L.P., Gilbert, R.D., Ritchey, T.W., Monto, A.S., 2004. *Echinacea purpurea* for prevention of experimental rhinovirus colds. Clin. Infect. Dis. 38, 1367–1371.

Stanisavljević, I., Stojičević, S., Veličković, D., Veljković, V., Lazić, M., 2009. Antioxidant and antimicrobial activities of Echinacea (*Echinacea purpurea* L.) extracts obtained by classical and ultrasound extraction. Chinese J. Chem. Eng. 17, 478–483.

Sultan, M.T., Buttxs, M.S., Qayyum, M.M.N., Suleria, H.A.R., 2014. Immunity: Plants as effective mediators. Crit. Rev. Food Sci. Nutr. 54, 1298–308.

Thomford, N.E., Dzobo, K., Chopera, D., Wonkam, A., Skelton, M., Blackhurst, D., Chirikure, S., Dandara, C., 2015. Pharmacogenomics implications of using herbal medicinal plants on African populations in health transition. Pharmaceuticals 8, 637–663.

Tsai, Y.-L., Chiu, C.-C., Yi-Fu Chen, J., Chan, K.-C., Lin, S.-D., 2012. Cytotoxic effects of *Echinacea purpurea* flower extracts and cichoric acid on human colon cancer cells through induction of apoptosis. J. Ethnopharmacol. 143, 914–919.

Vimalanathan, S., Arnason, J.T., Hudson, J.B., 2009. Anti-inflammatory activities of *Echinacea* extracts do not correlate with traditional marker components. Pharm. Biol. 47, 430–435.

Vimalanathan, S., Kang, L., Amiguet, V.T., Livesey, J., Arnason, J.T., Hudson, J., 2005. *Echinacea purpurea* aerial parts contain multiple antiviral compounds. Pharm. Biol. 43, 740–745.

Wen, C.C., Chen, H.M., Yang, N.S., 2012. Developing Phytocompounds from Medicinal Plants as Immunomodulators, Advances in Botanical Research.

Yildiz, E., Karabulut, D., Yesil-Celiktas, O., 2014. A bioactivity based comparison of *Echinacea purpurea* extracts obtained by various processes. J. Supercrit. Fluids 89, 8–15.

Zhai, Z., Haney, D., Wu, L., Solco, A., Murphy, P.A., Wurtele, E.S., Kohut, M.L., Cunnick, J.E., 2007. Alcohol extracts of Echinacea inhibit production of nitric oxide and tumor necrosis factor-alpha by macrophages *in vitro*. Food Agric. Immunol. 18, 221–236.