Universidade do Minho
Escola de Ciências

Catarina Isabel Guedes Teixeira

Diet in prevention and treatment of colorectal cancer: the role of selected legumes of the Mediterranean diet

Dezembro de 2018

Diet in prevention and treatment of colorectal cancer: the role of selected legumes of the Mediterranean diet
Catarina Isabel Guedes Teixeira
UMinho | 2018

Universidade do Minho
Escola de Ciências

Catarina Isabel Guedes Teixeira

Diet in prevention and treatment of colorectal cancer: the role of selected legumes of the Mediterranean diet

Tese de Doutoramento em Ciências
Programa Doutoral em Cadeias de Produção Agrícola - da mesa ao campo (Agrichains)

Trabalho efetuado sob a orientação da:
Professora Doutora Cristina Pereira Wilson
Professora Doutora Ana Isabel Barros

Dezembro 2018

DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados.

Mais declaro que tomei conhecimento do código de ética da Universidade do Minho.

Universidade do Minho, 19 / 12 / 2018

Assinatura: Catarina Isabel Guedes Teixeira

Acknowledgements

First of all, I would like to thank Professor Doctor Cristina Pereira Wilson, my main supervisor, for giving me the opportunity to do this work, for all the scientific support, fruitful discussions, encouragement and scientific acknowledgement. I am grateful for the opportunity to learn from you and for the enormous privilege of being your student.

I also like to thank my supervisor, Professor Doctor Ana Barros for accepting me in her laboratory and for introducing me to the colorimetrical and analytical analysis of phenolic compounds.

I would also like to thank the Directors of the PhD Program Agricultural Production Chains – From Fork to Farm (Agrichains) and the Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB-UTAD) and (CITAB-UM) for allowing me to develop this research project and for having provided the resources needed.

I would also like to extend my thanks to all the people of the Department of Biology including Professors, technicians and colleagues for always helping me when I needed and providing a good environment.

To all my laboratory colleagues (also for all that already passed in the lab) for the stimulating discussions and for all the fun we have had in the last years. A special thanks to Anabela, Carla and Cristina for the conversations, great working environment in the laboratory, and most important for your friendship.

I would like to thank all my PhD colleagues, with whom I have shared moments of deep anxiety but also of big excitement.

To all the people of the "lunch team", for their good disposition, company and friendship.

A warm word for my great friends Cátia, Iva, Weina, Nikola, Leslie and Isabelle, that always managed to make me feel special, your unconditional support, companionship and friendship.

Finally, I must express my profound gratitude to my parents for providing me with constant support and encouragement throughout all my years of study. To my brothers, Bruno and Alex, for all support and love.

Thank you all!

The author Catarina Isabel Guedes Teixeira acknowledges the financial support provided FCT-Portuguese Foundation for Science and Technology (SFRH/BD/52544/2014), under the Doctoral Program "Agricultural Production Chains-from fork to farm" (PD/00122/2012).

This work was supported by the European Investment Funds FEDER/COMPETE/POCI– Operational Competitiveness and Internationalization Programme [POCI-01-0145-FEDER-006958] and Portuguese Foundation for Science and Technology (FCT) [UID/AGR/04033/2013].

Project I&D Interact - Integrative Research in Environment, Agro-Chain and Technology [NORTE-01-0145-FEDER-000017], co-financed by European Regional Development Fund (FEDER) through NORTE 2020 (Programa Operacional Regional do Norte 2014/2020).

Abstract

Although legume intake is associated with health benefits, the consumption of *Vigna unguiculata* (cowpea) and several *Phaseolus vulgaris* varieties (common beans) has been decreasing in Mediterranean countries, where they used to be an important part of the diet, due to increasing western influence on dietary habits. In contrast with soy, which anticancer activity has been reported in different studies, little is known about legume mechanisms of health improvement, especially regarding colorectal cancer. This thesis aims therefore to provide molecular characterization of the anti-colorectal cancer of extracts from several legumes (4 varieties of *Phaseolus vulgaris*, *Vigna unguiculata* and *Glycine max*) using raw, processed (boiled and pressure cooked) and as 5-day old cowpea sprouts.

The phenolic characterization revealed that both cooking methods (boiling and pressure) increase the total phenolic content and antioxidant activity in methanolic extracts of the four *Phaseolus* varieties and cowpea, comparing with the raw extracts. In contrast, cooking of soy did not increase the antioxidant activity, but led to an increment of isoflavone content in the methanolic extracts. Accordingly, through high-performance liquid chromatography (HPLC) characterization, we found that boiling and pressure cooking increased by 41 and 27% the phenolic content in cowpea, 17 and 13% in black bean, respectively. In soy, the only sample containing isoflavones, daidzein and particularly genistein content increased several folds. In cowpea, sprouting increased total phenolic, flavonoid and *ortho*-diphenol content and also the antioxidant activity. The HPLC analysis showed a 75% increase in flavonoids, mostly quercetin glucosides.

To ascertain whether extracts of 5-day old cowpea sprouts could enhance the efficacy of 5-fluorouracil (5-FU), the most frequently used chemotherapeutic drug for colorectal cancer treatment, KRAS (HCT116 and HCT15) and BRAF (RKO and HT29) mutated cell lines were used. Although a more significant decrease in cell viability and increase in apoptosis induced by the sprout extract was observed in KRAS mutated cells, it was in BRAF mutated cells that the combination treatment of 5-FU and sprout extract was more promising. In fact, the presence of the extract significantly increased the 5-FU cytotoxicity. This effect did not seem to be due to quercetin alone, but it most likely results from the complex combination of phenolic compounds in the extract.

Globally, this study shows for the first time the effect of legume extracts on cell cycle arrest and associated pathways, further highlighting their anti-colorectal cancer

potential. The novel combination treatment of cowpea sprout extract and 5-FU may provide an innovative colorectal cancer therapy that can be explored in animal models and, later, in preclinical studies. Cowpea sprout extract can thus be explored as an added value food product and/or ingredient for functional foods and nutraceuticals. In conclusion, if deeper explored, the results gathered in this work may be used to provide guidelines for dietary recommendations regarding the inclusion of legumes for cancer prevention as well as for boosting the efficiency of conventional cancer treatments.

Resumo

Embora o consumo de leguminosas esteja associado a benefícios para a saúde, o consumo de feijão-frade bem como de enumeras variedades de *Phaseolus vulgaris* tem vindo a diminuir nos países Mediterrâneos. Este decréscimo é essencialmente devido à influência do estilo de vida ocidental, particularmente nos hábitos alimentares. Em contraste com a soja, cuja atividade tem sido reportada em vários estudos, pouco se sabe sobre os mecanismos de ação das leguminosas na saúde, particularmente no cancro colorretal. Esta tese visa, portanto, fornecer uma caracterização molecular dos efeitos anti-cancro colorretal de extratos de várias leguminosas, nomeadamente de *Vigna unguiculata*, *Glycine max* e de quatro variedades de *Phaseolus vulgaris*, cruas, processadas (cozidas e cozidas sob pressão) e com rebentos de feijão frade com 5 dias de germinação.

A caracterização fenólica revelou que ambos os métodos de processamento (cozedura e cozedura sob pressão) aumentaram o teor total de fenólicos e a atividade antioxidante nos extratos metanólicos das quatro variedades de Phaseolus e feijão-frade, em comparação com os extratos de feijão cru. Em contraste, o processamento de soja não aumentou a atividade antioxidante, mas levou a um incremento do teor de isoflavona nos extratos metanólicos. Em concordância, através da caracterização por cromatografia líquida de alta eficiência (HPLC), verificou-se que a cozedura sem e sob pressão aumentaram em 41 e 27% o conteúdo fenólico no feijão-frade, 17 e 13% no feijão preto, respetivamente. Na soja, a única amostra que apresentou isoflavonas, o conteúdo em daidzeína e particularmente em genisteína aumentou várias vezes. No feijão-frade, a germinação aumentou o teor total de fenólicos, flavonoides e orto-difenol e também a atividade antioxidante. A análise por HPLC mostrou um aumento de 75% nos flavonoides, principalmente quercetinas glicosiladas.

Para perceber se os extratos de rebentos de feijão frade com 5 dias poderiam aumentar a eficiência do 5-fluouracilo (5-FU), o fármaco quimioterapêutico mais utilizado na terapia do cancro colorretal, foram utilizadas linhas celulares com mutações no KRAS (HCT116 e HCT15) e no BRAF (RKO and HT29). Apesar de o extrato induzir uma diminuição na viabilidade celular e um aumento da apoptose mais significativo nas células com mutação no KRAS, foi nas células com mutação no BRAF que o tratamento de combinação com o 5-FU e extratos de rebentos foi mais promissor. De facto, a presença do extrato aumentou significativamente a citotoxicidade do 5-FU.

Este efeito não parece ser devido à quercetina apenas, mas resultaram provavelmente de uma combinação complexa de compostos fenólicos presente no extrato.

Globalmente, este estudo demonstrou pela primeira vez o efeito de extratos enriquecidos em fenólicos de leguminosas na paragem do ciclo celular e nas vias de sinalização associadas, reforçando o seu potencial anti-cancro colorretal. O novo tratamento combinado de extratos de rebentos de feijão frade com 5-FU poderá constituir como uma terapia inovadora para o cancro colorretal que poderá ser explorado em modelos animais e, mais tarde, em estudos pre-clínicos. Os extratos de rebentos de feijão frade poderão assim ser explorados como um produto alimentar de valor acrescentado e/ou ingrediente para alimentos funcionais e nutracêuticos. Em conclusão, se melhor explorados, os resultados obtidos neste estudo poderão ser usados em guias para recomendação nutricional no que diz respeito à inclusão de leguminosas para a prevenção do cancro, bem como, para melhor a eficiências das terapias de cancro convencionais.

Index

Index of Figures

Index of tables

List of abbreviations

5-FU - 5-fluorouracil

ACE – Angiotensin-1 converting enzyme

ABTS - 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid

ACF - aberrant crypt foci

AIF - apoptosis-inducing factor

AKT - protein kinase B

AMPK - AMP-activated kinase

AOM - azoxymethane

Apaf-1 - apoptotic protease activating factor-1

APC - adenomatous polyposis coli

BAD - Bcl-xL/Bcl-2-associated death promoter

BAK - Bcl-2-antagonist/ killer-1

BAX - Bcl-2-associated X protein

Bcl-xL - basal cell lymphoma-extra large

Bcl-2 - B-cell lymphoma protein-2

BCR - B cell receptor

BID - BH3-interacting death domain agonist

BIM - Bcl-2 interacting mediator of cell death

BRAF - v-raf murine sarcoma viral oncogene homolog B1

CDKs - cyclin-dependent kinases

CRC - colorectal cancer

CHD - coronary heart disease

CVD - cardiovascular diseases

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

EGF - epidermal growth factor

BIM - Bcl-2 interacting mediator of cell death

FADD - FAS-associated death domain protein

FAP - familial adenomatous polyposis

FRAP - ferric reduction antioxidant power

GI- glycemic index

GPCR - G protein-coupled receptor

GTPases - guanosine triphosphatases

HPLC - high performance liquid chromatography

IBD - inflammatory bowel disease

IC50 - half maximal inhibitory concentration

IDF - insoluble dietary fiber

JNK - c-Jun N-terminal kinase

KRAS - v-ki-ras 2 Kirsten rat sarcoma viral oncogene homolog

LDL - low-density lipoproteins

MAPK - mitogen-activated protein kinase

MLH1 - human mutL homolog 1

MSH2 - human mutS homolog 2

MSI - microsatellite instability

mTOR - mammalian target of rapamycin

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB - Nuclear factor kappa B

oDC - *ortho*-diphenol content

PDK1 - 3-phosphoinositide-dependent protein kinase 1

PH - pleckstrin homology

PI3K - phosphatidylinositol 3-kinase

PIK3CA - phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit-alpha

PIP2 - phosphatidylinositol-4,5-bisphosphate

PIP3 - phosphatidylinositol-3,4,5-trisphosphate

PTEN - phosphatase and tensin homolog

pRB – phosphor-retinoblastoma protein

qPCR - quantitative PCR

RB - retinoblastoma protein

ROS - reactive oxygen species

RTK - receptor tyrosine kinase

SCFA - short-chain fatty acid

SDF - soluble dietary fiber

SMAC - second mitochondria-derived activator of caspases

T2D - type 2 diabetes

TDF- total dietary fiber

TdT - terminal deoxynucleotidyl transferase

TFC - total flavonoid content

TGFBR2 - transforming-growth factor-beta receptor 2

TGF-β - transforming growth factor beta

TPC - total phenolic content

TRAIL - TNF-related apoptosis-inducing ligand

TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling

WNT - wingless-int

XIAP - X linked inhibitor of apoptosis protein

List of publications

The work performed during this PhD thesis resulted in the following publications:

Manuscripts

Teixeira-Guedes, C.I., Oppolzer, D., Barros, A. I., & Pereira-Wilson, C. 2018. Impact of cooking method on phenolic composition and antioxidant potential of four varieties of *Phaseolus vulgaris* L. and *Glycine max* L. *LWT - Food Science and Technology*. **Minor revisions for acceptance**

Teixeira-Guedes, C.I., Carvalho, A.C. & Pereira-Wilson, C. 2018. Anticancer effects by cell cycle modulation of polyphenolic-rich extracts from cooked cowpea, black bean, and soy on colorectal cancer cell lines. **Submitted**

Teixeira-Guedes, C.I., Oppolzer, D., Barros, A. I., & Pereira-Wilson, C. 2018. Sprouting of cowpea increases its phenolic content, antioxidant activity and anti-colorectal cancer properties. **Submitted**

Teixeira-Guedes, C.I., & Pereira-Wilson, C. 2018. Phenolic rich extracts from cowpea sprouts decrease cell proliferation and enhance of 5-fluorouracil effects in human colorectal cancer cell lines. **Submitted**

Abstracts in indexed journal

Teixeira-Guedes, C.I., & Pereira-Wilson, C. 2016. Methanolic extracts of cooked black bean and cowpea produce G0/G1 arrest in colorectal cancer cells independently of p53 status. 5th European Nutrition and Dietetics Conference. Rome, Italy. J Nutr Food Sci 6:3 p100. doi:10.4172/2155-9600.C1.021

Communications as an invited speaker

Teixeira-Guedes, C. 2017. The role of selected bean species of the Mediterranean diet in prevention and treatment of colorectal cancer. Advance course - molecular nutrition. University of Minho, Braga

Teixeira-Guedes, C. 2017. Comet assay – Ensaio Cometa. Advance course - molecular nutrition. University of Minho, Braga

Teixeira-Guedes, C. 2016. The role of selected bean species of the Mediterranean diet in prevention and treatment of colorectal cancer.10as Jornadas de Biologia. University of Trás -os Montes and Alto Douro, Vila real

International oral presentations

Teixeira-Guedes, C.I., Matos, C., Pereira-Wilson, C. & Barros, A.I. 2016. Effect of heat processing on selenium content and antioxidant activity of several beans. XXII Encontro Luso-Galego de Química. QAMA30, p100. Instituto Politécnico de Bragança, Bragança

Teixeira-Guedes, C.I., Barros A., & Pereira-Wilson, C. 2015. Legumes as a source of phenols and natural antioxidants: impact of heat processing. XXI Encontro Galego Portugués de Química. AMA 06, p98. Pontevedra (España)

National oral presentation

Teixeira-Guedes, C. I., Pereira-Wilson, C. 2015. Impact of legumes processing on the anti-colorectal cancer effects. 2º Simpósio Nacional, Promoção de uma Alimentação Saudável e Segura Qualidade Nutricional e Processamento Alimentar. CO3, p45. Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisboa

International poster presentation

Teixeira-Guedes, C., Pereira-Wilson, C. 2016. Methabolic extracts of cooked black bean and cowpea produce G0/G1 arrest in colorectal cancer cells independently of p53 status. 5th European Nutrition and Dietetics Conference. Rome, Italy. J Nutr Food Sci 6:3 p100

National poster presentation

Teixeira-Guedes, C.I., Pereira-Wilson C. 2017. Polyphenolic-rich extracts from cooked black bean and cowpea induce cell cycle G1 arrest in HCT116 human colorectal cancer cells independently of p53 status. 9º Simpósio de Metabolismo, p27. Faculdade de Medicina Universidade do Porto. Porto

Teixeira-Guedes, C., Barros, A., Pereira-Wilson, C. 2016. Legumes of Mediterranean diet produce G0/G1 arrest in colorectal cancer cells independently of p53 status. XIX national Congress of Biochemistry. SPB2016 – Sociedade Portuguesa de Bioquímica. S6 Cell Biology: Signaling and Regulation, p92. University of Minho, Guimarães.

CHAPTER 1

GENERAL INTRODUCTION

1. General introduction

Nutrition plays an important role in the etiology, prevention and progression of several diseases (Reedy *et al*., 2014). Diets rich in fruits, vegetables, legumes, whole grains, proteins from plant foods and low in meat and animal products have been shown to have long term beneficial effects on health (Lopez-Garcia *et al*., 2014). The Mediterranean diet is inspired by the eating habits of countries of the Mediterranean basin and is based on this type of foods (Willett *et al*., 1995). Ingestion of high levels of dietary fiber, antioxidants, unsaturated fatty acids and of low glycemic index (low-GI) foods are some of the features of this diet (Schroder, 2007).

Legumes are produced worldwide, including the Mediterranean Region being an important ingredient of the Mediterranean cuisine. Historically this food has been a major food ingredient, however, its consumption has been decreasing in the last decades, especially in populations that adopted the western life-style. The consumption of beans has been undervalued in North America and North Europe (Messina, 2014). The same trend was observed in countries of the Mediterranean Region and this has led to an increased incidence of chronic diseases such as, obesity, cardiovascular diseases and cancer (Chávez-Mendoza & Sánchez, 2017).

Emerging research demonstrates that legumes have enormous potential to help prevent or manage chronic disease such as diabetes, cardiovascular, obesity and prevent cancer development especially colorectal cancer (CRC), contributing to overall health and wellness (Bassett *et al*., 2010; Jenkins *et al*., 2012; Papanikolaou and Fulgoni, 2008). Recently, this food is emerging as a functional and nutraceutical, due to its richness in nutrients and variety of phytochemicals with potential health benefits (Jenkins *et al*., 2012; Mollard *et al*., 2012; Papanikolaou and Fulgoni, 2008).

Several studies have demonstrated the anti CRC effects of legumes in human trials, in animal models and *in vitro* models (using colorectal cancer cell lines). However, the mechanism undergoing this effect need to be studied to better understand the diet-gene interactions which may modulate colorectal cancer.

1.1. Legumes for human nutrition

Economically, the Leguminosae are the most important family in the Dicotyledonae class, with many cultivated species over the world. Legumes, one of the first crops cultivated by humans, belong to a large botanical family, consisting of more than 18 000 species classified into approximately 650 genera (Duranti, 2006). Legumes include alfalfa, clover, lupin, green beans and peas, peanuts, chickpeas, lentils, dry peas, faba bean, cowpea, common beans and soy. Legumes are mainly cultivated for human consumption; however, some species such as lupin, alfalfa and clover are principally grown as green manures and forages. The words "legumes" and "pulses" are normally used as synonyms, however, pulses in specific are dry seeds of annual leguminous crops yielding from one to 12 seeds of variable shape, size and color within a pod. This denomination excludes green beans and green peas (considered vegetables), peanut and soy (grown principally for oil extraction) and clovers (used exclusively for sowing) (Campos-Vega *et al*., 2010). Legumes are the second most important food crop produced and consumed worldwide after cereal grains. In comparison to cereal grains, legume seeds are richer in high-quality protein, providing a highly nutritious food resource (Duranti, 2006; Rebello *et al*. 2014). Approximately 20 leguminous species are used as dry grains for human nutrition, within these, the pea (*Pisum sativum*) is highly consumed in Asian countries, common bean (*Phaseolus vulgaris*) in Latin America and African countries, chickpea (*Cicer arietinum*) in India, lentil (*Lens culinaris*) in countries of the Middle East and cowpea (*Vigna unguiculata*) in Southeast Africa and Mediterranean countries (Bouchenak & Lamri-Senhadji, 2013; Gonçalves *et al*., 2016).

The World Health Organization (WHO) recommends the consumption of 30 g of legumes per day to aid the prevention of chronic diseases. Among European countries, higher legume consumption is observed around the Mediterranean area, with daily consumptions of 8 to 23 g per capita, while in Northern Europe, the daily consumptions are approximately 5 g per capita (Bouchenak & Lamri-Senhadji, 2013).

Table 1 displays the list of main edible legumes consumed worldwide and region of consumption.

Table 1. List of main edible legume seeds.

Legumes	*Latin name*	*Region of consumption*
Dry beans		
Kidney, haricot, pinto, navy, borlotti, black beans	*Phaseolus vulgaris*	World-wide
Lima bean, butter bean	*Vigna lunatus*	América, Africa
Adzuki bean	*Vigna angularis*	Asia
Mung bean, golden gram	*Vigna radiate*	Asia
Black gram	*Vigna mungo*	Asia
Rice bean	*Vigna umbellate*	Asia
Moth bean	*Vigna acontifolia*	India
Tepary bean	*Vigna acutifolius*	Southwest USA, Central America
Broad bean, faba bean	*Vicia faba*	Temperate regions
Peas		
Garden pea	*Pisum sativum var. sativum*	World-wide
Protein pea	*Pisum sativum var. arvense*	World-wide
Chickpea	*Cicer arietinum*	Asia, Middle East
Cowpea, black eye pea	*Vigna unguiculata* ssp.	Africa, Asia, South America
Lentil	*Lens culinaris*	World-wide
Lupins	*Lupinus* spp.	Australia, Mediterranean region
Non-pulses		
Soy	*Glycine max*	World-wide, especially Asia
Peanut	*Arachis hypogaea*	World-wide

Adapted from Campos-Vega *et al*., 2010).

1.1.1. Common beans

Common beans (*Phaseolus vulgaris* L.) are annual plants, cultivated for their edible dry seeds in temperate and semitropical regions. They are many varieties such as navy, kidney, red, black, pinto, butter and borlotti beans. Common beans have been domesticated in two separate events, in Mexico and Peru, 7 000 and 8 000 years ago, respectively and now consumed worldwide (Ganesan & Xu, 2017; Hayat *et al*. 2014). In temperate regions, the immature pod and green leaves are edible and consumed as vegetables, while young leaves are also eaten as salads in some parts of Asian. Dry beans are mainly consumed in low- and middle-class families, as well as people with vegetarian lifestyle, being a large portion of proteins in their diets. Common bean is produced worldwide but the biggest producer is Myanmar followed by India, Brazil, USA, Mexico, Tanzania and China (Ganesan & Xu, 2017).

1.1.2. Cowpea

Cowpea (*Vigna unguiculata* L. Walp) originated in the African continent where it contributes to the diet of millions of people (Gonçalves *et al*., 2016; Karapanos *et al*., 2017; Phillips *et al*., 2003). Its cultivation has spread worldwide, but is especially cultivated across Southeast Asia, Africa, Southern United States and Latin America, as well as in some Mediterranean countries, although it is not widespread in Europe (Domínguez-Perles *et al*., 2016; Gonçalves *et al*., 2016). Compared to other legumes, cowpea is very versatile and drought tolerant, adapting well to different types of soil and arid conditions, producing significant yields under low-input farming systems (Gonçalves *et al*., 2016; Karapanos *et al*., 2017).

1.1.3. Soy

Glycine max L., known as soy, is an annual legume of the Fabaceae family. This legume is native to East Asia (including China, Japan and Korea) and has been domesticated more than 3 000 years ago for its edible seeds and young pods. It is today the world's most important legume crop growing in diverse climates and the most widely used as oilseed (Waqas *et al*., 2015). Soy pods may be harvested when still green for use as a fresh vegetable in the Japanese cuisine or fully mature and dried for numerous Asian dishes. Soy sprouts are also commonly used as a fresh vegetable. Soy is high in both protein and fat, it can be processed into diverse food products, including, fermented (tofu and tempeh) and hydrolyzed (textured soy) products, soy sauce, soy paste (miso) and soy milk.

1.2. Chemical composition of dry legumes

Pulses are recognized as a beneficial source of nutrients and are recommended by health organizations and dieticians as an essential food. They represent an important source of vegetarian protein and have low-glycemic index (low-GI), being recognized as an important food choice with significant potential health benefits (Bouchenak & Lamri-Senhadji, 2013). The bean's nutritional properties are linked to their nutritional content. Beans are rich sources of macronutrients (carbohydrate and protein), micronutrients (vitamins and minerals) and phenolic compounds. In table 2 are shown the nutritional composition of macro and micronutrients of several legumes.

Table 2. Nutritional composition of several legumes, values expressed per 100 g of dry weight.

	Common bean	Cowpea	Soy	Pea	Faba bean	Chickpea	Lentils
Protein (g)	21,4	22,6	32,8	22,7	25,8	19	25,2
Fat (g)	1,4	1,3	19,3	1,3	1,3	5	0,7
Total CH (g)	43,3	55,3	18,3	49,4	32,8	51,4	47,6
Mono-disaccharides (g)	2,6	3,1	6,6	2,3	6,2	2,8	1,2
Oligosaccharides (g)	3,7	3,9	6,3	1,7	2,3	3,4	3,1
Starch (g)	37	48,3	5,4	45,4	24,3	45,2	43,3
Vitamin A (µg)	0	5	17	44	5	10	10
Carotene (mg)	0	30	103	62	30	60	60
α- Tocopherol (mg)	0,3	0,3	2,9	1	0,46	2,7	0
Thiamine (mg)	0,50	0,54	1,2	0,88	0,43	0,41	0,43
Riboflavin (mg)	0,18	0,18	0,49	0,3	0,26	0,15	0,27
Niacin (mg)	4,8	6,9	7	6,6	6,7	4,4	1,9
Vitamin C (mg)	0,5	0,37	0,6	0,13	0,37	0,5	0,93
Folates (µg)	330	630	328	33	145	180	110
Ashe (g)	2,9	3,2	5	2,3	3,5	3	2,4
Na (mg)	33	18	4	40	13	6	12
K (mg)	1260	1100	1750	1036	1086	980	940
Ca (mg)	171	81	246	61	105	136	74
P (mg)	310	410	667	336	334	241	360
Mg (mg)	160	130	254	121	196	100	114
Fe (mg)	6,2	5,2	8	3,7	5	6,3	6.8
Zn (mg)	3,8	3,5	3,8	3,7	3,2	2,5	3,9

Source (Instituto Nacional de Saúde Dr. Ricardo Jorge, 2015). CH - Carbohydrates.

1.2.1. Carbohydrates

Carbohydrates are the major component of beans, accounting for 55-75% of the dry matter. Their major constituents are starch, dietary fiber (non-starch polysaccharides) and mono, di and oligosaccharides. Among all carbohydrates, starch is the major carbohydrate found in beans, accounting approximately 50% of total seed weight. Starch is mostly constituted by amylose (linear molecule) and amylopectin (branched

molecule) (Chávez-Mendoza & Sánchez, 2017; Ganesan & Xu, 2017). Legumes contain between 30-40% of amylose (5-10% more amylose than cereals). Higher amylose than amylopectin is correlated with higher resistant starch content. The starch of beans can be degraded into glucose and oligodextrin by different enzymes namely, α- and β-amylases. According to the amylose susceptibility, the rate of glucose release and its absorption, the starches can be characterized as resistant starch, slowly digestible starch and rapidly digestible starch (Tharanathan & Mahadevamma, 2003).

The ingestion of rapidly digestible starch results in rapid increase of glucose in the blood while slowly digestible starch results in a slower rate. Resistant starch is the fraction that resists hydrolysis by the amylopectin enzymes and consists of insoluble and soluble dietary fiber as well as non-digestible oligosaccharides that resist in the small intestine to the hydrolysis but are fermented in the large intestine by the colonic microflora (Tiwari *et al*., 2011). The type and quantity of resistant starch from pulses have a major influence on the intestinal microbiota composition, and thereby the production, content and distribution of short-chain fatty acid (SCFA), such as acetic, propionic and butyric acids (den Besten *et al*., 2013). Different factors such as amylose/amylopectin ratio, molecular structure of amylopectin, amylose chain length, degree of crystallinity and size of granules might influence the digestibility of starch (Hayat *et al*., 2014).

Dietary fiber is essentially composed by cellulose, hemicelluloses, pectic polysaccharides and lignin. The soluble sugar fraction includes monosaccharides (ribose, glucose, galactose and fructose) and disaccharides (sucrose and maltose) (Chávez-Mendoza & Sánchez, 2017; Ganesan & Xu, 2017).

1.2.2. Proteins

During the maturation, pulses accumulate a large amount of proteins, being the richest plant source of dietary protein for human nutrition. In the dry weight, the protein of legumes seeds represents around 20% in pea and beans and 32 to 40% in soy and lupine (Duranti, 2006). Proteins are classified as albumin, globulins, prolamin, glutelin and residue, based on sequential extraction using distilled water, dilute salt solution, dilute alkali and 70% ethanol. Globulins constitute 50-70% of total protein and are classified as 7S and 11S globulins according to their sedimentation coefficients (Tang & Sun, 2011). Legume also contains essential amino acids very important to the human's nutrition such as lysine, tyrosine, phenylalanine, arginine, isoleucine; histidine and

methionine, providing balanced amino acid profiles. Legume seeds also contain several minor proteins called anti-nutrient, that are relevant to the nutritional/functional quality of the seed, those proteins including protease and amylase inhibitors, lectins, lipoxygenase and defense proteins (Duranti, 2006; Ganesan & Xu, 2017; Rebello *et al*., 2014).

1.2.3. Lipids

In the lipid fraction, bean seeds contain 2-21% of fat. It is composed mainly of mono- and polyunsaturated fatty acids, accounting for 62 % of the total fat content. The predominant unsaturated fatty acids are linoleic (21–53%) and linolenic (4–22%) acids (Campos-Vega *et al*. 2010b).

1.2.4. Vitamins and minerals content

Vitamins are micronutrients organic compounds that cannot be synthetized by humans. Pulses are good sources of B-vitamins, namely riboflavin, thiamin, niacin, pyridoxine, biotin and folic acid and of E-vitamin (tocopherol) (Campos-Vega *et al*., 2010a). Niacin is a coenzyme of the energy metabolism and fatty acid metabolic pathways, it is also involved in the modification of chromosomal proteins that takes part in DNA repair as well as cell differentiation. In addition, niacin is required for mobilization of calcium from intracellular stores. Regular consumption of some pulses can provide 100% of the folate daily requirements. Folate modulates DNA methylation, being an important tool for maintaining good health. Global DNA hypomethylation and targeted hypermethylation are considered irregular patterns of DNA methylation found in many human diseases, such as cancer, developmental disabilities and imprinting disorders (Rebello *et al*., 2014).

Minerals are essential micronutrients that plays essential role in human's health. Legume are also an important source of minerals, having high levels of iron, zinc, calcium, phosphorous, selenium, and magnesium, while other minerals are also found in appreciable amounts. Iron is essential for respiration and energy metabolism. Zinc is a component of numerous metalloenzymes having a catalytic activity or playing a role in the structural integrity of the enzymes. Zinc deficiency has been associated with the development of cardiovascular and kidney diseases. Calcium is an important component of the structure of bones and teeth and it is involved in blood clotting, enzyme

regulation, nervous impulse transmission and muscle contraction. Selenium is an essential micronutrient in human nutrition, it is cofactor for glutathione peroxidase, an enzyme that protects against oxidative damage (Rebello *et al*., 2014).

1.3. Non-nutrient compounds

Legumes possess valuable nutritional composition; however, they also contain non-nutrient compounds. They are elements present in some foods that are not strictly required by the body to function. Their intake may have a positive or negative impact, depending on the dose consumed. The seeds accumulate these compounds as a defense mechanism against the attack of insects, fungi, and herbivorous animals in order to grow in adverse conditions (Rebello *et al*., 2014). The non-nutrient compounds can be classified into two groups: the first corresponds to compounds of protein nature, including lectins and protease inhibitors (like trypsin and chymotrypsin inhibitors); and the second of non-protein nature, which include alkaloids, phytic acid, phenolic compounds and saponins. Some bioactive compounds found in legumes and their biological activities are present in table 3.

1.3.1. Lectins

Lectins are a diverse group of proteins with great affinity for carbohydrates or biomolecules that contain bond carbohydrates. This complementarity is highly specific and allows lectins to have a wide range of biological activities. These activities include, cell agglutination, toxicity and inhibition of cell growth (Kumar *et al*., 2012; Yue *et al*., 2016). It has been reported that lectins contained in the common bean impair the growth of the MCF7 breast cancer cell line and HepG2 hepatocarcinoma cell line. Carcinogenic cells secrete or express gluco-conjugates with abnormal glycan structure, lectins can detect such changes and may be used for cancer diagnosis and/or treatment (Hashim, Jayapalan, & Lee, 2017; Kumar *et al*., 2012). They may also potentially be used as nutraceuticals to control obesity, attributed to their ability to resist gastric digestion, be absorbed by the bloodstream while remaining biologically active and decreases insulin levels (Chávez-Mendoza & Sánchez, 2017; Roy *et al*., 2010).

1.3.2. Protease Inhibitors

Protease Inhibitors are defined as thermo-sensitive compounds of protein nature that modify protein digestion by inhibiting the activity of digestive enzymes involved in the hydrolysis of those proteins consumed in the diet. Those inhibiting serine-proteases, such as trypsin and chymotrypsin are the best known (Guillamón *et al*., 2008). These inhibitors are resistant to pepsin and the pH of the gastric tract. They also act by suppressing the negative feedback regulation of pancreatic secretions through the release of the cholecystokinin hormone from intestinal mucosa. This may stimulate a pancreatic hypertrophy, decreasing the hydrolyze of dietary proteins and consequently reduces the amino acid absorption and on de novo protein synthesis (Chávez-Mendoza & Sánchez, 2017; Guillamón *et al*., 2008). In spite of being considered anti-nutritional factors, several reports have mentioned their potential as anti-inflammatory agents, anti-hypertension, anti-cancer and against neurodegenerative diseases (Roy *et al*., 2010).

1.3.3. Saponins

Saponins are characterized by possessing a structure containing a steroidal aglycone or a triterpenoid bound to one or more sugar chains. These bioactive compounds possess a complex structure comprised of a hydrophobic steroidal nucleus and a hydrophilic moiety constituted by monosaccharide units. The main saponins contained in common bean cotyledons and seed coats are soyasaponin A, soyasaponin B and phaseoside I, however soyasaponin I has been reported as the predominant form (Figure 1) (Chávez-Mendoza & Sánchez, 2017).

Several epidemiological and experimental studies have demonstrated the beneficial effects of saponin on humans. It has been found that these compounds have hypocholesterolemic, immunostimulant, anti-carcinogenic, hypoglycemic, anti-thrombotic, diuretic and anti-inflammatory effects, they reduce the risk of heart diseases and possess an elevated antioxidant capacity (Chávez-Mendoza & Sánchez, 2017; Ramírez-Jiménez *et al*., 2015; Shi *et al*., 2004).

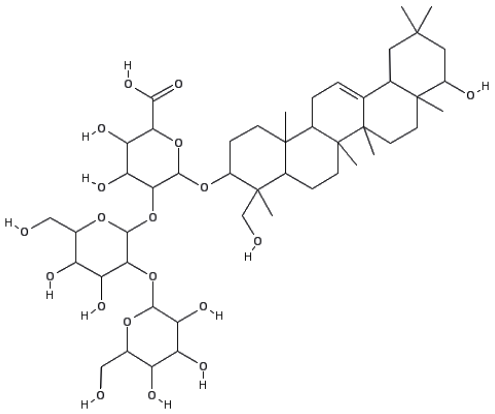

Figure 1. Chemical structure of soyasaponin I of *Phaseolus vulgaris* L.

1.3.4. Phytic acid

Phytic acid is the major form of phosphorus storage in plants and its salts are known as phytates. It has been recognized as an anti-nutrient due to its ability to decrease the bioavailability of protein- or starch-bound divalent and trivalent cations such as iron and zinc thus reducing their absorption (Campos-Vega *et al*., 2010a; Díaz-Batalla *et al*., 2006). Therefore, a diet with a high-energy content obtained from legumes and grains may lead to nutritional deficits. However, their interaction with starch and divalent cations cause a low glycemic index and it reduces iron participation in metal oxidation reaction thus mediating the effects correlated with a decreased risk of diabetes and cardiovascular diseases. Additionally, the antioxidant and anti-DNA damage effects have been reported for phytic acid (Campos-Vega *et al*., 2010a; Chávez-Mendoza & Sánchez, 2017).

1.3.5. Alkaloids

Alkaloids constitute a group of very diverse compounds consisting of a heterocycle with a nitrogen atom within the cycle. This conformation confers a basic character on the molecule, which tends to acquire a proton in aqueous solution (e.g. ricinine) (Tiwari *et al*., 2011). The majority of alkaloids from edible legumes have been reported from lupins. It has been documented that lupin and quinolizidine alkaloids may play a role in helping to facilitate the secretion of insulin, having a potential role for the management of type II diabetes (Bouchenak & Lamri-Senhadji, 2013; Tiwari *et al*., 2011).

1.3.6. Phenolic compounds

Common beans contain a large amount of polyphenols or phenolic compounds. Phenolic compounds are secondary metabolites that have different structure and function, it consists of an aromatic ring generally containing one or more hydroxyl groups. They are classified according to their chemical structure, ranging from simple molecules, such as phenolic acids, to complex polymers such as tannins and lignin. Phenolic content can be affected by external factors related to crop location, such as temperature, fertilization levels, occurrence of pests and diseases, time since harvest, farming practices and food preparation methods (Hayat *et al*., 2014).

Polyphenols are bioactive compounds widely known for their antioxidant properties, having the ability to neutralize free radicals and to chelate transition metals, thus contradicting the initiation and propagation of oxidative reactions. They, therefore, have a very important role in decreasing the risk of cardiovascular diseases, diabetes, cancer, Alzheimer's and Parkinson's diseases (Chávez-Mendoza & Sánchez, 2017).

Legume seeds are divided into three main parts, cotyledon, seed coat and embryonic axe. The flavonoid compounds are located especially in the seed coat, whereas non-flavonoid phenolic compounds such as hydroxybenzoic acid and hydroxycinnamic acid derivatives are mostly found in the cotyledon (Chávez-Mendoza & Sánchez, 2017).

1.3.6.1. Flavonoids

Flavonoids share a common structure consisting of two aromatic rings that are linked through three carbons, forming an oxygenated heterocycle. These are classified in six subclasses, depending on their heterocycle: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (catechin and proanthocyanidin or condensed tannins) (Figure 2). The main flavonoids contained in both raw and cooked beans are catechin, quercetin, kaempferol, myricetin and procyanidin (Akillioglu & Karakaya, 2010; Hayat *et al*., 2014; Huber, 2016). The chemical representation is presented in Figure 3.

Flavonoid biological activity depends on the type of phytochemical constituents and the complexity of their structure and the composition of the flavonoids mixture, since it has been well established that phytochemical mixtures of fruits and legumes may provide protecting benefits to health, mainly through synergic effects between them (Aparicio-Ernandez *et al*,. 2005). The consumption of these flavonoids has been

inversely correlated with cancer and the risk of cardiovascular diseases (CVD). The mechanism of action involves the modulation of detoxifying enzymes and the inhibition of cell proliferation, although its most recognized effect is their antioxidant capacity (Chávez-Mendoza & Sánchez, 2017).

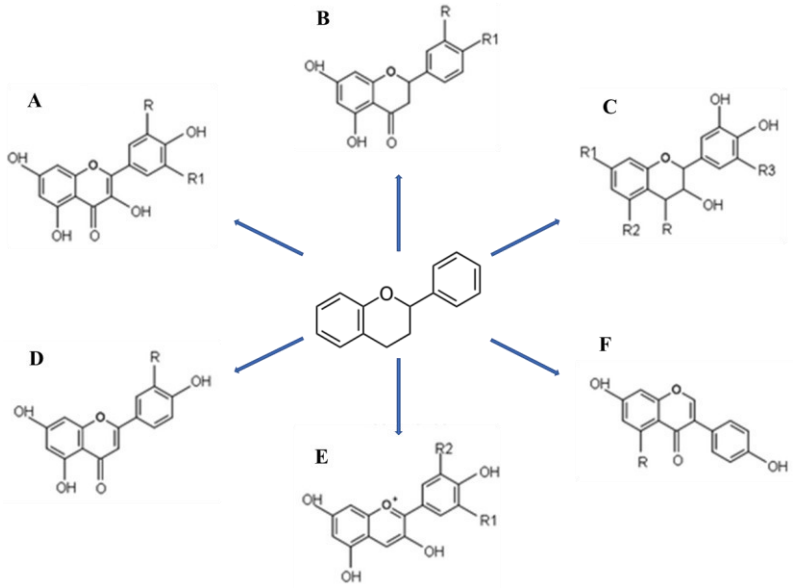

Figure 2. Chemical structure of A - flavonols, B - flavanones, C - flavanols, D - flavones, E - anthocyanins and F - isoflavones.

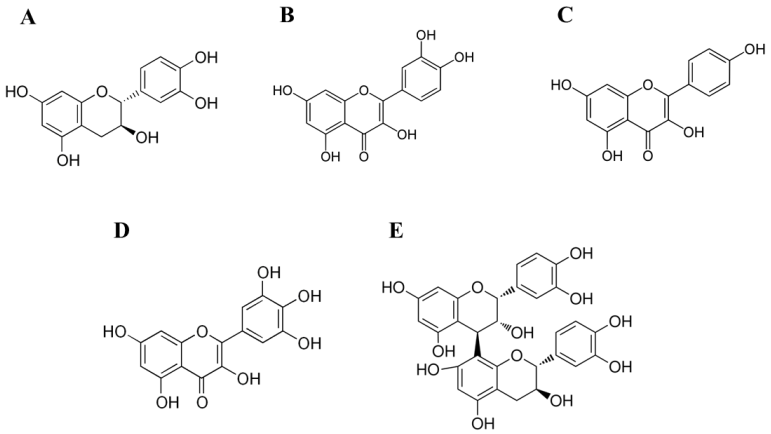

Figure 3. Chemical structure of the most common legume flavonoids**.** A - catechin, B - quercetin, C - kaempferol, D - myricetin and E - procyanidin.

1.3.6.1.2. Condensed tannins

The color of the bean seed coat is attributed to the presence and the amount of polyphenols such as flavonols glucosides, condensed tannins and anthocyanins. Their function is to provide protection against pathogens. Tannins are polymeric flavonoids that comprise a small part of the widely diverse group of phenolic compounds produced

by vegetables as secondary metabolites. Along with oxalates and phytates, they are considered as anti-nutritional by their effect in the nutrient bioavailability for the consumer. However, they are also considered as nutritionally important as they are antioxidants and have anti-mutagenic and anti-carcinogenic potential (Chávez-Mendoza & Sánchez, 2017; Díaz *et al*., 2010)

1.3.6.1.2. Anthocyanins

Anthocyanins are mostly present in colored beans such us black, red and pinto beans, while proanthocyanidins exists in almost all varieties of beans (Hayat *et al*., 2014). These compounds possess a high antioxidant activity and they are known to help prevent diseases such as cancer, atherosclerosis and inflammation (Chávez-Mendoza & Sánchez, 2017).

1.3.6.1.3. Isoflavones

Isoflavones are a subgroup of flavonoids, known as phytoestrogens because they exhibit pseudo-hormonal properties interacting with estrogen receptors, a property that results from their structural and functional similarity to the natural estrogen. Isoflavone content is variable among plant species and may even vary among genotypes of the same species, being particularly abundant in the Fabaceae/Leguminosae family. Chickpeas contain daidzein, genistein, formononetin and biochanin A. Soy have significantly higher levels of daidzein and genistein but contain less formononetin and biochanin A compared to chickpeas (Campos-Vega *et al*., 2010ab).

There are many health improving effects attributed to isoflavones including a reduction in osteoporosis, prevention cardiovascular disease and treatment of menopause symptoms. The main beneficial effects attributed to isoflavones are the prevention of breast cancer. This statement is based on observational studies and clinical trials (Chen *et al*., 2014; Messina, 2014). It has been described that isoflavones can block tumorigenesis by inhibiting enzymes required for DNA replication, metastasis, signal transduction; inactivating growth factors that promote angiogenesis, such as VEGF; and activating the immune system (Ziaei & Halaby, 2017).

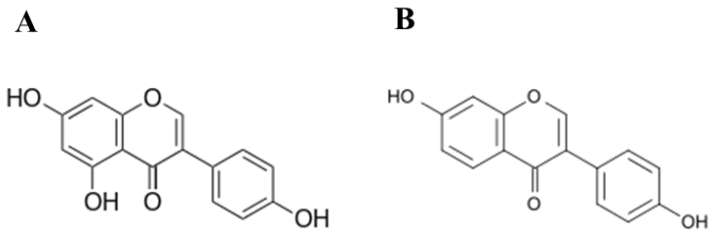

Figure 4. Chemical structure of A – genistein and B – daidzein.

1.3.6.2. Phenolic acids

Phenolic acids (shown in Figure 5) are of greatest importance in vegetables as they are precursors of other more complex phenolic compounds. These may be classified in two types: those derived from benzoic acid (e.g., *p*-hydroxybenzoic, vanillic and gallic acids) and those derived from cinnamic acid (e.g., ferulic, *p*-coumaric, chlorogenic, caftaric and caffeic acids). Studies have reported that phenolic acids have antioxidant, anti-inflammatory and immunostimulant properties (Campos-Vega *et al*., 2010a; Chávez-Mendoza & Sánchez, 2017).

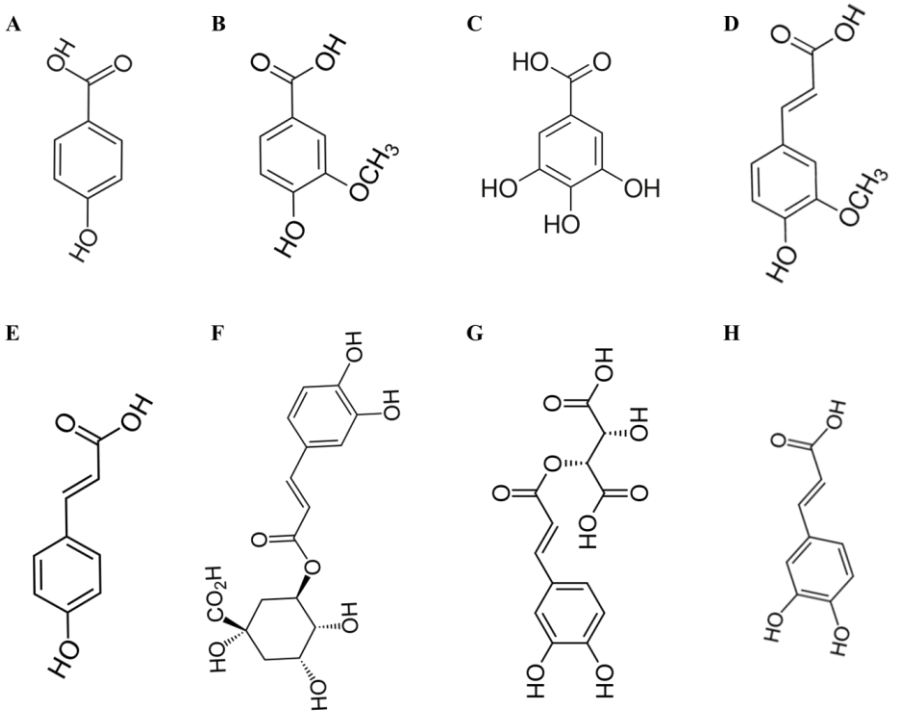

Figure 5. Chemical structure of phenolic acids: A - p-hydroxybenzoic, B – vanillic, C - gallic, D - ferulic, E - p-coumaric, F - chlorogenic, G - caftaric and H - caffeic acids.

Table 3. Bioactive compounds in legumes and their biological activities.

Bioactive compounds	Chemical Nature	Sources	Biological activity
Enzymatic inhibitors	Proteins	Soy, common bean, chickpea, faba bean, pea, lentil, peanut	Inhibition of proteolytic enzymes and α-amylase; Decrease of blood glucose (amylase inhibitor); Anticancer activity;
Lectins	Proteins or glycoproteins	Soy, common bean, jack bean, chickpea, faba bean, pea, lentil, peanut	Reduced intestinal absorption of nutrients; Decrease of blood glucose; Anticancer properties;
Polyphenols	Tannins, flavonoids, phenolic acids, lignins	Common beans, black gram, faba bean, colored pea, lentil, soy	Reduced food intake, protein and mineral utilization (tannins); Inhibition of digestive enzymes; Antioxidant and anticancer activity;
Phytic acid	Myo-inositol hexaphosphate	Soy, common bean, chickpea, faba bean, pea, lentil	Reduced mineral absorption; Antioxidant and anticancer activity.
Saponins	Steroidal or triterpenic glycosides	Soy, chickpea, faba bean, pea, lentil, peanut	Decrease of enzymatic activity; Anticancer properties; Reduction of blood cholesterol.
Alkaloids	Amino acids/purine/ pyrimidine	Lupin	Inhibition of digestion; Decrease of blood glucose.

Adapted from Tiwari *et al*., (2011).

1.4. Health benefits of legume consumption

Many components of legumes have been demonstrated to exert beneficial effects on human health, such us dietary fiber, proteins, peptides, lipids, phenolics, vitamins and minerals. The consumption of legumes has been strongly associated with numerous physiological and health promoting effects such as prevention and management of type 2 diabetes (T2D), hypercholesterinemia, cardiovascular diseases, obesity and cancer, same of these effects are summarized in table 4. In Figure 7, are presented the mechanisms involving reduction of cardiovascular diseases, obesity and diabetes mellitus by legumes.

1.4.1. Effects of legume consumption on obesity and diabetes management

Overweight and obesity are key features of the metabolic syndrome, a cluster of disease that results from deregulation of overall metabolism. Preventing overweight is a public health international priority. Patients with metabolic syndrome have an increased risk of developing T2D, cardiovascular disease and cancer. The role of legumes in weight management programs has been investigated. It has been suggested diets high in legumes results in low-GI, stimulating greater weight loss and reduce insulin resistance when compared with other weight reduction diets (Rebello *et al*., 2014). One study performed in the United, States (Haveman-Nies *et al*., 2001) found that individuals with diet rich in fiber, whole grains, and beans, had lower body mass index and smallest waist circumference. Wong *et al*., (2009) demonstrated that a diet containing pulses may reduce the risk of overweight or obesity, possibly by improving satiety and lowering energy intake (Figure 6).

Epidemiological studies strongly suggest that legumes protect against the development of T2D as well as being useful in the management of blood glucose in people who already have developed T2D. As previously mentioned, legumes have low-GI, due to their content of slow-release carbohydrate, resistant starch and high fiber content, benefiting glycemic and lipid control (Rebello *et al*., 2014). Several mechanisms have been suggested in order to explain the protective effects of dietary fiber, one of each is the increase of stool volume and decreasing transit time among the intestine allowing slow glucose absorption and improving insulin sensitivity (Chávez-Mendoza & Sánchez, 2017). Beans starch is characterized by high amylose/amylopectin ratio, amylose has lower digestibility and it is an important factor in the modulation of blood glucose (Hayat *et al*., 2014; Rebello *et al*., 2014). In Figure 6 are reported the physiological effects of dietary fiber in the human gut.

Table 4. Reported health effects of legumes.

Source	Involved metabolism	Beneficial effect	Reference
Legumes (including some pulses)	Cardiovascular	Lower risk of coronary heart disease and cardiovascular disease	(Bazzano *et al*., 2001)
	Cardiovascular and diabetes	Modulation of glucose, insulin and homocysteine concentrations and lipid peroxidation in coronary artery disease patients	(Jang *et al*., 2001)
Azuki bean juice	Hypertriglyceridemia	Decreased triglyceride concentrations by inhibited pancreatic lipase activity	(Maruyama *et al*., 2008)
Legumes	Type 2 diabetes mellitus	Risk reduction to develop type 2 diabetes	(Venn and Mann, 2004)
Legumes	Endometrial cancer	Low risk of endometrial cancer	(Tao *et al.*, 2005)
	Breast cancer	Low breast cancer risk	(Velie *et al*., 2005)
	Colon cancer	Low risk of colorectal adenoma	(Agurs-Collins *et al.*, 2006)
Legumes	Type 2 diabetes mellitus	Low glycemic index and lower systolic blood pressure	(Jenkins *et al*., 2012)
Legumes and cereals	Obesity	Low average body mass index and low risk of obesity	(Greenwood *et al*., 2000)
Common bean	Obesity	Low body mass index and lower systolic blood pressure	(Papanikolaou and Fulgoni, 2008)
Pulses	Obesity	Low waist-to-hip ratio	(Williams *et al*., 2000)
	Obesity	Low energy intake, waist circumference, systolic blood pressure, glycosylated hemoglobin and glucose	(Mollard *et al*., 2012)
Whole grains, beans and legumes	Obesity	Low body mass index and waist circumference	(Haveman-Nies *et al*., 2001)
Whole-grain bread and beans	Glycemia and obesity	Glycemic control and weight loss	(Jimenez-Cruz *et al*., 2003)
Chickpeas	Hypertriglyceridemia	Reductions in serum total and low-density lipoprotein cholesterols	(Pittaway *et al.*, 2006)
Vegetables (including some seeds of pulses	Lymphoblastic leukemia	Low risk of lymphoblastic leukemia	(Petridou *et al*., 2005)

Source: Adapted and updated from Campos-Vega *et al.* (2010b).

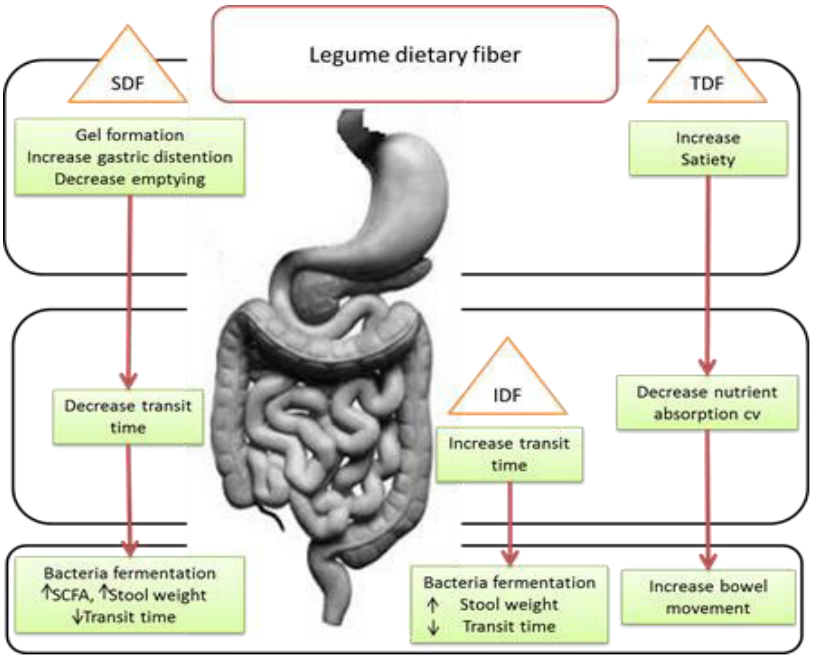

Figure 6. Physiological effects of dietary fiber in the human gut. Total dietary fiber (TDF), soluble dietary fiber (SDF) and insoluble dietary fiber (IDF). Adapted from Tiwari *et al*., (2011).

1.4.2. Effects of legume consumption in blood cholesterol and cardiovascular disease

Studies indicate that consumption of beans has a beneficial effect on lipids metabolism and a regular intake of beans may contribute to lowering the plasma cholesterol levels and therefore prevent the development of cardiovascular disease. Dietary fiber has been shown to have important health implications in the prevention of chronic disease. In the small intestine, dietary fiber is not digested but may be fermented in the colon into SCFA such as acetate, propionate and butyrate. Propionate has been shown to inhibit the activity of the enzyme hydroxy-3-methylglutaryl-CoA reductase, the limiting enzyme for cholesterol synthesis. Dietary fiber also enhances water absorption in the colon, and thus prevent constipation. Fiber also has the ability to bind bile acids thereby preventing their colonic reabsorption, contributing to cholesterol excretion (Trinidad *et al*., 2010).

Legumes are cholesterol free, have low fat content and are good sources of phytosterols and dietary fiber. It has been found that legumes have cholesterol-lowering properties, regulating body weight and preventing coronary heart disease (Tiwari *et al*., 2011). Some legumes such us chickpeas and peas contain Angiotensin-1 converting enzyme (ACE) inhibitor peptides, which causes an antihypertensive effect if reaches the systemic circulation (Vermeirssen *et al*., 2005). A large study conducted by (Bazzano *et al*., 2001) demonstrated that eating legumes four or more times a week may reduce by 22% the risk of coronary heart disease (CHD) and by 11% the risk of cardiovascular events. Legume

consumption may also influence cardiovascular well-being, SCFA production by the colonic bacterial populations, decreasing serum cholesterol and low-density lipoproteins (LDL) (Rebello *et al*., 2014) (Figure 7).

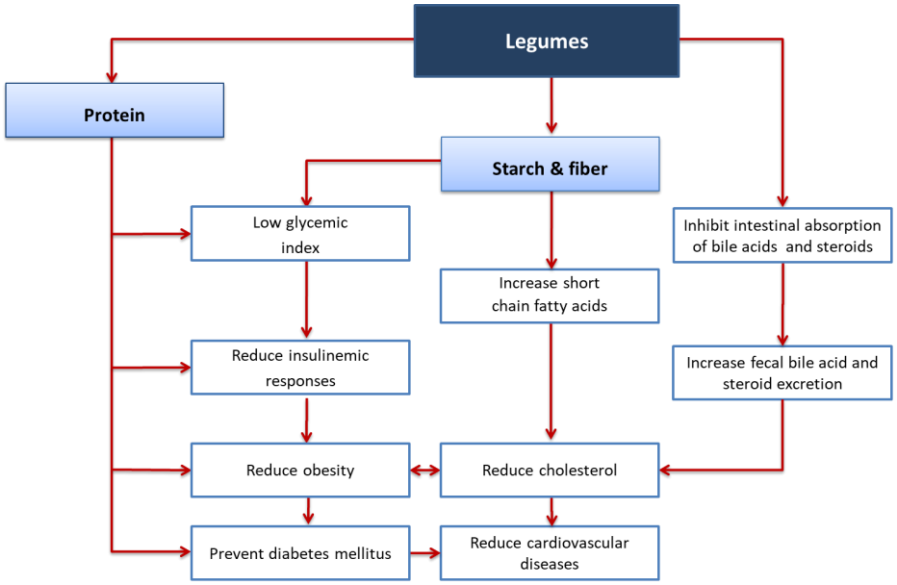

Figure 7. Mechanisms involving reduction of cardiovascular diseases, obesity and diabetes mellitus by dry legumes. Adapted from Hayat *et al*., (2014).

1.4.3. Effects of legume consumption in cancer

The generation of reactive oxygen species (ROS) and oxidative stress results in damage of macromolecules such as lipids, proteins, RNA, and DNA, which may cause chronic degenerative diseases, including cancer. Studies also suggests that the occurrence of cancer can be decreased by lifestyle and dietary changes. Diets rich in common beans reduce risk of developing various types of cancers including colon, breast and prostate (Campos-Vega *et al*., 2010b; Thompson *et al*., 2008; Vergara-Castañeda *et al*., 2010). The anti-carcinogenic and anti-mutagenic activities of beans are highly associated with the presence of phenolic compounds as well as other bioactive compounds (fiber and resistant starch). Phenolic compounds have the potential to inhibit mutagenic agents including polycyclic aromatic hydrocarbons, nitrosamines, and mycotoxins by inhibiting activation enzymes and promoting detoxification (Ganesan & Xu, 2017). Folate, which can influence DNA stability via synthesis of nucleotides and the DNA methylation, it is also found in high amount in legumes. Folate is very important in cancer preventing initiation and progression phase of carcinogenesis (Campos-Vega *et al*., 2010b).

In Chinese population in a case-control study, an inverse association between fruit, vegetable and legumes intake with the development of endometrial cancer risk in in comparison with United States population, where consumption of these foods is low (Tao *et al*., 2005). In a large prospective study, a reduced risk of invasive breast cancer in postmenopausal women was associated with high intake of legumes (Velie *et al*., 2005).

It has been suggested that soy isoflavones may also have anticarcinogenic properties reducing prostate cancer risk. Both genistein and daidzein induced cell death (apoptosis) in prostate cancer cells (Hsu *et al*., 2010). In addition, phenolic extract from legumes also demonstrated antiproliferative effects in ovarian cancer cells (SK-OV-3), colon adenocarcinoma cells (SW480), oral adenosquamous carcinoma cells (CAL 27), hepatocarcinoma cells (HepG2), epithelial colorectal adenocarcinoma cells (Caco-2), human breast cancer cells (MCF-7), lung adenocarcinoma cells (A549 and NSCLC) (Ombra *et al*., 2016; Zou *et al*., 2014).

1.4.3.1. Effects of legume consumption on colorectal cancer

In Table 5 are listed recent researches that demonstrate the anti CRC effects of legumes in human studies. Numerous epidemiological studies suggest that high consumption of legumes could help to decrease the risk and reduce the mortality from colorectal cancer (Azzeh *et al*., 2017; Freudenheim *et al*., 1990; Zhu *et al*., 2015) and are inversely associated with the development of colorectal adenoma (Agurs-Collins *et al*., 2006).

The protective effect of legumes on colorectal cancer is in part associated with their high fiber content. Dietary fiber promotes the excretion and decrease of carcinogens, pro-carcinogens and tumors promoter in the fecal stream, thus reducing access of these substances to the colonic mucosa. Resistant starch also increases fecal bulking, lowers fecal pH, alters bile acid metabolism and increases the production of SCFA, promoting a healthy colonic mucosa (Lupton, 2004; Wang, Yao *et al*., 2017). Phenolic compounds present in legumes are also associated with the prevention of CRC by their antioxidant activity, decreasing ROS generation and oxidative stress that may cause cancer (Ganesan & Xu, 2017).

A trial study conducted by Lanza *et al*., (2006) investigating the effect of diet on patients with adenomatous polyps (benign colon tumors with a glandular structure) showed an inverse correlation between the consumption of dry beans and the recurrence of advanced adenoma in humans. In addition, a controlled trial performed by Borresen *et al*., (2016) showed a reduced risk of colorectal cancer development in the group with high

intake of navy bean and rice bran. Relative to soy bean consumption, soy products and dietary isoflavones were also associated with a reduced risk of colorectal cancer, especially in distal colon or rectal in Asian population (Shin *et al*., 2015).

In Table 6 are listed recent studies that demonstrate the anti CRC effects of legumes in *in vivo* models.

In animal models, the consumption of dry beans such as pinto, navy and black beans, increased the production of SCFA and reduced significantly colon cancer incidence and multiplicity in the F344 rats azoxymethane-induced (AOM-induced) colon cancer model (Hangen & Bennink, 2009; Hughes, Ganthavorn, & Wilson-Sanders, 1997). The polysaccharides extracted form cooked common beans showed an increase in SCFA production and a concomitant decreasing in the number of aberrant crypt foci (ACF). Additionally, an increased apoptosis in colonic cells (increasing bax and caspase-3 expression) and cell cycle arrest (decreasing the RB expression) was observed in rat colons of AOM models of induced CRC (Feregrino-Pérez *et al*., 2008). Koratkar & Rao, (1997) showed that saponins from legume significantly reduced the incidence of total colonic ACF in both AOM-induced colon cancer CF1 mine and F344 rats (Raju *et al*., 2004). In a study conducted by Bobe *et al*., (2008) in AOM-induced CRC, the feeding mice with cooked, insoluble fraction and phenolic extract of navy beans resulted in lower hyperplasia, dysplasia, adenomas or adenocarcinoma when compared to AOM group. Research performed by Feregrino-Perez *et al*., (2014) and Vergara-Castañeda *et al*., (2012) demonstrated that non-digestible fraction from bean prevented the development of ACF, induces cell cycle arrest and apoptosis in Sprague-Dawley rats with AOM-induced colon cancer.

In Table 7 are listed studies that demonstrate the anti CRC activities of legumes extracts and isolated compound from legumes using *in vitro* models.

Legumes containing polyphenols have demonstrate high antioxidant activity measured by *in vitro* methods of 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS), oxidant radical absorbing capacity (ORAC) and Ferric reduction antioxidant power (FRAP) (Ombra *et al*., 2016; Siah *et al*., 2012; Siah *et al*., 2014; Zou & Chang, 2014). Polyphenolic enriched extracts of several legumes have been demonstrated to have anti-colorectal cancer effects through inhibition of cell viability, induction of apoptosis and inhibition of cell migration (Lee *et al*., 2009; Lima *et al*., 2016; Ombra *et al*., 2016; Siah *et al*., 2012; Tsai *et al*., 2010),

Isoflavones and saponins are the most discussed compounds related with the beneficial effect of soy in colon cancer (MacDonald *et al*., 2005). Research has shown that steroid saponins can inhibit cell proliferation, induce apoptosis, affect cell morphology and induce differentiation of HT29 human colon cancer cells (Oh & Sung, 2001; Tsai *et al*., 2010). Diosgenin (saponin) from fenugreek (*Trigonella foenum graecum*) also shows anticancer effects against HT29, inhibiting cell proliferation and inducing apoptosis by suppression of Bcl-2 and induction of caspase-3 protein expression (Raju *et al*., 2004). Mizushina *et al*., 2013) investigated the effects of incubation of HCT116 and HT29 with genistein and daidzein and observed a decreased of cell viability, cell cycle arrest at the G2/M phase and inhibition of topoisomerase II. These isoflavones also decreased cell adhesion by down-regulation of β-catenin expression in HT29 (Lepri *et al*., 2014). Isoflavones may also arrest the cell cycle G1(Guo *et al*., 2004).

Extracts obtained from fermented soymilk also exhibited a suppression effect on the proliferation of HT29 and Caco-2 cells (Lai *et al*., 2013). The effects of bean extracts subjected to gut fermentation were also investigated and showed to induce an increase of SCFA production and inhibition of HT29 cells proliferation (Campos-Vega *et al*., 2012).

Table 5. Summary of clinical and observational studies of effects of legumes in colorectal cancer.

	Human	Anti CRC beneficial effect/activities	References
Dietary fiber	Case-control study in both men and women	Dietary fiber intake decreased the risk of developing colorectal cancer	(Freudenheim *et al*., 1990)
Dry bean	Dietary intervention in patients with colorectal adenomas	Inverse correlation between dry beans consumption and the recurrence of advanced adenoma	(Lanza *et al*., 2006)
Legumes	Case-control study in African-Americans population	Legume intake reduced the risk of colorectal adenoma in African-Americans	(Agurs-Collins *et al*., 2006)
Legume fiber	Dietary intervention in both men and women	Legume intake increased mineral availability; Reduced the LDL and total cholesterol; Reduced the risk of colon cancer development	(Trinidad *et al*., 2010)
Legumes	Meta-analysis of observational studies	High intake of legume reduced risk of colorectal cancer	(Zhu *et al*., 2015)
Isoflavone and soy food Intake	Case-control study in Korea in both men and women	Soy products and dietary isoflavones were associated with a reduced risk of colorectal cancer, especially in distal colon or rectal	(Shin *et al*., 2015)
Navy bean and rice bran	Dietary intervention in both men and women	Intake of navy bean and rice bran reduce the risk of colorectal cancer; Decreased serum amyloid A (serum inflammatory biomarker)	(Borresen *et al*., 2016)
Legumes	Case-control study in Saudi Arabia	Intake of legumes decreased the risk of colorectal cancer development	(Azzeh *et al*., 2017)

Table 6. Summary of *in vivo* studies of effects of legumes in colorectal cancer.

	Animal model	Anti CRC beneficial effect	References
Dry beans	AOM-induced colon cancer in F344 Rats	Reduced the number and multiplicity (adenomas and adenocarcinomas) of tumors	(Hughes *et al*., 1997)
Soy saponins	AOM-induced colon cancer in CF1 mice	Soya saponin significantly reduced the incidence of total colonic aberrant crypt foci	(Koratkar & Rao, 1997)
Diosgenin from Trigonella foenum graecum	AOM-induced colon cancer in F344 Rats	Suppressed total number and multiplicity of colonic aberrant crypt foci	(Raju *et al*., 2004)
Cooked, insoluble fraction and phenolic extract of navy bean	AOM-induced colon cancer in ob/ob mice.	Lower hyperplasia, dysplasia, adenomas or adenocarcinoma in bean fed mice compared with control AOA group; Ethanolic extract showed the highest preventive effect	(Bobe *et al*., 2008)
Polysaccharides extracts form cooked common beans (Negro 8025)	AOM-induced colon cancer in Sprague-Dawley rats	Increased SCFA production; Decreased the number of aberrant crypt foci; Increased the transcriptional expression of Bax and Caspase-3 and decreased the RB expression	(Feregrino-Pérez *et al*., 2008)
Navy and black beans	AOM-induced colon cancer in male F344 rats	Both beans increased SCFA especially in distal colon; Reduced significantly the colon cancer incidence and multiplicity	(Hangen & Bennink, 2009)
Cooked beans and non-digestible fraction of common bean	AOM-induced colon cancer in Sprague-Dawley rats	Both coked and non-digestible fraction suppressed the formation of aberrant crypt foci	(Vergara-Castañeda *et al*., 2010)
Non-digestible fraction of common beans	AOM-induced colon cancer in Sprague–Dawley rats	Prevented the development of aberrant crypt foci; Induced cell-cycle arrest in G1/S and G2/M phases and cell death by apoptotic; Inhibited cell proliferation, inflammation and induced DNA repair	(H. Vergara-Castañeda *et al*., 2012)
Non-digestible fraction of common beans	AOM-induced colon cancer in Sprague-Dawley rats	Decreased the number of total colonic aberrant crypt foci; Increase number of cells in G1 phase; Decreased proliferation index and increased apoptotic cells	(Feregrino-Perez *et al*., 2014)

Table 7. Summary of *in vitro* studies of effects of legumes in colorectal cancer cell lines.

	Cell lines	Anti CRC beneficial effect/activities	References
Soy saponins	HT29	Inhibited cell viability; Suppressed PKC activity and induced differentiation	(Oh & Sung, 2001)
Diosgenin of trigonella foenum graecum	HT29	Inhibited cell viability; Induced apoptosis by suppression of Bcl-2 and the induction of caspase-3 proteins	(Raju *et al*., 2004)
Phenolic extract of kidney bean husk	HT29	Inhibited cell viability; Induced apoptosis and activation of AMPK	(Lee *et al*., 2009)
Saponins	HT29	Inhibited cell viability; Induced the development cytoplasmic vesicles and irregular cell membrane	(Tsai *et al*., 2010)
Phenolic extracts from raw and roast faba bean	HT29	Antioxidant effect against ABTS radical and ORAC; Cellular protection against H_2O_2-induced DNA damage; Inhibited cell viability	(Siah *et al*., 2012)
Bean extracts subjected to fecal microbiota fermentation	HT29	Increased SCFA production; Inhibited cell viability by induction of apoptosis	(Campos-Vega *et al*., 2012)
Extract from non-fermented and fermented soymilk	HT29 and Caco-2	Fermentation increased phenolic content, decreased saponins and phytic acid; Extract of fermented soymilk higher decreased the cell viability of both HT29 and Caco-2	(Lai *et al*., 2013)
Daidzein, genistein and glycitein	HCT116	Inhibited cell viability; Promoted cell cycle arrest at G2/M phase; Genistein inhibited human topo II activity	(Mizushina *et al*., 2013)
Daidzein, genistein	HT29	Decreased cell viability; Down-regulated β-catenin expression	(Lepri *et al*., 2014)
Aqueous soy, mistletoe and red clover extracts	Gastric and colon tissues	Increased xanthine oxidase enzymes activity in cancerous colon tissue and inhibited in non-cancerous gastric tissue	(Namuslu *et al*., 2014)
Phenolic extract and fractions from small red bean	SW480	Antioxidant effect against DPPH and ABTS radicals; Decreased cell viability	(Zou *et al*., 2014)
Phenolic extracts of raw and cooked beans	Caco-2	Antioxidant effect against DPPH radical; Decreased cell viability	(Ombra *et al*., 2016)
Legume phenolic extracts	HT29	Decreased cell viability and cell migration; Inhibited of MMP-9 protein	(Lima *et al*., 2016)

1.5. Colorectal cancer

Colorectal cancer (CRC) is one of the major causes of cancer-related mobility and mortality worldwide being the second- and the third-most common cancer in women and men respectively. In 2012, 746,000 new cases were diagnosed in men (10 % of the total cancer cases) and 614,000 cases in women (9 % of the total cancer cases), last data available. The global colorectal cancer burden is expected to increase by 60 % in the next 15 years to more than 2.2 million new cases and 1.1 million deaths per year (World Cancer Research Fund, 2017).

There is a large geographic difference in the global distribution of colorectal cancer but affect similarly both sexes. The incidence rates vary ten-fold worldwide, being the highest estimated rates in Australia, New Zealand, Canada, USA and parts of Europe and the lowest in Western Africa, China, India and South America (Haggar & Boushey, 2009; Kuipers *et al*., 2015). The incidence and mortality rates of CRC worldwide are shown in Figure 7 and 8.

About two-thirds of colorectal cancer cases and about 60 % of colorectal cancer deaths occur in developed countries. The age-standardized mortality rate of CRC reflects the disease incidence. The mortality also depends on the stage and the distribution at the time of diagnosis, which is influenced by the availability of population-screening programs and by the level of health care in each country (Kuipers *et al*., 2015; World Cancer Research Fund, 2017).

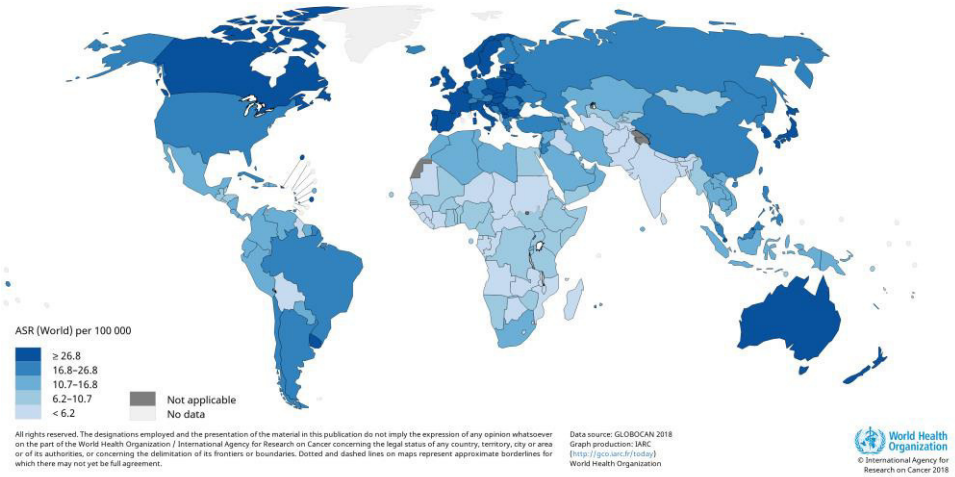

Figure 8. Worldwide colorectal cancer incidence (age adjusted according to the world standard population, per 100 000) (GLOBOCAN, 2018).

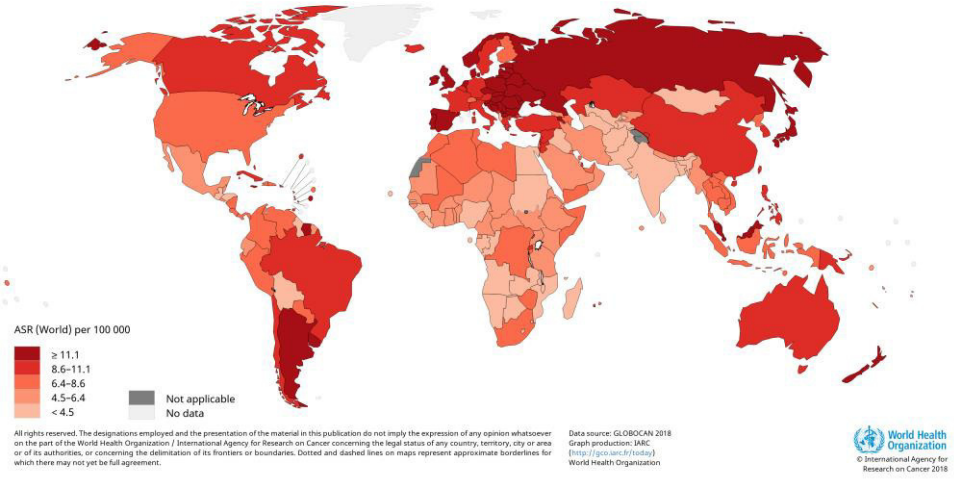

Figure 9. Worldwide colorectal cancer mortality rates (age adjusted according to the world standard population, per 100 000) (GLOBOCAN, 2018).

1.5.1. Risk factors

Both genetic and environmental factors play important roles in the etiology of CRC. About, 5 to 10% of CRC all patients' have a hereditary predisposition of development colorectal cancer, being Lynch syndrome and familial adenomatous polyposis (FAP) the most common. The majority of CRC cases are sporadic and occur in individuals that have no inherited predisposition.

Lynch syndrome is caused by a mutation in one of the DNA mismatch-repair genes such as MLH1, MSH2, MSH6, PMS2 or EPCAM. During replication, impaired mismatch repair gives rise to accumulation of DNA mutations, occurring often in microsatellite DNA fragments with a repetitive nucleotide sequence, resulting in microsatellite instability (MSI) (Vasen *et al*., 2015).

The second most common hereditary colorectal cancer syndrome is familial adenomatous polyposis (FAP). This syndrome is caused by mutations in the adenomatous polyposis coli (APC) gene, which controls the activity of the Wnt signalling pathway. Most patients with familial adenomatous polyposis develop very large numbers of colorectal adenomas at a young age that developed into colorectal cancer (Kuipers *et al*., 2015; Vasen *et al*., 2015).

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease are also associated with an increased risk of CRC. IBD is responsible for 1% of CRC in western populations. It has been suggested that the incidence of CRC in those with IBD

is decreasing by the use of anti-inflammatory drugs (Jess *et al*., 2012; Kuipers *et al*., 2015).

A range of environmental modifiable lifestyle factors influences the development of CRC. CRC is considered one of the clearest markers of epidemiological and nutritional transition, with incidence rates linked to Western lifestyles (Arnold *et al*., 2017; Doubeni *et al*., 2012; Marley & Nan, 2016). The risk is increased by smoking, alcohol intake, increased body weight, T2D, high intake of red meat and processed meat (Huxley *et al*., 2009; Luna-Vital *et al*., 2016; World Cancer Research Fund International, 2015). By contrast, consumption of milk, whole grains, fruits, vegetables, legumes as well as intake of calcium, dietary fiber, multivitamins, folate and vitamin D, decrease the risk (Ceter *et al*., 2009; Haggar & Boushey, 2009; World Cancer Research Fund, 2017).

It is estimated that up to 80% of CRC in Europe and USA are attributable to lifestyle factors namely, diet rich in saturated fatty acids and red meet and low in fruits and vegetables (Aleksandrova *et al*., 2014; Erdrich *et al*., 2015). The variety of environmental factors that influence colorectal carcinogenesis is reflected in the heterogeneity of CRC. This fact has been stimulated research in the field of 'molecular pathological epidemiology', which focuses on the correlation between environmental and genetic factors, and between molecular tumor characteristics and disease progression (Ogino *et al*, 2012).

1.5.2. Mechanisms of pathophysiology

The environmental and genetic factors that cause colorectal cancer, promote the acquisition of hallmark behaviors by normal colon epithelial cells and transformation into tumor cells (Hanahan & Weinberg, 2011) (Figure 10). These hallmarks of cancer are acquired by progressive accumulation of genetic and epigenetic alterations that activate oncogenes and inactivate tumor suppressor genes, leading to genetic and epigenetic instability, which drive the malignant transformation of colon cells (Kuipers *et al*., 2015).

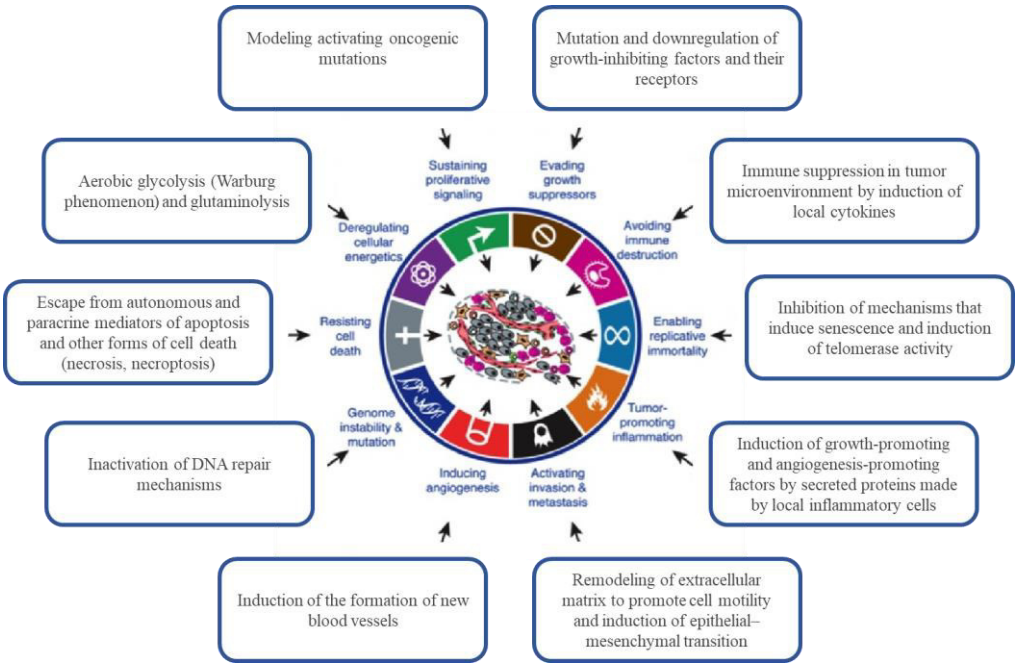

Figure 10. This illustration encompasses the hallmarks of capabilities of cancer. Adapted from Hanahan & Weinberg, (2011).

The evolution of normal epithelial cells to adenocarcinoma follows a predictable progression of epigenetic, genetic and histological changes that are described in Figure 11.

In colorectal cancer, substantial heterogeneity in the specific mutations is evident between tumors, although the mutations seem to cluster in related groups of certain signaling pathways. The most common gene alterations seen in colorectal cancer include those in the genes APC, catenin-beta1 (CTNNB1), KRAS, BRAF, SMAD4, transforming-growth factor-beta receptor 2 (TGFBR2), TP53, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit-alpha (PIK3CA), epidermal growth factor (EGF). Mutations in these genes affects the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and transforming growth factor beta (TGF-β) signaling pathways. Besides may also occur in DNA repair genes which accelerates the rate of which cell accumulate mutation (Bracht *et al*., 2010; Grady & Pritchard, 2014; Kuipers *et al*., 2015). In addition to gene mutations, epigenetic alterations are common in CRC and seem to cooperate with gene mutation to drive the polyp to cancer progression. Modification in DNA methylation involves two fundamental changes, hypermethylation of CpG islands of gene promoters, resulting in transcriptional silencing of tumors suppressor genes and hypomethylation of oncogenes promoters leading to genomic instability (Zeki *et al*., 2011).

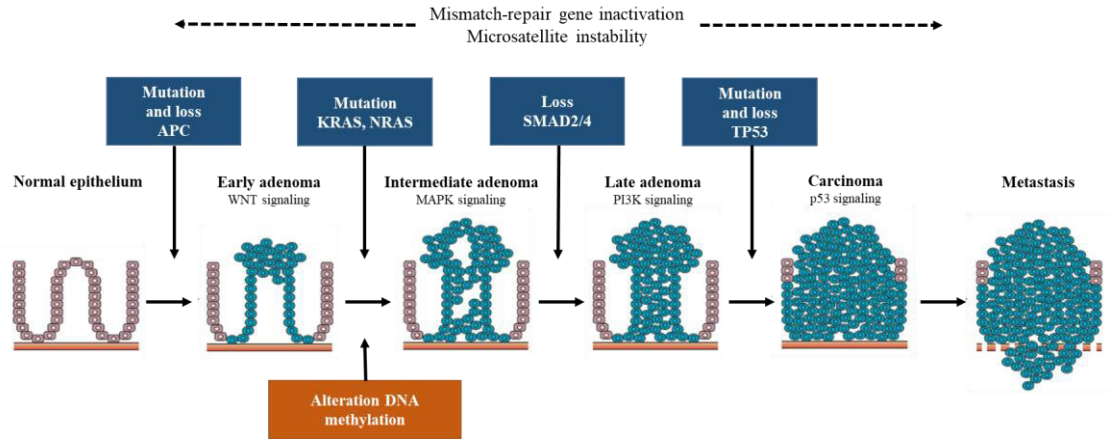

Figure 11. Mechanisms and molecular events that characterize the transition for normal colonic epithelial cell to CRC. Colon carcinogenesis is a complex multistep process, the adenoma–carcinoma sequence, from small benign precursor lesions to metastatic carcinomas. The progression of normal epithelium to adenoma and to colorectal carcinoma is characterized by the accumulating of abnormal genes. Mutations in mismatch-repair genes cause microsatellite instability and the successive mutation of target cancer genes. The initial step in tumorigenesis is that of aberrant crypt foci (ACF) formation, associated with loss of APC. Early carcinomas acquire accumulation of mutations in additional oncogenes or tumor suppressor genes, such as KRAS, TP53, and SMAD4.

1.5.3. Cell proliferation, cell cycle regulation and apoptosis in colorectal cancer

Under normal conditions, the production and propagation of growth signals and cellular proliferation are highly regulated. Stringent control of proliferative processes supports the maintenance of healthy tissue structure and function. Signaling pathways associated with proliferation are activated when required and switched off when no longer needed. During cancer development occurs accumulation of genetic mutations ultimately disrupts mechanisms required for cell proliferation, cell cycle regulation and apoptosis.

1.5.3.1. *The PI3K/AKT/mTOR signaling pathway*

The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is a major signaling pathway required for a wide range of normal cellular functions. These signaling pathways play critical roles in controlling most hallmarks of cancer namely, metabolism, growth, proliferation, and metastasis. It is also linked to tumor-promoting processes such as tumor angiogenesis, apoptosis and cancer-associated inflammation (Figure 12).

PI3K is a signaling protein that phosphorylates the 3'-hydroxyl group of phosphatidylinositol a plasma membrane component and a metabolic precursor of phosphoinositides bioactive lipid molecules that regulate cell survival and death by modulating a wide range of cellular activities. PI3K can be activated through multiple upstream signals, such as cytokines, integrins, B cell receptor (BCR) activation, or G protein-coupled receptor (GPCR) ligands. Upon ligand binding to receptor tyrosine kinase (RTK) and GPCR, PI3K activation is induced at the plasma membrane. Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3). Subsequently, accumulation of PIP3 and recruitment of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and AKT activate a cascade that affects cell growth and survival and a host of other critical cellular functions including: calcium mobilization, cell motility, vesicle transport and cellular proliferation, apoptosis, and other functions (Liu *et al*., 2009).

AKT, a serine/threonine kinase, is a central and convergence protein in one of the most influential signaling networks. AKT activation serves as a master regulator of this cellular signaling pathways, generating multitude of intracellular responses through a variety of downstream targets and interacting partners. Because of its pivotal role in cell signaling, AKT is one of the most actively studied kinases in both basic research and anti-cancer drug development. AKT is expressed as three isoforms AKT1, AKT2, and AKT3. All isoforms present an amino-terminal pleckstrin homology (PH) domain, a central serine/threonine catalytic domain and a small C-terminal regulatory domain. The PH domain binds PIP2 and PIP3, both products of PI3K. These interactions induce translocation of AKT to the plasma membrane, where it is phosphorylated by PDK1 and by mTOR complex 2 (mTORC2). AKT substrates are also involved with regulation of cellular proliferation and include p21 and p27 (Arencibia *et al*., 2013; Davies, 2011).

Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene through the action of its phosphatase protein product. Disruptions in PTEN are tightly linked with uncontrolled activation of AKT, leading to tumor cell proliferation (Crowell *et al*., 2007).

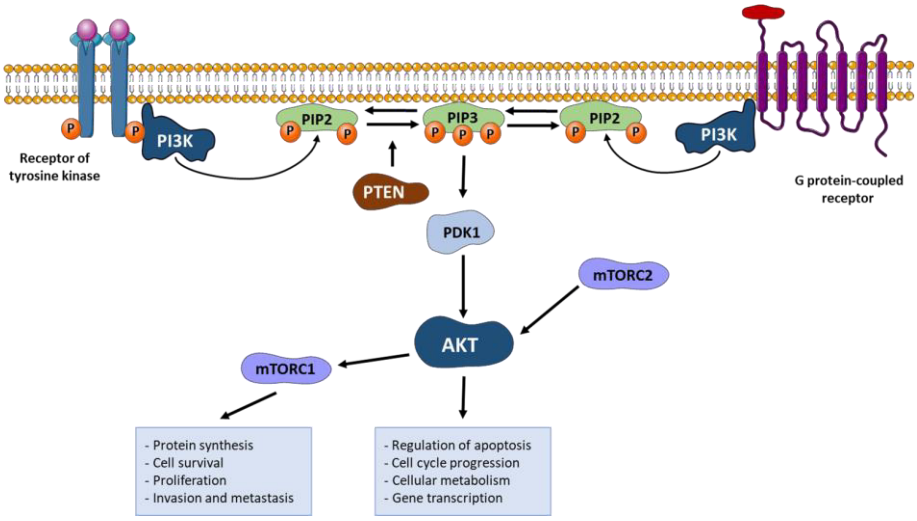

Figure 12. PI3K/AKT/mTOR signaling pathway.

1.5.3.2. RAS/RAF/MEK/ERK (MAPK) signaling pathway

Mitogen-activated protein kinase (MAPK) pathway dysregulation has been implicated as driver of tumorigenesis and enables cancer cells to grow independently of mitogenic signals, sustaining the proliferative signaling, the ability to evade apoptosis, the insensitivity to anti-growth signals, the ability to metastasize and to induce angiogenesis. There are 4 major branches of the MAPK pathway (Figure 13). The ERK pathway is induced by growth factors typically associated with proliferative signaling, whereas the JNK, p38, and ERK5 pathways are primarily activated by stimuli associated with cellular stress response, apoptosis, growth inhibition and innate immunity (Cargnello & Roux, 2011; Roberts & Der, 2007).

Classical MAPK pathway activation begins at the cell membrane in response to growth cytokines and cytosines, where small guanosine triphosphatases (GTPases) and various protein kinases phosphorylate and activate MAPKKKs. Consequently, MAPKKs phosphorylate MAPKKs, which once activated phosphorylate the MAPKs. Activated MAPKs interact with and phosphorylate numerous cytoplasmic substrates and ultimately modulate transcription factors that drive specific gene expression. This, in turn, results in various biological responses, for example, cell cycle progression or induction of interferon production (Cargnello & Roux, 2011).

The RAS proteins are small GTPases associated with a number of signal transduction pathways involved in cell cycle progression, cell motility, apoptosis, senescence and other vital functions (Santarpia *et al.*, 2012). RAS was the first human oncogene identified and mutations in RAS family members are detected in

approximately 50% of colorectal cancers. Numerous cell surface molecules activate RAS proteins, which in turn, activate MAPK cascades including RAF/MEK/ERK a major effector pathway of RAS with a well-described role in cancer. The PI3K/AKT/mTOR pathway, which is activated by RAS-dependent and RAS independent mechanisms, shares points of convergence with the MAPK pathway downstream of growth factor and G protein-coupled receptors. Signaling events initiated by RAS that are transduced through RAF, MEK and ERK1/2 are transmitted to the nucleus, where they regulate transcription factors of gene expression required for cellular proliferation and survival (Figure 13) (Castellano & Downward, 2011).

The BRAF proto-oncogene encodes the RAF family members that include three known isoforms, ARAF, BRAF, and CRAF. These highly conserved serine/threonine kinases are mediators of the MAPK (RAS/RAF/MEK/ERK) pathway. The RAF proteins serve as substrates for the upstream kinase RAS and direct activators of MEK, which functions downstream. Hyperactivation of RAF leads to dysregulated proliferation, differentiation, and apoptosis. The BRAF mutation isoform is the most frequently associated with malignancy especially in colorectal carcinomas. Approximately 80 % of the BRAF mutation results of a single-base missense substitution of valine to glutamic acid at codon 600 (V600E). These BRAF mutations compromise BRAF kinase activity and is a persistent activator of the MAPK pathway (Figure 12) (Davies *et al*., 2002).

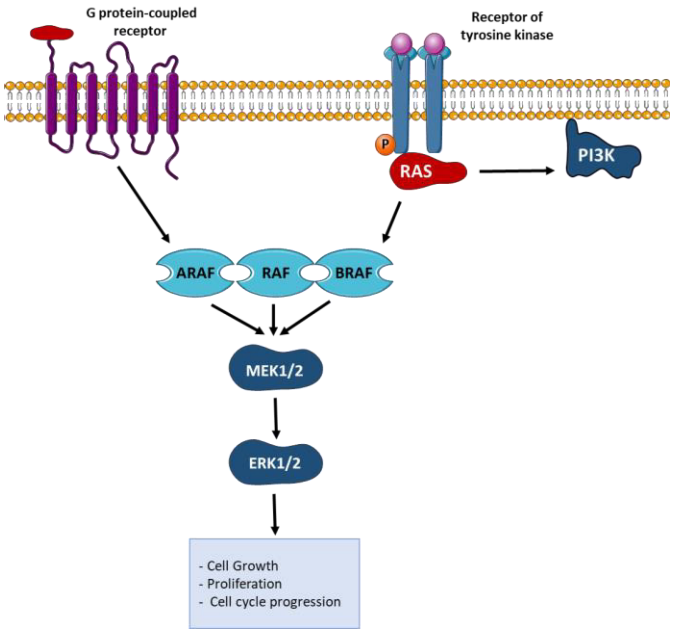

Figure 13. RAS/RAF/MEK/ERK (MAPK) signaling pathway.

1.5.3.3. Cell cycle regulation

The cell cycle progression is a highly organized and regulated process that ensures duplication of genetic material and cell division. This regulation involves growth-regulatory signals as well as monitorization of the genetic integrity to ensure the absence of DNA damage. Proliferation depends on progression through four distinct phases of the cell cycle namely, G0/G1, S, G2 and M. As shown in Figure 14, the cell cycle progression is regulated by several cyclin-dependent kinases (CDKs) that act in complexes with their cyclin partners. The activity of CDKs involved in cell cycle regulation is tightly controlled by mitogenic signals and can be inhibited by the activation of cell cycle checkpoints in response to DNA damage (Otto & Sicinski, 2017).

More specifically, mitogenic signals activate complexes of cyclins and CDKs that promote progression from the G1 phase into S phase by phosphorylating (P) several cellular targets, including the retinoblastoma protein (RB). The complex formed by D-type cyclin and cyclin-dependent kinases 4 and 6 (cyclin D-CDK4/6) hyperphosphorylate the retinoblastoma protein (RB) during G1. When hyperphosphorylated, RB is further phosphorylated by the complex cyclin E-CDK2, resulting in the release and nuclear translocation of E2F transcription factor that is crucial for entry in S phase. Progression through S phase and from G2 phase into mitosis (M phase) is also under the control of other cyclin-CDK complexes but not under the extracellular mitogenic influence. In addition, DNA damage is sensed by several specialized proteins and triggers cell cycle arrest via p53 in G1 phase or G2 phase. The INK4 proteins, including p15, p16, inhibit cyclin D-CDK4/6 activity, CIP/KIP family such p21 and p27 inhibit later stages cyclins (Figure 13) (Hydbring *et al*., 2016; Otto & Sicinski, 2017).

One hallmark of malignant cells is the loss of homeostatic regulation and sustained, or uncontrolled proliferation. This occurs either as a result of mutations in upstream signaling pathways or by mutations of genes encoding cell cycle proteins namely cyclins, CDK4/6 and RB. For example, the cyclin D1 gene (CCND1) represents the second most frequent amplified locus in all human cancers types. CDK4 is amplified in several tumors and consequently activated and insensitive to inhibition of INK4 (p15, p16). Furthermore, CDKN2A gene (which encodes tumor suppressors p16 and p14) represent the most frequent deleted locus in human cancers. Finally, deletion of RB 1

occurs frequently in many tumors enabling the proliferation independently of CDC4/6. The inhibition of cell cycle progression in cancer cells is, therefore, an important strategy to control cancer progression (Chen *et al*., 2009; Otto & Sicinski, 2017).

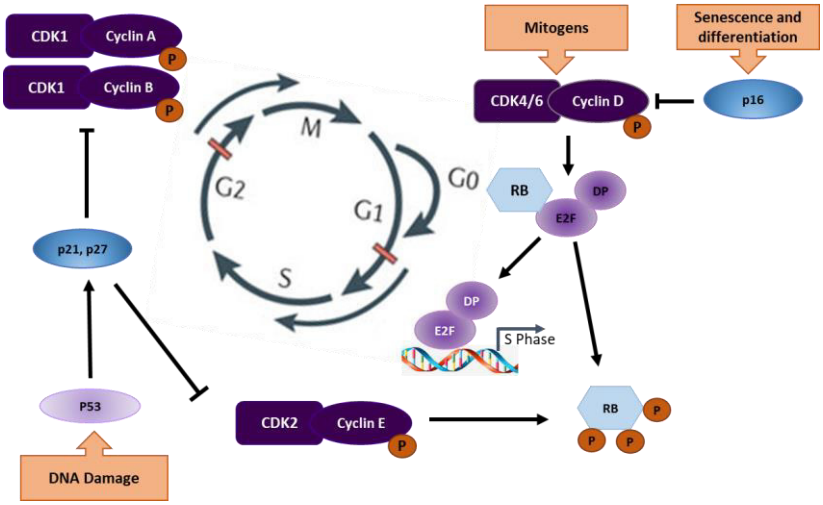

Figure 14. Cell cycle progression and major regulatory proteins. Figure adapted from (Otto & Sicinski, 2017).

1.5.3.4. Apoptosis in cancer

Apoptosis or controlled cell death is a major type of cell death in our body. It is a very conserved process that plays crucial roles during embryogenesis (Ichim & Tait, 2016). Abundant evidence supports the role of apoptosis in cancer development namely in the initiation, promotion and therapeutic responses (Colangelo, 2015; Letai, 2008). Consequently, apoptosis has been one of the most studied topics in the scientific community surrounding the therapeutic potential of engaging apoptosis in cancer treatment (Ichim & Tait, 2016).

The protease activity of caspases is essential for the morphological and biochemical hallmarks of apoptosis. More specifically, caspases can be activated through one of two pathways, the extrinsic (also called death receptor pathway) and the intrinsic pathway (called mitochondrial pathway) as shown in Figure 15.

The extrinsic apoptotic pathway is activated upon a ligand bind a cognate death receptor, such as (TNF)-related apoptosis-inducing ligand (TRAIL) and FAS-related apoptosis inducer receptor, then they activate caspase-8 and caspase-10 by the FAS-associated death domain protein (FADD). Active caspase-8 and caspase-10 cleaves and activates caspase-3 and caspase-7, leading to apoptosis (Taylor *et al*., 2008).

The intrinsic apoptotic pathway, which is often deregulated in cancer, involves a wide array of intracellular stimuli including cytokine deprivation, DNA damage and endoplasmic reticulum stress. These stresses trigger the mitochondrial outer membrane permeabilization (MOMP) (Czabotar *et al.*, 2014). Cell stresses involve BCL-2 homology domain 3 (BH3) only protein activation, leading to BAX and BAK activity that triggers MOMP. The BCL-2 family proteins counteract this process being anti-apoptotic. Subsequent MOMP, the second mitochondria-derived activator of caspases (SMAC) and cytochrome *c* are released into the cytosol. Cytochrome *c* interacts with apoptotic protease activating factor 1 (APAF1), triggering apoptosome assembly, which activates caspase-9. Active caspase 9 then activates caspase 3 and caspase 7, which in turn leads to apoptosis. The release of mitochondrial SMAC facilitates apoptosis by blocking the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP). Caspase-8 cleavage of the BH3-only protein BH3-interacting death domain agonist (BID) enables crosstalk between the extrinsic and intrinsic apoptotic pathways (Ichim & Tait, 2016; Taylor *et al.*, 2008).

Apoptosis has long been considered a process that must be evaded to enable cancer development. This view is supported by several researchers that demonstrate that inhibition of cell death, in combination with mitogenic oncogenes, can promote cancer; the discovery that many oncogenic pathways inhibit apoptosis, whereas tumor suppressors, such as p53, can engage apoptosis; the frequently observed upregulation of anti-apoptotic proteins in cancer and the positive correlation between apoptotic sensitivity and therapeutic efficacy in patient-derived cancer cells (Ichim & Tait, 2016).

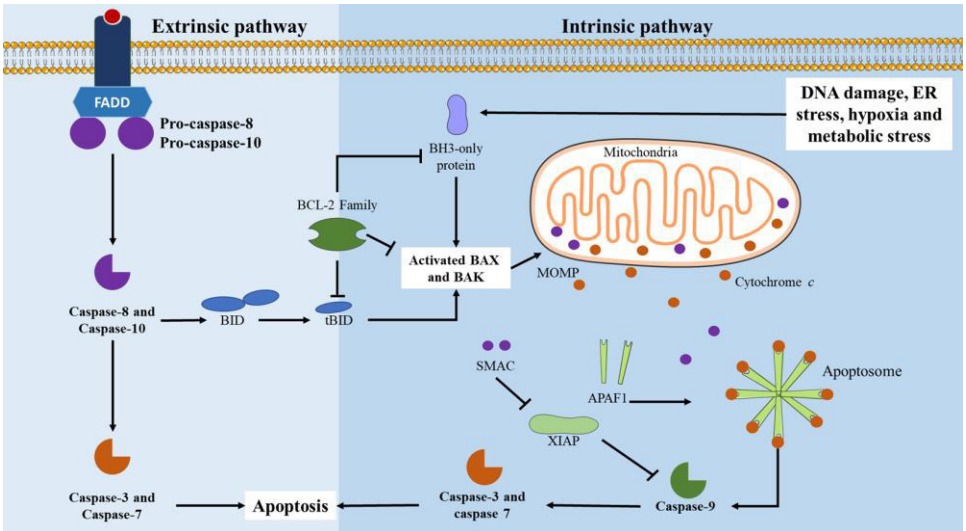

Figure 15. Extrinsic and intrinsic apoptotic signaling pathways.

1.5.4. Conventional treatment

Currently, treatment of CRC is based on surgery and chemotherapy. The chemotherapeutic agents are classified according to their mechanism of action on DNA replication inhibitors, mitosis inhibitors and other cytotoxic agents. The chemotherapeutic adjuvants are given in order to maximize the effectiveness of the chemotherapeutical drug (Lindskog *et al*., 2014; Saridaki & Souglakos, 2013).

The most commonly used chemotherapeutic agents are antimetabolites (capecitabine, 5-fluorouracil (5-FU)), platinum derivatives (oxaliplatin), topoisomerase inhibitors (irinotecan) and Tegafur/uracil (UFT), which inhibit the catabolism of 5-fluorouracil. In advanced CRC, the first line chemotherapy is based on 5-FU alone or combined with oxaliplatin (de Gramont *et al*., 2000; Rejhová *et al*., 2018). The overall response rate to 5-FU monotherapy in advanced CRC is limited in 10 to 15% of the cases (Pardini *et al*., 2011).

The non-specific action of chemotherapeutics is connected with a number of side effects. Chemotherapeutic agents often destroy rapidly dividing cells such as tumor cells, but also hair follicle cells, mucous membranes of the oral cavity and gastrointestinal tract, erythrocytes and leukocytes (Gerber, 2008). The adverse effects of the treatment differ among patients, depending on the type and dose chemotherapeutic agent. As mentioned above, the first line chemotherapeutics in CRC therapy is 5-FU (de Gramont *et al*., 2000). The most common adverse effects of 5-FU are nausea, vomiting, diarrhea, mucositis of the oral cavity, headaches, skin pruritus, myelosuppression, anemia, cardiotoxicity, agranulocytosis, alopecia, depression and anxiety (Rejhová *et al*., 2018).

A potential way to improve therapy and reduce adverse effects is the combination of chemotherapeutic drugs or multicomponent therapy. The combination of 5-FU with other cytotoxic drugs may not only improves the response rates but also reduce the undesirable reaction of these drugs (Assed Bastos *et al*., 2010). In many cases, the combination therapy shows a better outcome by enhancing the cytotoxic effect of conventional therapy to cancer cells (Rejhová *et al*., 2018).

In the present study, we hypothesize, that combination of conventional chemotherapeutics with well-tolerated natural non-toxic compounds such as polyphenols present in bean extracts produced in different conditions may also contribute to the better response to therapy and better patient's quality life.

1.6. References

Agurs-Collins, T., Smoot, D., Afful, J., Makambi, K., & Adams-Campbell, L. L. (2006). Legume intake and reduced colorectal adenoma risk in African-Americans. *Journal of National Black Nurses' Association*, *17*, 6–12.

Akillioglu, H. G., & Karakaya, S. (2010). Changes in total phenols, total flavonoids, and antioxidant activities of common beans and pinto beans after soaking, cooking, and in vitro digestion process. *Food Science and Biotechnology*, *19*(3), 633–639. https://doi.org/10.1007/s10068-010-0089-8

Aleksandrova, K., Pischon, T., Jenab, M., Bueno-de-Mesquita, H. B., Fedirko, V., Norat, T., … Boeing, H. (2014). Combined impact of healthy lifestyle factors on colorectal cancer: A large European cohort study. *BMC Medicine*, *12*(1). https://doi.org/10.1186/s12916-014-0168-4

Aparicio-Ernandez, X., Yousef, G. G., Lorca-Pina, G., Mejla, E., & Lila, M. A. (2005). Characterization of Polyphenolics in the Seed Coat of Black Jamapa Bean (*Phaseolus vulgaris* L .). *Journal of Agricultural and Food Chemistry*, *53*, 4615–4622. https://doi.org/10.1021/jf047802o

Arencibia, J. M., Pastor-Flores, D., Bauer, A. F., Schulze, J. O., & Biondi, R. M. (2013). AGC protein kinases: From structural mechanism of regulation to allosteric drug development for the treatment of human diseases. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1834*(7), 1302–1321. https://doi.org/10.1016/j.bbapap.2013.03.010

Arnold, M., Sierra, M. S., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2017). Global patterns and trends in colorectal cancer incidence and mortality. *Gut*, *66*(4), 683–691. https://doi.org/10.1136/gutjnl-2015-310912

Assed Bastos, D., Freitas, D., Coelho Ribeiro, S., & Hoff, P. M. (2010). Review: Combination therapy in high-risk stage II or stage III colon cancer: Current practice and future prospects. *Therapeutic Advances in Medical Oncology*, *2*(4), 261–272. https://doi.org/10.1177/1758834010367905

Azzeh, F. S., Alshammari, E. M., Alazzeh, A. Y., Jazar, A. S., Dabbour, I. R., El-Taani, H. A., … Tashtoush, S. H. (2017). Healthy dietary patterns decrease the risk of colorectal cancer in the Mecca Region, Saudi Arabia: A case-control study. *BMC Public Health*, *17*(1), 1–8. https://doi.org/10.1186/s12889-017-4520-4

Bazzano, L., He, J., Ogden, L., Loria, C., Vupputuri, S., Myers, L., & Whelton, P.

(2001). Legume consumption and risk of coronary heart disease in US men and women: NHANES I Epidemiologic Follow-up Study. *Archives of Internal Medicine*, *161*(21), 2573–25738.

Bobe, G., Barret, K. G., Mentor-Marcel, R., Saffiotti, U., Young, M. R., Colburn, N. H., … Lanza, E. (2008). Dietary cooked navy beans and their fractions attenuate colon carcinogenesis in azoxymethane-induced Ob/Ob mice. *Nutrition and Cancer*, *60*(3), 373–381. https://doi.org/10.1016/j.pestbp.2011.02.012.Investigations

Borresen, E. C., Brown, D. G., Harbison, G., Taylor, L., Fairbanks, A., O'Malia, J., … Ryan, E. P. (2016). A randomized controlled trial to increase navy bean or rice bran consumption in colorectal cancer survivors. *Nutrition and Cancer*, *68*(8), 1269–1280. https://doi.org/10.1080/01635581.2016.1224370

Bouchenak, M., & Lamri-Senhadji, M. (2013). Nutritional quality of legumes, and their role in cardiometabolic risk prevention: A review. *Journal of Medicinal Food*, *16*(3), 1–14. https://doi.org/10.1089/jmf.2011.0238

Bracht, K., Nicholls, A. M., Liu, Y., & Bodmer, W. F. (2010). 5-Fluorouracil response in a large panel of colorectal cancer cell lines is associated with mismatch repair deficiency. *British Journal of Cancer*, *103*(3), 340–346. https://doi.org/10.1038/sj.bjc.6605780

Campos-Vega, R., García-Gasca, T., Guevara-Gonzalez, R., Ramos-Gomez, M., Oomah, B. D., & Loarca-Piña, G. (2012). Human gut flora-fermented nondigestible fraction from cooked bean (*Phaseolus vulgaris* L.) modifies protein expression associated with apoptosis, cell cycle arrest, and proliferation in human adenocarcinoma colon cancer cells. *Journal of Agricultural and Food Chemistry*, *60*(51), 12443–12450. https://doi.org/10.1021/jf303940r

Campos-Vega, R., Guevara-Gonzalez, R. G., Guevara-Olvera, B. L., Dave Oomah, B., & Loarca-Piña, G. (2010a). Bean (*Phaseolus vulgaris* L.) polysaccharides modulate gene expression in human colon cancer cells (HT29). *Food Research International*, *43*(4), 1057–1064. https://doi.org/10.1016/j.foodres.2010.01.017

Campos-Vega, R., Loarca-Piña, G., & Oomah, B. D. (2010b). Minor components of pulses and their potential impact on human health. *Food Research International*, *43*(2), 461–482. https://doi.org/10.1016/j.foodres.2009.09.004

Cargnello, M., & Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Journal of Microbiology and Molecular Biology*, *75*(1), 50–83. https://doi.org/10.1128/MMBR.00031-10

Castellano, E., & Downward, J. (2011). Ras interaction with PI3K: More than just another effector pathway. *Genes and Cancer*, *2*(3), 261–274. https://doi.org/10.1177/1947601911408079

Ceter, M. M., Jemal, A., Smith, R. A., & Ward, E. (2009). Worldwide variations in colorectal cancer. *CA Cancer Journal for Clinicians*, *59*, 366–378. https://doi.org/10.3322/caac.20038.Available

Chávez-Mendoza, C., & Sánchez, E. (2017). Bioactive compounds from mexican varieties of the common bean (*Phaseolus vulgaris*): Implications for health. *Molecules*, *22*(8), 1–32. https://doi.org/10.3390/molecules22081360

Chen, H. Z., Tsai, S. Y., & Leone, G. (2009). Emerging roles of E2Fs in cancer: An exit from cell cycle control. *Nature Reviews Cancer*, *9*(11), 785–797. https://doi.org/10.1038/nrc2696

Chen, M., Rao, Y., Zheng, Y., Wei, S., Li, Y., Guo, T., & Yin, P. (2014). Association between soy isoflavone intake and breast cancer risk for pre- and post-menopausal women: A meta-analysis of epidemiological studies. *PLoS ONE*, *9*(2), 1–10. https://doi.org/10.1371/journal.pone.0089288

Colangelo, L. (2015). La traduzione delle fonti del diritto romano e la formazione di un linguaggio giuridico cinese: Possibili interferenze grammaticali dal latino. *Rivista Degli Studi Orientali*, *88*(1–4), 285–312. https://doi.org/10.1038/nrc.2015.17

Crowell, J. A., Steele, V. E., & Fay, J. R. (2007). Targeting the AKT protein kinase for cancer chemoprevention. *Molecular Cancer Therapeutics*, *6*(8), 2139–2148. https://doi.org/10.1158/1535-7163.MCT-07-0120

Czabotar, P. E., Lessene, G., Strasser, A., & Adams, J. M. (2014). Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. *Nature Reviews Molecular Cell Biology*, *15*(1), 49–63. https://doi.org/10.1038/nrm3722

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., … Futreal, P. A. (2002). Mutations of the BRAF gene in human. *Nature*, *417*(6892), 949–954. https://doi.org/10.1038/nature00766

Davies, M. A. (2011). Regulation, role, and targeting of Akt in cancer. *Journal of Clinical Oncology*, *29*(35), 4715–4717. https://doi.org/10.1200/JCO.2011.37.4751

de Gramont, A., Figer, A., Seymour, M., Homerin, M., Hmissi, A., Cassidy, J., … Bonetti, A. (2000). Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *Journal of Clinical Oncology*, *18*(16),

2938–2947. https://doi.org/10.1200/JCO.2000.18.16.2938

den Besten, G., van Eunen, K., Groen, A., Venema, K., Reijngoud, D., & Bakker, B. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, *54*, 2325–2340. https://doi.org/10.1194/jlr.R036012

Díaz-Batalla, L., Widholm, J. M., Fahey, G. C., Castaño-Tostado, E., & Paredes-López, O. (2006). Chemical components with health implications in wild and cultivated Mexican common bean seeds (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry*, *54*(6), 2045–2052. https://doi.org/10.1021/jf051706l

Díaz, A. M., Caldas, G. V., & Blair, M. W. (2010). Concentrations of condensed tannins and anthocyanins in common bean seed coats. *Food Research International*, *43*(2), 595–601. https://doi.org/10.1016/j.foodres.2009.07.014

Domínguez-Perles, R., Machado, N., Abraão, A. S., Carnide, V., Ferreira, L., Rodrigues, M., … Barros, A. I. R. N. A. (2016). Chemometric analysis on free amino acids and proximate compositional data for selecting cowpea (*Vigna unguiculata* L.) diversity. *Journal of Food Composition and Analysis*, *53*, 69–76. https://doi.org/10.1016/j.jfca.2016.09.006

Doubeni, C. A., Major, J. M., Laiyemo, A. O., Schootman, M., Zauber, A. G., Hollenbeck, A. R., … Allison, J. (2012). Contribution of behavioral risk factors and obesity to socioeconomic differences in colorectal cancer incidence. *Journal of the National Cancer Institute*, *104*(18), 1353–1362. https://doi.org/10.1093/jnci/djs346

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia*, *77*, 67–82. https://doi.org/10.1016/j.fitote.2005.11.008

Erdrich, J., Zhang, X., Giovannucci, E., & Willett, W. (2015). Proportion of colon cancer attributable to lifestyle in a cohort of US women. *Cancer Causes and Control*, *26*(9), 1271–1279. https://doi.org/10.1007/s10552-015-0619-z

Feregrino-Pérez, A. A., Berumen, L. C., García-Alcocer, G., Guevara-Gonzalez, R. G., Ramos-Gomez, M., Reynoso-Camacho, R., … Loarca-Piña, G. (2008). Composition and chemopreventive effect of polysaccharides from common beans (*Phaseolus vulgaris* L.) on azoxymethane-induced colon cancer. *Journal of Agricultural and Food Chemistry*, *56*(18), 8737–8744. https://doi.org/10.1021/jf8007162

Feregrino-Perez, A. A., Piñol-Felis, C., Gomez-Arbones, X., Guevara-González, R. G.,

Campos-Vega, R., Acosta-Gallegos, J., & Loarca-Piña, G. (2014). A non-digestible fraction of the common bean (*Phaseolus vulgaris* L.) induces cell cycle arrest and apoptosis during early carcinogenesis. *Plant Foods for Human Nutrition*, 248–254. https://doi.org/10.1007/s11130-014-0428-7

Freudenheim, J. L., Graham, S., Marshall, J. R., Horvath, P. J., Haughey, B. P., & Wilkinson, G. (1990). Risks associated with source of fiber and fiber components in cancer of the colon and rectum. *Cancer Research*, *50*(11), 3295–3300.

Ganesan, K., & Xu, B. (2017). Polyphenol-rich dry common beans (*Phaseolus vulgaris* L.) and their health benefits. *International Journal of Molecular Sciences*, *18*(11), 1–26. https://doi.org/10.3390/ijms18112331

Gerber, D. E. (2008). Targeted therapies: A new generation of cancer treatments. *American Family Physician*, *77*(3), 311–319.

GLOBOCAN. (2018). World Health Organization. Retrieved from http://gco.iarc.fr/today

Gonçalves, A., Goufo, P., Barros, A., Domínguez-Perles, R., Trindade, H., Rosa, E. A. S., … Rodrigues, M. (2016). Cowpea (*Vigna unguiculata* L. Walp), a renewed multipurpose crop for a more sustainable agri-food system: Nutritional advantages and constraints. *Journal of the Science of Food and Agriculture*, *96*(9), 2941–2951. https://doi.org/10.1002/jsfa.7644

Grady, W. M., & Pritchard, C. C. (2014). Molecular alterations and biomarkers in colorectal cancer. *Toxicologic Pathology*, *42*(1), 124–139. https://doi.org/10.1177/0192623313505155

Guillamón, E., Pedrosa, M. M., Burbano, C., Cuadrado, C., Sánchez, M. de C., & Muzquiz, M. (2008). The trypsin inhibitors present in seed of different grain legume species and cultivar. *Food Chemistry*, *107*(1), 68–74. https://doi.org/10.1016/j.fooDChem.2007.07.029

Guo, J. M., Xiao, B. X., Liu, D. H., Grant, M., Zhang, S., Lai, Y. F., … Liu, Q. (2004). Biphasic effect of daidzein on cell growth of human colon cancer cells. *Food and Chemical Toxicology*, *42*(10), 1641–1646. https://doi.org/10.1016/j.fct.2004.06.001

Haggar, F., & Boushey, R. (2009). Colorectal cancer epidemiology : Incidence , mortality , survival , and risk factors. *Clinics in Colon and Rectal Surgery*, *6*(212), 191–197. https://doi.org/10.1055/s-0029-1242458.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(1), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hangen, L., & Bennink, M. R. (2009). Consumption of black beans and navy beans (*Phaseolus vulgaris*) reduced azoxymethane-induced colon cancer in rats. *Nutrition and Cancer*, *44*(1), 60–65. https://doi.org/10.1207/S15327914NC441

Hashim, O. H., Jayapalan, J. J., & Lee, C. S. (2017). Lectins: an effective tool for screening of potential cancer biomarkers. *PeerJ*, *5*, e3784. https://doi.org/10.7717/peerj.3784

Haveman-Nies, A., Tucker, K., de Groot, L., Wilson, P., & Staveren, W. (2001). Evaluation of dietary quality in relationship to nutritional and lifestyle factors in elderly people of the US Framingham Heart Study and the European SENECA study. *European Journal of Clinical Nutrition*, *55*, 870–880. https://doi.org/0954–3007/01

Hayat, I., Ahmad, A., Masud, T., Ahmed, A., & Bashir, S. (2014). Nutritional and health perspectives of beans (*Phaseolus vulgaris* L.): An overview. *Critical Reviews in Food Science and Nutrition*, *54*(5), 580–592. https://doi.org/10.1080/10408398.2011.596639

Hsu, A., Bray, T. M., Helferich, W. G., Doerge, D. R., & Ho, E. (2010). Differential effects of whole soy extract and soy isoflavones on apoptosis in prostate cancer cells, *235*(1), 90–97. https://doi.org/10.1016/j.pmrj.2014.02.014.Lumbar

Huber, K. (2016). Phenolic acid, flavonoids and antioxidant activity of common brown beans (*Phaseolus vulgaris* L.) before and after cooking. *Journal of Nutrition & Food Sciences*, *06*(05), 1–7. https://doi.org/10.4172/2155-9600.1000551

Hughes, J., Ganthavorn, C., & Wilson-Sanders, S. (1997). Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats. *The Journal of Nutrition*, *127*(June), 2328–2333.

Huxley, R. R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C. L., & Woodward, M. (2009). The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *International Journal of Cancer*, *125*(1), 171–180. https://doi.org/10.1002/ijc.24343

Hydbring, P., Malumbres, M., & Sicinski, P. (2016). Non-canonical functions of cell cycle cyclins and cyclin-dependent kinases. *Nature Reviews Molecular Cell Biology*, *17*(5), 280–292. https://doi.org/10.1038/nrm.2016.27

Ichim, G., & Tait, S. W. G. (2016). A fate worse than death: Apoptosis as an oncogenic process. *Nature Reviews Cancer*, *16*(8), 539–548. https://doi.org/10.1038/nrc.2016.58

Jess, T., Simonsen, J., Jorgensen, K. T., Pedersen, B. V., Nielsen, N. M., & Frisch, M. (2012). Decreasing risk of colorectal cancer in patients with inflammatory bowel disease over 30 years. *Gastroenterology*, *143*(2), 375–381. https://doi.org/10.1053/j.gastro.2012.04.016

Karapanos, I., Papandreou, A., Skouloudi, M., Makrogianni, D., Fernández, J. A., Rosa, E., … Savvas, D. (2017). Cowpea fresh pods – a new legume for the market: assessment of their quality and dietary characteristics of 37 cowpea accessions grown in southern Europe. *Journal of the Science of Food and Agriculture*, *97*(13), 4343–4352. https://doi.org/10.1002/jsfa.8418

Koratkar, R., & Rao, A. V. (1997). Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions in the colon of mice. *Nutrition and Cancer*, *27*(October), 206–209. https://doi.org/10.1080/01635589709514526

Kuipers, E. J., Grady, W. M., Lieberman, D., Seufferlein, T., Sung, J. J., Boelens, P. G., … Watanabe, T. (2015). Colorectal cancer. *Nat Rev Dis Primers*, *1*(15065), 1–51. https://doi.org/10.1038/nrdp.2015.65.COLORECTAL

Kumar, K. K., Reddy, G. S., Reddy, B., Shekar, P. C., Sumanthi, J., & Chandra, K. L. P. (2012). Biological role of lectins: A review. *Journal of Orofacial Sciences*, *4*(1), 20. https://doi.org/10.4103/0975-8844.99883

Lai, L. R., Hsieh, S. C., Huang, H. Y., & Chou, C. C. (2013). Effect of lactic fermentation on the total phenolic, saponin and phytic acid contents as well as anti-colon cancer cell proliferation activity of soymilk. *Journal of Bioscience and Bioengineering*, *115*(5), 552–556. https://doi.org/10.1016/j.jbiosc.2012.11.022

Lanza, E., Hartman, T. J., Albert, P. S., Shields, R., Slattery, M., Caan, B., … Schatzkin, A. (2006). High dry bean intake and reduced risk of advanced colorectal adenoma recurrence among participants in the polyp prevention trial. *Journal of Nutrition*, *136*(7), 1896–1903. https://doi.org/10.1093/jn/136.7.1896

Lee, Y. K., Hwang, J. T., Lee, M. S., Kim, Y. M., & Park, O. J. (2009). Kidney bean husk extracts exert antitumor effect by inducing apoptosis involving AMP-activated protein kinase signaling pathway. *Annals of the New York Academy of Sciences*, *1171*, 484–488. https://doi.org/10.1111/j.1749-6632.2009.04697.x

Lepri, S. R., Zanelatto, L. C., da Silva, P. B. G., Sartori, D., Ribeiro, L. R., & Mantovani, M. S. (2014). The effects of genistein and daidzein on cell proliferation kinetics in HT29 colon cancer cells: The expression of CTNNBIP1 (β-catenin), APC (adenomatous polyposis coli) and BIRC5 (survivin). *Human Cell*, *27*(2), 78–84. https://doi.org/10.1007/s13577-012-0051-6

Letai, A. G. (2008). Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nature Reviews Cancer*, *8*(2), 121–132. https://doi.org/10.1038/nrc2297

Lima, A. I. G., Mota, J., Monteiro, S. A. V. S., & Ferreira, R. M. S. B. (2016). Legume seeds and colorectal cancer revisited: Protease inhibitors reduce MMP-9 activity and colon cancer cell migration. *Food Chemistry*, *197*, 30–38. https://doi.org/10.1016/j.fooDChem.2015.10.063

Lindskog, E. B., Gunnarsdóttir, K. Á., Derwinger, K., Wettergren, Y., Glimelius, B., & Kodeda, K. (2014). A population-based cohort study on adherence to practice guidelines for adjuvant chemotherapy in colorectal cancer. *BMC Cancer*, *14*(1), 1–9. https://doi.org/10.1186/1471-2407-14-948

Liu, P., Cheng, H., Roberts, T. M., & Zhao, J. J. (2009). Targeting the phosphoinositide 3-kinase (PI3K) pathway in cancer. *Nature Reviews. Drug Discovery*, *8*(8), 627–644. https://doi.org/10.1038/nrd2926.Targeting

Luna-Vital, D. A., Liang, K., González de Mejía, E., & Loarca-Piña, G. (2016). Dietary peptides from the non-digestible fraction of: *Phaseolus vulgaris* L. decrease angiotensin II-dependent proliferation in HCT116 human colorectal cancer cells through the blockade of the renin-angiotensin system. *Food and Function*, *7*(5). https://doi.org/10.1039/c6fo00093b

Lupton, J. R. (2004). Diet Induced Changes in the Colonic Environment and Colorectal Cancer. *Journal of Nutrition*, 479–482. https://doi.org/0022-3166/04

Marley, A. R., & Nan, H. (2016). Epidemiology of colorectal cancer. *International Journal of Molecular Epidemiology and Genetics*, *7*(3), 105–114. ISSN:1948-1756/IJMEG0036410

Messina, V. (2014). Nutritional and health benefits of dried beans.: Discovery Service for Endeavour College of Natural Health Library. *The American Journal of Clinical Nutrition*, *100*(1), 437. https://doi.org/10.3945/ajcn.113.071472.2

Mizushina, Y., Shiomi, K., Kuriyama, I., Takahashi, Y., & Yoshida, H. (2013). Inhibitory effects of a major soy isoflavone, genistein, on human DNA topoisomerase II activity and cancer cell proliferation. *International Journal of*

Oncology, *43*(4), 1117–1124. https://doi.org/10.3892/ijo.2013.2032

Namuslu, M., Kocaoglu, H., Celik, H., Avci, A., Devrim, E., Genc, Y., … Durak, I. (2014). Effects of aqueous soy, mistletoe and red clover extracts on activities of adenosine deaminase and xanthine oxidase enzymes. *Bratisl Lek Listy*, *115*(6), 367–371. https://doi.org/10.4149/BLL_2014_072 CLINICAL

Ogino, S., Chan, A. T., Fuchs, C. S., & Giovannucci, E. (2012). NIH Public Access, *60*(3), 397–411. https://doi.org/10.1136/gut.2010.217182.Molecular

Oh, Y. J., & Sung, M. K. (2001). Soy saponins inhibit cell proliferation by suppressing PKC activation and induce differentiation of HT29 human colon adenocarcinoma cells. *Nutrition and Cancer*, *39*(1), 132–138. https://doi.org/10.1207/S15327914nc391_18

Ombra, M. N., D 'Acierno, A., Nazzaro, F., Riccardi, R., Spigno, P., Zaccardelli, M., … Fratianni, F. (2016). Phenolic composition and antioxidant and antiproliferative activities of the extracts of twelve common bean (*Phaseolus vulgaris* L.) endemic ecotypes of southern Italy before and after cooking. *Oxidative Medicine and Cellular Longevity*, *2016*, 1–11. https://doi.org/10.1155/2016/1398298

Otto, T., & Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. *Nature Reviews Cancer*, *17*(2), 93–115. https://doi.org/10.1038/nrc.2016.138

Pardini, B., Kumar, R., Naccarati, A., Novotny, J., Prasad, R. B., Forsti, A., … Lorenzo Bermejo, J. (2011). 5-Fluorouracil-based chemotherapy for colorectal cancer and MTHFR/MTRR genotypes. *British Journal of Clinical Pharmacology*, *72*(1), 162–163. https://doi.org/10.1111/j.1365-2125.2010.03892.x

Phillips, R. D., McWatters, K. H., Chinnan, M. S., Hung, Y. C., Beuchat, L. R., Sefa-Dedeh, S., … Saalia, F. K. (2003). Utilization of cowpeas for human food. *Field Crops Research*, *82*(2–3), 193–213. https://doi.org/10.1016/S0378-4290(03)00038-8

Raju, J., Patlolla, J. M. R., Swamy, M. V, Raju, J., Patlolla, J. M. R., Swamy, M. V, & Rao, C. V. (2004). Diosgenin , a Steroid Saponin of Trigonella foenum graecum (Fenugreek), Inhibits Azoxymethane-Induced Aberrant Crypt Foci Formation in F344 Rats and Induces Apoptosis in HT29 Human Colon Cancer Cells Diosgenin , a Steroid Saponin of Trigonella foenum g. *Cancer Epidemiology, Biomarkers & Prevention*, *13*(1), 1392–1399.

Ramírez-Jiménez, A. K., Reynoso-Camacho, R., Tejero, M. E., León-Galván, F., & Loarca-Piña, G. (2015). Potential role of bioactive compounds of *Phaseolus vulgaris* L. on lipid-lowering mechanisms. *Food Research International*, *76*(P1), 92–104. https://doi.org/10.1016/j.foodres.2015.01.002

Rebello, C. J., Greenway, F. L., & Finley, J. W. (2014). Whole grains and pulses: A comparison of the nutritional and health benefits. *Journal of Agricultural and Food Chemistry*, *62*(29), 7029–7049. https://doi.org/10.1021/jf500932z

Rejhová, A., Opattová, A., Čumová, A., Slíva, D., & Vodička, P. (2018). Natural compounds and combination therapy in colorectal cancer treatment. *European Journal of Medicinal Chemistry*, *144*, 582–594. https://doi.org/10.1016/j.ejmech.2017.12.039

Roberts, P., & Der, C. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of RAS mutant cancers. *Oncogene*, *26*, 3291–3310. https://doi.org/10.1038/sj.onc.1210422

Roy, F., Boye, J. I., & Simpson, B. K. (2010). Bioactive proteins and peptides in pulse crops: Pea, chickpea and lentil. *Food Research International*, *43*(2), 432–442. https://doi.org/10.1016/j.foodres.2009.09.002

Santarpia, L., Lippman, S. M., & El-Naggar, A. K. (2012). Targeting the MAPK–RAS–RAF signaling pathway in cancer. *Expert Opinion on Therapeutic Targets*, *16*(1), 103–119. https://doi.org/10.1517/14728222.2011.645805

Saridaki, Z., & Souglakos, J. (2013). Genetic alterations in colorectal cancer in older patients. *Management of Colorectal Cancers in Older People*, (February), 9–20. https://doi.org/10.1007/978-0-85729-984-0_2

Shi, J., Arunasalam, K., Yeung, D., Kakuda, Y., Mittal, G., & Jiang, Y. (2004). Saponins from Edible Legumes: Chemistry, Processing, and Health Benefits. *Journal of Medicinal Food*, *7*(1), 67–78. https://doi.org/10.1089/109662004322984734

Shin, A., Lee, J., Lee, J., Park, M. S., Park, J. W., Park, S. C., … Kim, J. (2015). Isoflavone and soyfood intake and colorectal cancer risk: A case-control study in Korea. *PLoS ONE*, *10*(11), 1–17. https://doi.org/10.1371/journal.pone.0143228

Siah, S. D., Konczak, I., Agboola, S., Wood, J. A., & Blanchard, C. L. (2012). In vitro investigations of the potential health benefits of Australian-grown faba beans (Vicia faba L.): chemopreventative capacity and inhibitory effects on the angiotensin-converting enzyme, α-glucosidase and lipase. *British Journal of*

Nutrition, *108*(S1), S123–S134. https://doi.org/10.1017/S0007114512000803

Siah, S., Wood, J. A., Agboola, S., Konczak, I., & Blanchard, C. L. (2014). Effects of soaking, boiling and autoclaving on the phenolic contents and antioxidant activities of faba beans (*Vicia faba* L.) differing in seed coat colours. *Food Chemistry*, *142*, 461–468. https://doi.org/10.1016/j.fooDChem.2013.07.068

Tang, C. H., & Sun, X. (2011). Structure-physicochemical function relationships of 7S globulins (vicilins) from red bean (Phaseolus angularis) with different polypeptide constituents. *Food Hydrocolloids*, *25*(3), 536–544. https://doi.org/10.1016/j.foodhyd.2010.08.009

Tao, M. H., Xu, W. H., Zheng, W., Gao, Y. T., Ruan, Z. X., Cheng, J. R., … Shu, X. O. (2005). A case-control study in Shanghai of fruit and vegetable intake and endometrial cancer. *British Journal of Cancer*, *92*(11), 2059–2064. https://doi.org/10.1038/sj.bjc.6602609

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: Controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology*, *9*(3), 231–241. https://doi.org/10.1038/nrm2312

Tharanathan, R. N., & Mahadevamma, S. (2003). Grain legumes - A boon to human nutrition. *Trends in Food Science and Technology*, *14*(12), 507–518. https://doi.org/10.1016/j.tifs.2003.07.002

Thompson, M. D., Thompson, H. J., Brick, M. A., McGinley, J. N., Jiang, W., Zhu, Z., & Wolfe, P. (2008). Mechanisms Associated with Dose-Dependent Inhibition of Rat Mammary Carcinogenesis by Dry Bean (*Phaseolus vulgaris* L.). *The Journal of Nutrition*, *138*(11), 2091–2097. https://doi.org/10.3945/jn.108.094557

Tiwari, B., Gowen, A., & McKnna, B. (2011). *Pulse Foods Processing, Quality and Nutraceutical Applications*. (B. Tiwari, A. Gowen, & B. McKenna, Eds.) (1st Editio). Academic Press.

Trinidad, T. P., Mallillin, A. C., Loyola, A. S., Sagum, R. S., & Encabo, R. R. (2010). The potential health benefits of legumes as a good source of dietary fibre. *The British Journal of Nutrition*, *103*(4), 569–574. https://doi.org/10.1017/S0007114509992157

Tsai, C. Y., Chen, Y. H., Chien, Y. W., Huang, W. H., & Lin, S. H. (2010). Effect of soy saponin on the growth of human colon cancer cells. *World Journal of Gastroenterology : WJG*, *16*(27), 3371–6.

https://doi.org/10.3748/wjg.v16.i27.3371

Vasen, H. F. A., Tomlinson, I., & Castells, A. (2015). Clinical management of hereditary colorectal cancer syndromes. *Nature Reviews Gastroenterology & Hepatology*, *12*(2), 88–97. https://doi.org/10.1038/nrgastro.2014.229

Velie, E. M., Schairer, C., Flood, A., He, J. P., Khattree, R., & Schatzkin, A. (2005). Empirically derived dietary patterns and risk of postmenopausal breast cancer in a large prospective cohort study. *American Journal of Clinical Nutrition*, *82*(6), 1308–1319. https://doi.org/82/6/1308 [pii]

Vergara-Castañeda, H. A., Guevara-González, R. G., Ramos-Gómez, M., Reynoso-Camacho, R., Guzmán-Maldonado, Feregrino-Pérez, A. A., … Lorca-Piña, G. (2010). Non-digestible fraction of cooked bean (*Phaseolus vulgaris* L .) cultivar Bayo Madero suppresses colonic aberrant crypt foci in azoxymethane-induced rats, 294–300. https://doi.org/10.1039/c0fo00130a

Vergara-Castañeda, H., Guevara-González, R., Guevara-Olvera, L., Oomah, B. D., Rosalía, R.-C., Wiersma, P., & Loarca-Piña, G. (2012). Non-digestible fraction of beans (*Phaseolus vulgaris* L .) modulates signalling pathway genes at an early stage of colon cancer in Sprague – Dawley rats British Journal of Nutrition. https://doi.org/10.1017/S0007114512000785

Vermeirssen, V., Augustijns, P., Morel, N., Van Camp, J., Opsomer, A., & Verstraete, W. (2005). In vitro intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests. *International Journal of Food Sciences and Nutrition*, *56*(6), 415–430. https://doi.org/10.1080/09637480500407461

Wang, B., Yao, M., Lv, L., Ling, Z., & Li, L. (2017). The Human Microbiota in Health and Disease. *Engineering*, *3*(1), 71–82. https://doi.org/10.1016/J.ENG.2017.01.008

Waqas, M. K., Akhtar, N., Mustafa, R., Jamshaid, M., Khan, H. M. S., & Murtaza, G. (2015). Dermatological and cosmeceutical benefits of *Glycine max* (soy) and its active components. *Acta Poloniae Pharmaceutica - Drug Research*, *72*(1), 3–11.

Wong, C. L., Mollard, R. C., Zafar, T. A., Luhovyy, B. L., & Anderson, G. H. (2009). Food intake and satiety following a serving of pulses in young men: Effect of processing, recipe, and pulse variety. *Journal of the American College of Nutrition*, *28*(5), 543–552. https://doi.org/10.1080/07315724.2009.10719786

World Cancer Research Fund. (2017). Diet, nutrition, physical activity and colorectal cancer. Retrieved from https://www.wcrf.org/sites/default/files/Colorectal-Cancer-2017-Report.pdf

World Cancer Research Fund International. (2015). Worldwide data | World Cancer Research Fund International. Retrieved August 28, 2017, from http://www.wcrf.org/int/cancer-facts-Figures/worldwide-data

Yue, R., Shen, B., & Morrison, S. J. (2016). Clec11a/osteolectin is an osteogenic growth factor that promotes the maintenance of the adult skeleton. *ELife*, *5*, 27. https://doi.org/10.7554/eLife.18782

Zeki, S. S., Graham, T. A., & Wright, N. A. (2011). Stem cells and their implications for colorectal cancer. *Nature Reviews Gastroenterology and Hepatology*, *8*(2), 90–100. https://doi.org/10.1038/nrgastro.2010.211

Zhu, B., Sun, Y., Qi, L., Zhong, R., & Miao, X. (2015). Dietary legume consumption reduces risk of colorectal cancer: Evidence from a meta-analysis of cohort studies. *Scientific Reports*, *5*, 27–33. https://doi.org/10.1038/srep08797

Ziaei, S., & Halaby, R. (2017). Dietary Isoflavones and Breast Cancer Risk. *Medicines*, *4*(2), 18. https://doi.org/10.3390/medicines4020018

Zou, Y., & Chang Sam, K. (2014). Antioxidant and Antiproliferative Properties of Extract and Fractions from Small Red Bean (*Phaseolus vulgaris* L .). *Journal of Food Nutrition*, *1*, 1–11.

CHAPTER 2

Objectives

2.1. General objectives

The main purpose of the present PhD project is to study the effect of selected leguminous foods of the Mediterranean diet on prevention and treatment of CRC. In this sense, we propose to:

i) Perform the extraction and characterization of phenolic compounds from different legumes;
ii) Characterize the chemoprotective effects of legumes extracts in different colon cancer cell lines;
iii) Identify molecular targets in signaling pathways related to proliferation and cell cycle;
iv) Study effects on the sensitivity to drugs used in chemotherapy;

2.2. Specific objectives

This project is structured into four specific related aims that pursue the non-pharmacological and combination with pharmacological therapeutic approaches to prevent and treat colon cancer. The experiments were performed in cell derived from human colon cancer with different mutation in PI3KCA, KRAS, BRAF, PTEN and TP53 signaling pathways: HCT116 p53 wild-type, HCT116 p53 null, HCT15, CO115, RKO and HT29.

i. Perform the extraction and characterization of phenolic compounds from different legumes

It was investigated the *in vitro* antioxidant properties, phenolic content and phytochemical composition of raw, cooked and pressure-cooked hydromethanolic extracts obtained from four varieties of *Phaseolus vulgaris* L. (common beans), *Vigna unguiculata* L. (cowpea) and *Glycine max* L (soy). For these propose it was used colorimetric assays for investigate the total phenolic content (TPC), total flavonoid content (TFC) and *ortho*-diphenol content (oDC) and the antioxidant activity using DPPH radical scavenging activity, ABTS radical scavenging activity and ferric reduction antioxidant power (FRAP). It was also performed the phytochemical characterization of all different extracts using high performance liquid chromatography (HPLC).

ii. Characterize the anticancer effects of pulses extracts in colon cancer cell lines

To evaluate the cell cytotoxicity of the extracts it was used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. MTT is a colorimetric assays that involve the conversion of MTT, by dehydrogenase enzymes in metabolic active cells, into formazan crystals. With this experiment it was also determine the IC_{50} that corresponded to minimal concentration that inhibit 50% of cell viability.

The apoptosis was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. It is a method for detecting DNA fragmentation generated during apoptosis. The assay is based on the use of terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes attachment of deoxynucleotides, tagged with a fluorochrome to 3'-hydroxyl termini in the double-strand DNA breaks. Fluorescence microscopy was used to quantify the cell death by apoptosis.

Flow cytometry analysis was performed to study if the extracts can induce cell cycle arrest.

iii. Identify molecular targets in signaling pathways related to proliferation and cell cycle modulation

Western-blot and quantitative PCR (qPCR) were used to analyze the involvement of the PI3K-AKT and MAPK/ERK signaling pathways and the expression of proteins involved cell cycle progression.

iv. Study the effects on the sensitivity to drugs used in chemotherapy

In this task was use the different cell lines (harboring different gene mutation representatives of several CRC) to evaluate the effect of extracts in combination with chemotherapeutic drugs 5-FU to evaluate possible synergisms. For this, it was assessed the sensibility of the cell lines to the chemotherapeutic drugs and determine the half maximal inhibitory concentration IC_{50}. After that, cells were co-incubate with the drug and extracts. The effect of the co-incubation on cell viability was performed using MTT reduction assay.

CHAPTER 3

Effect of cooking on phenolic composition and antioxidant potential of selected common bean and soy

This chapter enclose the following manuscript:

Teixeira-Guedes, C. I., Oppolzer, D., Barros, A. I. & Pereira-Wilson, C. (2018). Impact of cooking method on phenolic composition and antioxidant potential of four varieties of *Phaseolus vulgaris* L. and *Glycine max* L. Submitted to *LWT: Food Science and Technology.* Minor Revision for Acceptance.

Impact of cooking method on phenolic composition and antioxidant potential of four varieties of *Phaseolus vulgaris* L. and *Glycine max* L.

Catarina I. Teixeira-Guedes[ab]*, David Oppolzer[c], Ana I. Barros[c], Cristina Pereira-Wilson[abd]

[a]*Department of Biology, University of Minho, Braga, Portugal*

[b]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Department of Biology, University of Minho, Braga, Portugal*

[c]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

[d]*Centre of Biological Engineering, University of Minho, Braga, Portugal*

Abstract

The present study aimed to investigate the effects of different cooking conditions - atmospheric (100°C) and pressure cooking (115°C) - on the phenolic composition and antioxidant activity of methanolic extracts of four *Phaseolus vulgaris* varieties and soy (*Glycine max*). Contrary to soy, in *P. vulgaris* varieties both cooking methods increased drastically the total phenolic, flavonoid, and *ortho*-diphenol content, as well as antioxidant capacity. These results were corroborated by HPLC analysis, where an overall increase of phenolic acids and flavonoids was detected in processed samples. However, draining the cooking water significantly decreased phenolic acids, flavonoids and antioxidant activity in all *P. vulgaris* varieties and as well as soy. The hypothesis that cooking increases the compound accessibility and nutritional value through increased release of phytochemicals was verified in the present study for *P. vulgaris* varieties. Keeping the cooking water is crucial to the increased nutritional value of all Phaseolus varieties. Overall, compared with the tested varieties of Phaseolus, soy, to which many health benefits are attributed, is not the best legume source of antioxidants.

Keywords: *Phaseolus vulgaris*; soy, cooking; phenolic; antioxidant

3.1. Introduction

Legumes are the second most important food crop produced and consumed worldwide after cereal grains. They are a significant dietary protein source in several regions of the world (Kalogeropoulos *et al*., 2010) and constitute an important ingredient of the Mediterranean diet (Rosato *et al*., 2016).

Most dietary beans, also known as common beans, are varieties of *Phaseolus vulgaris* L., herbaceous annual plants of the Fabaceae (legume or bean) family that have been domesticated in two separate events, in Peru and Mexico, 8 000 and 7 000 years ago, respectively (Hayat *et al*., 2014). *Glycine max* L., known as soy bean, is a legume native to East Asia (including China, Japan and Korea) that has been domesticated more than 3 000 years ago for its edible seeds and young pods. It is today the world's most important legume crop growing worldwide in diverse climates and the most widely used as oilseed (Waqas *et al*., 2015).

Nutritionally, legumes are excellent and affordable sources of protein, complex carbohydrates and dietary fiber, and are low in fat (Chávez-Mendoza & Sánchez, 2017; Ganesan & Xu, 2017). They exhibit a lower glycemic index compared to other starchy foods, such as cereals and potatoes. Legumes have also additional nutritional benefits due to their micronutrients, including minerals and vitamins, as well as, bioactive compounds including oligosaccharides, lectins, saponins, phytates and phenolic compounds (Kalogeropoulos *et al*., 2010; Siah *et al*., 2012). These phytochemicals can have a health promoting effect. Legumes have gaining interest in the food market for their antioxidant and other beneficial properties, suggesting that legumes can became added value functional foods or nutraceuticals (Campos-Vega *et al*., 2017).

Bean phytochemicals are secondary metabolites that are synthesized during normal development and as a response to stress conditions (Chon, 2013). The major bean polyphenolic classes of compounds are tannins, phenolic acids and flavonoids. Phenolic extracts from different types of beans have been shown to have antioxidant properties (Açar *et al*., 2009; Siah *et al*., 2012), protection against DNA damage (Madhujith *et al*., 2004), being antimutagenic and chemopreventive (Siah *et al*., 2012). Legumes consumption may help reduce the risks associated with the consumption of animal proteins in Western countries, leading to the prevention and management of several disease such as hypertension, hypercholesterolemia, type II diabetes, cardiovascular

diseases and cancer, contributing to overall health and wellness (Campos-Vega *et al*., 2010; Chávez-Mendoza & Sánchez, 2017; Hayat *et al*., 2014).

Traditionally, dietary dry seed legumes are prepared by soaking in water, followed by thermal processing such as boiling or pressure cooking, with exception of peas, chickpeas and fava beans that occasionally are roasted and eaten as snacks. It has been described that thermal cooking of legumes improves their nutritional value by reduction of antinutrients such as phytic acid and tannins and increases protein and starch digestibility (Deol & Bains, 2010; Ranilla *et al*., 2009; Rehman *et al*., 2001). Furthermore, cooking procedures induce desirable sensory properties in beans such as sweet taste, flavor and soft texture (Mkanda, Minnaar, & de Kock, 2007; Ranilla *et al*., 2009).

There are abundant reports on the phenolic composition in raw beans (Açar *et al*., 2009; Itoh *et al*., 2005; Marathe *et al*., 2011; Siah *et al*., 2012), however, limited and contradictory information concerning the impact of heat processing of legumes on phytochemical composition and antioxidant activities are available (Eshraq *et al*., 2016; Kumar *et al*., 2012; Ranilla *et al*., 2009; Rocha-Guzmán *et al*., 2007; Siah *et al*., 2014).

The present study aims to investigate the *in vitro* antioxidant properties and phytochemical composition of raw, cooked and pressure-cooked hydromethanolic extracts obtained from four varieties of common beans in a comparative study with soy, which is the most consumed legume worldwide.

3.2. Material and methods

3.2.1. Chemicals and equipment

The reagents of aluminium chloride, sodium nitrite, sodium hydroxide, acetic acid, formic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Methanol, sodium carbonate, gallic acid, Folin–Ciocalteau, sodium molybdate, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetremethychroman-2-carboxylic acid (Trolox), 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid (ABTS), potassium persulfate, 2,4,6-Tripyridyl-s-Triazine (TPTZ), Iron(III) chloride, hydrochloric acid, gallic acid and catechin were purchased from Sigma–Aldrich (St. Louis, USA). All the chemicals used were of analytical grade. Filters of 11 µm pore size from Whatman No. 1 paper (Maidstone, UK) and 96-well plates from Frilabo (Portugal)

were used. The equipment used were an orbital shaker 501 (Bibby Stuart, UK), rotatory evaporator (BÜCHI 461 water bath REIII, Germany), freeze dryer Christ alpha 2-4 (Martin Christ, Germany), microplate reader (Infinite M200 microplate reader, Tecan, Austria), high performance liquid chromatography (HPLC) system with diode array detector (DAD) (Thermo-Fisher Scientific, Inc., Waltham, USA) and ACE 5 C18 column (Advanced Chromatography Technologies Ltd, Scotland).

3.2.2. Plant material

Different varieties of *Phaseolus vulgaris* L. (kidney bean, pinto bean, black bean and borlotti bean) and *Glycine max* L. (soy bean) were used in this work. All legumes were purchased dried from the Portuguese market.

3.2.3. Processing

Beans (10 g) were soaked in 100 mL of distilled water for 12 h at room temperature. Samples of each legume were subjected to atmospheric boiling (100 °C, for 50 min) or pressure cooking (115 °C, for 20 min). In each case samples were prepared by keeping or draining the cooking water.

3.2.4. Preparation of crude phenolic extract

All samples, including raw beans, were freeze-dried and ground into a powder. Extraction was carried out in methanol:water (80:20, v/v) at a solid to solvent ratio of 1:10, ultrasound assisted during 30 min at 30 °C and maintained for 24 h in orbital shaker in the dark. The extracts were then vacuum filtrated through Whatman No. 1 paper and the extracts were concentrated using a rotatory evaporator under reduced pressure at 40 °C and then freeze-dried and stored in the dark vials sealed with cap and parafilm at 4 °C.

3.2.5. Total phenolic content

The total phenolic content (TPC) was determined by the Folin–Ciocalteau method described Domínguez-Perles *et al*., (2014) with some modification and optimized to 96-well plates. In brief, 20 µL of samples or standard were added, followed by 100 µL of

Folin–Ciocalteau:H_20 (1:10) and 80 µL of sodium carbonate (7.5 %). The reaction was incubated during 30 min at 42 °C in the dark and the absorbance measured at 750 nm in a microplate reader. Results were calculated with the help of a standard curve and expressed as mg of gallic acid equivalents per g of dry weight (mg GAEq g^{-1} DW).

3.2.6. Total flavonoid content

The total flavonoid content (TFC) was determined by the aluminium chloride colorimetric method as described by Domínguez-Perles *et al*., (2014) with some modifications. To each of 96-wells, 24 µL of the sample or standard were added, followed by 28 µL of sodium nitrite (5 %), and 5 min later, 28 µL of aluminium chloride (10 %). The mixture was left to react for 6 min and then 120 µL sodium hydroxide (1 M) were added. Absorbance was read at 510 nm, results calculated with the help of a standard curve and expressed as mg catechin equivalents per g of dry weight (mg CTEq g^{-1} DW).

3.2.7. ortho-diphenol content

The *ortho*-diphenol content (oDC) was assessed by the sodium molybdate complexation method according to Domínguez-Perles *et al*., (2014). The samples or standard (160 µL) were pipetted to each well followed by 40 µL of Na_2MO_4 (5 %). The reaction was incubated for 15 min in the dark and the absorbance measured at 370 nm. Results were calculated with the help of a gallic acid standard curve, therefore oDC content was expressed in mg GAE g^{-1} DW.

3.2.8. DPPH radical scavenging activity

DPPH radical scavenging activity assay was carried out as in Domínguez-Perles *et al*., (2014) with some modifications. To 10 µL of sample or Trolox standard, 190 µL of the DPPH solution were added. The mixture was placed in the dark at room temperature for 30 min, and absorbance was measured at 520 nm in a microplate reader. Inhibition of free radical DPPH in percent (%) was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Abs blank} - \text{Abs sample}}{\text{Abs blank}} \text{x } 100$$

DPPH radical scavenging activity of the samples was determined by interpolation of the calibration curve for Trolox. Results were expressed in µM Trolox equivalent per g of dry weight (µM TEq g^{-1} DW).

3.2.9. ABTS radical scavenging activity

ABTS radical scavenging activity was performed as previously reported by Domínguez-Perles *et al*., (2014) with some modifications. To assess ABTS radical inhibition, 12 µL of sample or standard were placed in the microplate followed by 188 µL of ABTS working solution. The plate was incubated for 30 min at room temperature in the dark and the absorbance was measured at 734 nm. Inhibition of ABTS radicals was calculated as previously described for DPPH.

3.2.10. Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay was performed according to Bolanos De La Torre *et al*., (2015) with minor alterations. To the 96-well microplate 20 µL of sample was added followed by 280 µL of FRAP working solution. The reaction was incubated at 37 ºC in the dark for 30 min and read at 593 nm. Trolox was used as standard and results expressed in µM TEq g^{-1} DW.

3.2.11. Phytochemical characterization by high performance liquid chromatography (HPLC)

The phenolic compounds profile was analyzed according to Lin et a., (2008) in a HPLC system composed of a quaternary pump, auto sampler and diode array detector (DAD). Separation was performed on a ACE 5 C18 column (5 µm, 250 x 4.6 mm i.d.) at a flow rate of 1 ml/min. Column temperature was 25 °C and injection volume 20 µL. The mobile phase A consisted of water-formic acid (99.9:0.1; v/v) and B of acetonitrile-formic acid (99.9:0.1; v/v). The extract (10 mg/ml) was dissolved in water, injected and eluted using the gradient reported by Lin *et al*., (2008). DAD was set at 280, 310 and 520 nm while a UV/VIS spectrum from 200-600 nm was continuously collected. The chromatograms were analyzed with Xcalibur (Thermo Fisher Scientific, Inc., Waltham, USA).

3.2.12. Statistical analysis

Statistical analyses were performed using IBM SPSS statistics 21.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests was used to compare control and treated groups. Pearson's correlation coefficient *r* was used to quantify a relationship between 2 variables. A p value <0.05 was considered statistically significant.

3.3. Results and discussion

3.3.1. Effect of processing on extraction yield

The efficiency of hydromethanolic extraction, expressed in mg of lyophilized extracts per g of dry weight of the different raw samples was found to decrease in the following order: soy > black > borlotti > pinto > kidney bean (data not shown). The effect of boiling and pressure cooking on the extraction yields, relative to raw samples, is presented in Figure 1. In all *P. vulgaris*, boiling and pressure cooking significantly increased the extraction efficiency, while in soy only a slight increase was observed. The extraction yield in processed kidney and borlotti beans was found to be the highest, reaching an increase of 94 % for boiled and 130.42 % for pressure-cooked kidney bean, and 62 % for boiled and 83 % for the pressure-cooked borlotti bean. In pinto and black beans, boiling was more efficient than pressure cooking. In these samples, boiling generated an increase of 50 % and 30 %, while pressure cooking generated an increase of 20 % and 5 % for pinto and black bean, respectively.

A decrease in extraction yields in all processed samples was observed when the cooking water was discarded. This decrease was more evident in pressure-cooked kidney bean and in both boiled and pressure-cooked borlotti, black bean and soy bean.

These results are consistent with those of Siah *et al*., (2014) who found that boiling increased extraction yield by 2 to 34 % and pressure cooking by 265 to 275 %. The present study showed that heat treatment increased the extraction yield in broths after boiling and pressure cooking in *Phaseolus vulgaris*. This finding suggested that heat could cause destruction of seed structure enabling the release of phenolic compounds into the cooking broths, thereby increasing the extraction efficiency by the used solvent. Heat processing can also induce the release of other components, such as soluble sugars, proteins and soluble fibers, contributing to the increase of the generated material yields.

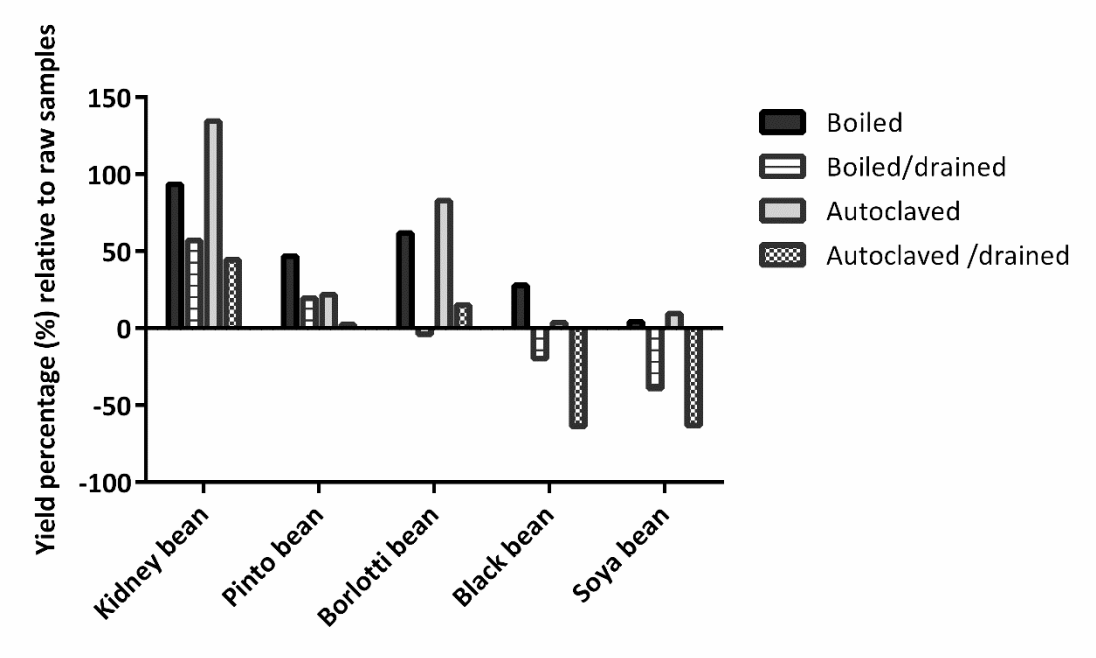

Figure 1. Percentage (%) of gain or loss on extraction yield of different cooking conditions. Values were calculated based on the yield of raw bean. Extraction yield of raw kidney bean - 39,83; pinto bean - 57,76; borlotti bean - 57,79; black bean - 61,06; soy bean - 106,44; expressed in mg of extract/g of dry weight (mg Ex. g^{-1} DW).

3.3.2. Total phenolic, flavonoid and ortho-diphenol content and antioxidant activity

3.3.2.1. Raw samples

Results of colorimetric assays used for assessment of TPC, TFC and oDC are presented in Table 1. Comparing between raw samples, TPC was found to be the highest in soy bean and in decreasing order in pinto bean > black bean > borlotti bean > kidney bean. Raw soy bean TPC was significantly higher ($p<0.05$) than any extracts of *Phaseolus vulgaris*.

Extraction of raw Phaseolus, all presented higher TFC content than soy bean extracts ($p<0.05$), being pinto bean the richest source. In *P. vulgaris* varieties, 40-70 % of the phenolic compounds were flavonoids whereas in soy flavonoids corresponded only to 20 % of total phenols.

The antioxidant activity results are present in Table 2. In all assays, (DPPH, ABTS and FRAP) the antioxidant activity of raw black beans and pinto beans were higher that all the other varieties ($p<0.05$). The antioxidant activity assessed by ABTS and FRAP

showed the same pattern with black bean extracts having higher activity, followed by pinto, kidney, soy and finally borlotti bean. The DPPH radical scavenging activity was higher in black bean, followed by pinto, kidney, borlotti and soy bean.

There are many classes of compounds with antioxidant activity of which *ortho*-diphenols have been recognized as the class with highest antioxidant activity (Soufi, Romero, & Louaileche, 2014). Regarding *ortho*-diphenols, black bean was the richest source ($p<0.05$) and kidney and borlotti beans showed the lowest levels ($p<0.05$) in line with the higher antioxidant activity present in black bean extracts.

Overall, the antioxidant capacity of soy bean was approximately 2 times lower than that of black and pinto beans. Although a higher phenolic content in raw soy bean was observed, this is not reflected in a higher antioxidant activity. In black bean the antioxidant activity seems to be correlated with the oDC, while in the remaining varieties with the TFC.

3.3.2.2. Effect of processing

The effect of boiling and pressure cooking on TPC, TFC and oDC are presented in Table 1 and the percentage of gain or loss relative to the raw samples are shown in Figure 2.

Overall, processing by both cooking methods increased all parameters in *P. vulgaris* varieties in contrast with the effects on soy, where cooking did not improve flavonoid content nor antioxidant activity of hydromethanolic extracts. In kidney, pinto and black beans boiling and pressure cooking had a positive impact on total phenolic, flavonoid and *ortho*-diphenol content. Pressure cooking produced the highest increase in TPC, TFC and oDC in kidney and borlotti beans, while in pinto and black varieties boiling was more efficient. Importantly, in both processing methods, a significant part of TPC, TFC and oDC, as well as antioxidant activity was lost by draining the cooking water.

The antioxidant capacities of extracts are presented in Table 2. The relative changes found in extracts of processed legumes compared to raw samples are presented in the Figure 2. In agreement with phenolic content, the antioxidant activity of the extracts of all varieties of *P. vulgaris* significantly increased after the different cooking conditions, in contrast to soy bean.

Similarly to the phenolic content of extracts, boiling also increased the antioxidant activity, particularly in pinto and black beans but not in soy. Pressure cooking seemed

to be more effective in the larger varieties, kidney and borlotti beans. Regardless of the processing method, discarding the cooking water significantly decreased the content in phenolics and in antioxidant activity ($p<0.05$) in all beans.

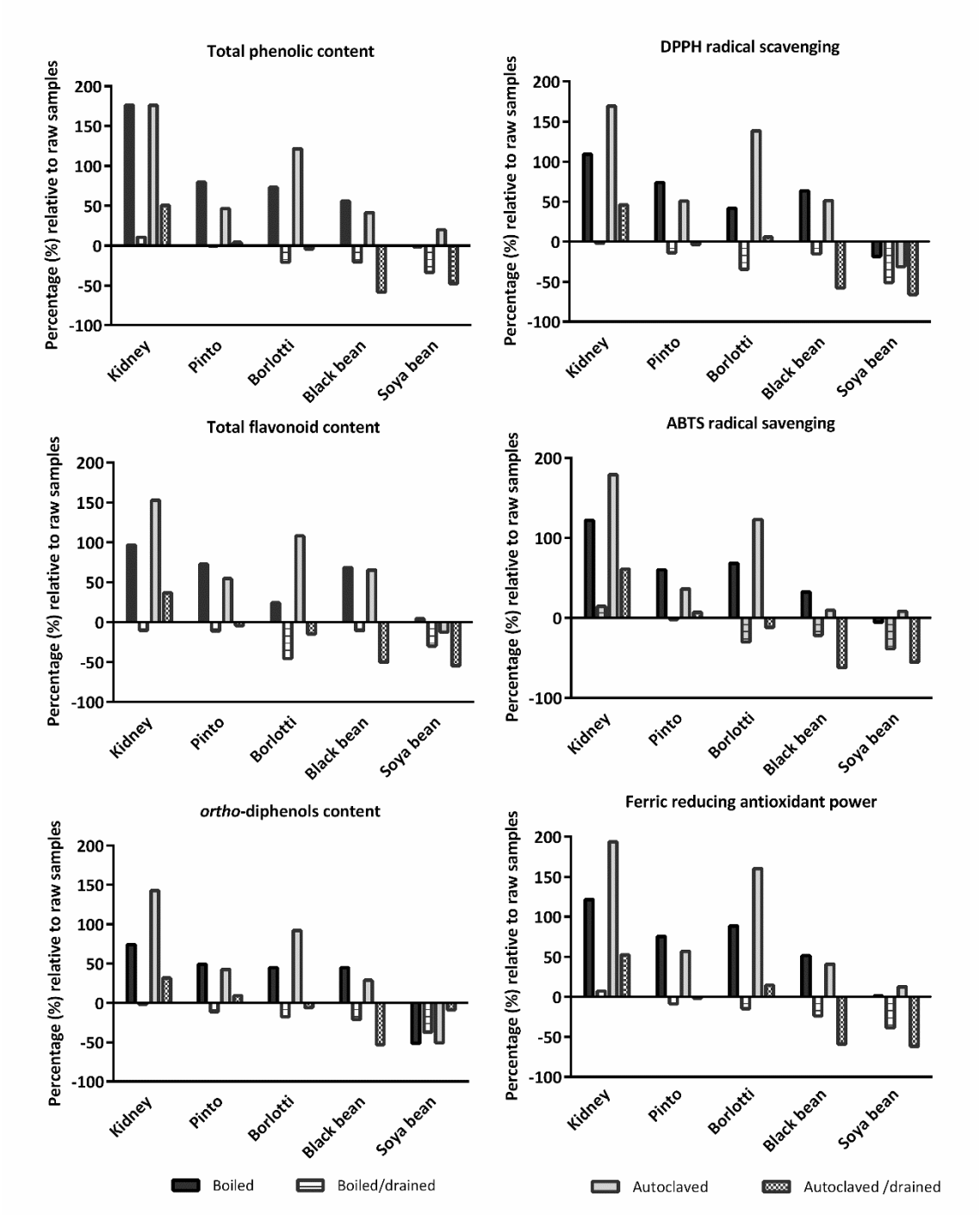

Figure 2. Percentage gain or loss in the total phenolic content, total flavonoid content, *ortho*-diphenol content and antioxidant capacity of the different cooking conditions. Values were calculated based on the initial contents in raw beans.

It has been demonstrated in same vegetable foods, that phenolic compounds are generally bound covalently to amine functional groups and therefore heat treatment can hydrolyze them, increasing the extractability (Jiratanan & Liut, 2004). Ranilla, Genovese, & Lajolo, (2009) reported a significant increase of TPC and antioxidant activity assessed by DPPH assay in Brazilian bean cultivars cooked at 100 and 121°C and without draining the cooking water. Rocha-Guzmán *et al*., (2007) also reported the increase of antioxidant activity in *Phaseolus vulgaris* cooked at 121°C. In agreement with this, Sarmento *et al*., (2015) found in *Cicer arietinum* L. and *Lathyrus sativus* L., an increase in tocopherols, bioactive compounds and antioxidant activity in seeds previously soaked and pressure-cooked for 15 min. In contrast, several researchers reported that thermal processing decrease the phenolic content and antioxidant activity in legumes. Granito *et al*., (2007) showed a decrease of TPC in boiled *Phaseolus lunatus*, suggesting that heat treatments could affect the aromatic rings of phenolic compounds making them more susceptible to polymerization and decomposition. Siah *et al*., (2014) also reported a decrease in TPC and TFC after boiling and pressure cooking *Vicia faba* L. In addition, in boiled black bean Eshraq *et al*., (2016) observed a decrease in TPC and TFC when compared with raw samples.

Our date shows a clear correlation between the levels of polyphenols and the free radical scavenging activities of the extracts. The Pearson's correlations between the parameters TPC, TFC, oDC and DPPH, ABTS and FRAP of all beans are shown in Figure 3. Correlation between polyphenol content and antioxidant activity was performed to evaluate which group of compounds are mainly responsible for the antioxidant activity. The strongest correlation was found between antioxidant activity (assessed by ABTS and TFC (Pearson $r = 0.829$), followed by FRAP activity) and oDC, as well as FRAP activity and TFC (Pearson $r = 0.796$ and 0.795, respectively).

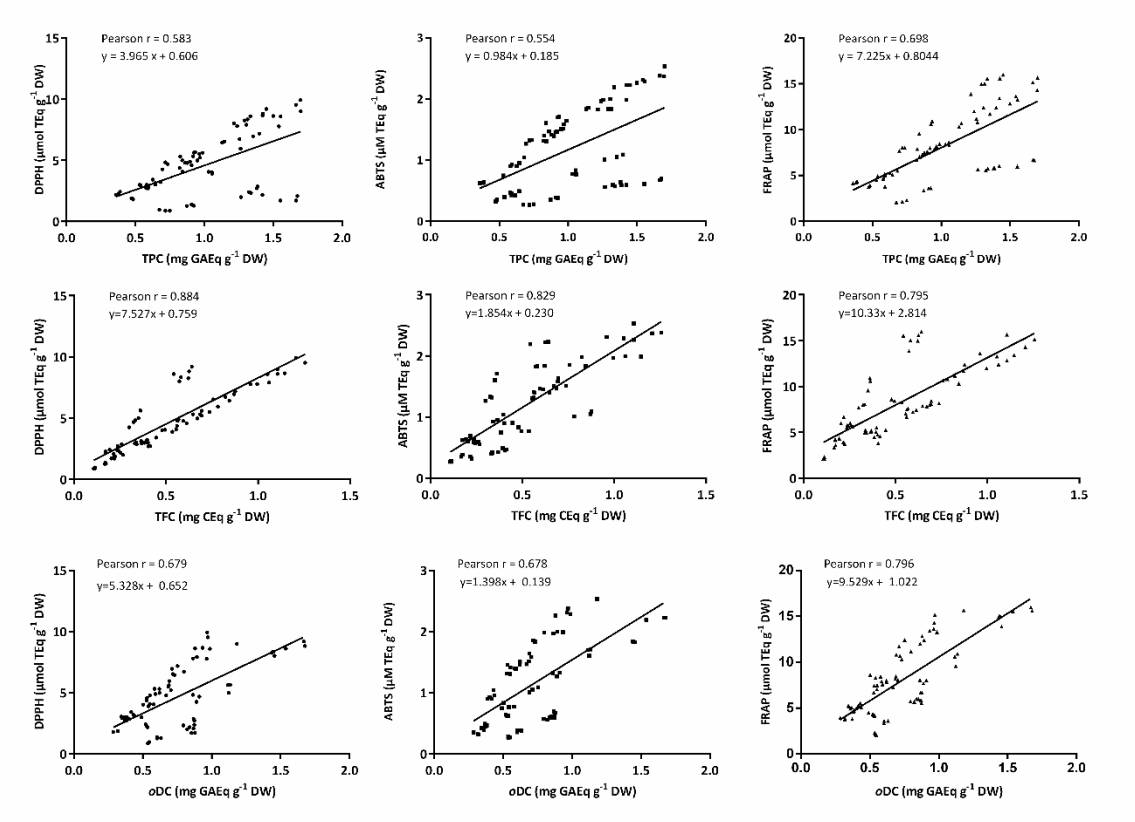

Figure 3. Correlation of the total phenolic content (TPC), total flavonoid content (TFC), *ortho*-diphenol content (oDC) and antioxidant activity using DPPH - 2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activity; ABTS - 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid radical-scavenging activity; FRAP - Ferric reducing antioxidant power. The results were based on three observations per legume by processing combination. Significant correlation at $p < 0.001$ were observed in all experiments.

Table 1. Effect of boiling and pressure cooking on the phenolic contents of crude methanolic extracts from the different beans.

	Raw	*Boiled*	*Boiled/drained*	*Press. cooked*	*Press. cooked /drained*
TPC (mg GAEq g^{-1} DW)					
Kidney bean	$0.58 \pm 0.05^{A,a}$	$1.60 \pm 0.07^{A,B,c}$	$0.64 \pm 0.05^{B,a}$	$1.60 \pm 0.09^{B,c}$	$0.87 \pm 0.03^{D,b}$
Pinto bean	$0.90 \pm 0.07^{B,a}$	$1.62 \pm 0.11^{D,c}$	$0.89 \pm 0.07^{C,a}$	$1.32 \pm 0.09^{A,b}$	$0.94 \pm 0.07^{D,a}$
Borlotti bean	$0.61 \pm 0.04^{A,b}$	$1.05 \pm 0.02^{A,c}$	$0.48 \pm 0.01^{A,a}$	$1.34 \pm 0.07^{A,d}$	$0.58 \pm 0.04^{B,a,b}$
Black bean	$0.90 \pm 0.05^{B,c}$	$1.40 \pm 0.06^{C,e}$	$0.72 \pm 0.02^{B,b}$	$1.27 \pm 0.05^{A,d}$	$0.38 \pm 0.02^{A,a}$
Soy bean	$1.36 \pm 0.04^{C,c}$	$1.34 \pm 0.08^{B,C,c}$	$0.90 \pm 0.03^{C,b}$	$1.63 \pm 0.07^{B,d}$	$0.71 \pm 0.04^{C,a}$
TFC (mg CEq g^{-1} DW)					
Kidney bean	$0.41 \pm 0.03^{B,a}$	$0.81 \pm 0.05^{C,c}$	$0.37 \pm 0.03^{B,a}$	$1.04 \pm 0.07^{D,d}$	$0.56 \pm 0.01^{C,b}$
Pinto bean	$0.69 \pm 0.05^{C,a}$	$1.19 \pm 0.08^{D,b}$	$0.61 \pm 0.06^{C,a}$	$1.07 \pm 0.08^{D,b}$	$0.66 \pm 0.06^{D,a}$
Borlotti bean	$0.41 \pm 0.01^{B,b}$	$0.50 \pm 0.03^{B,c}$	$0.22 \pm 0.00^{A,a}$	$0.84 \pm 0.05^{C,d}$	$0.34 \pm 0.02^{B,b}$
Black bean	$0.36 \pm 0.01^{B,b}$	$0.60 \pm 0.05^{B,c}$	$0.32 \pm 0.02^{B,b}$	$0.59 \pm 0.03^{B,c}$	$0.17 \pm 0.01^{A,a}$
Soy bean	$0.24 \pm 0.01^{A,d}$	$0.26 \pm 0.01^{A,d}$	$0.17 \pm 0.00^{A,b}$	$0.21 \pm 0.01^{A,c}$	$0.11 \pm 0.00^{A,a}$
oDC (mg GAEq g^{-1} DW)					
Kidney bean	$0.43 \pm 0.06^{A,a}$	$0.75 \pm 0.04^{B,b}$	$0.42 \pm 0.02^{B,a}$	$1.04 \pm 0.12^{C,c}$	$0.57 \pm 0.02^{C,a}$
Pinto bean	$0.63 \pm 0.05^{B,a,b}$	$0.94 \pm 0.05^{C,c}$	$0.56 \pm 0.05^{C,a}$	$0.90 \pm 0.04^{B,C,c}$	$0.69 \pm 0.02^{D,b}$
Borlotti bean	$0.37 \pm 0.01^{A,b}$	$0.54 \pm 0.04^{A,c}$	$0.31 \pm 0.02^{A,a}$	$0.72 \pm 0.03^{A,d}$	$0.35 \pm 0.01^{A,a,b}$
Black bean	$1.13 \pm 0.01^{D,c}$	$1.63 \pm 0.08^{D,e}$	$0.89 \pm 0.02^{D,b}$	$1.45 \pm 0.01^{D,d}$	$0.53 \pm 0.02^{B,a}$
Soy bean	$0.87 \pm 0.00^{C,d}$	$0.82 \pm 0.02^{B,C,c}$	$0.61 \pm 0.02^{C,b}$	$0.87 \pm 0.01^{A,B,d}$	$0.54 \pm 0.01^{B,C,a}$

Data presented as mean and SD from three replicates, n=3. The data marked by the same letters were not significantly different ($p>0.05$). Different uppercase letters for comparison within each column and lowercase letters for comparison within each row, indicating significant differences ($p<0.05$) between varieties and processing, respectively. TPC – Total phenolic content; Total flavonoid content; oDC – *ortho*-diphenol content; mg GAEq g-1 DW - mg gallic acid equivalents per gram of dry weight; mg CEq g-1 DW – mg of catechin equivalents per gram of dry weight.

Table 2. Effect of boiling and pressure cooking on the antioxidant activity of crude methanolic extracts.

	Raw	*Boiled*	*Boiled/drained*	*Press. cooked*	*Press. cooked /drained*
DPPH (µmol TEq g^{-1} DW)					
Kidney bean	$3.14 \pm 0.25^{A,a}$	$6.57 \pm 0.15^{C,c}$	$3.07 \pm 0.19^{B,a}$	$8.45 \pm 0.62^{C,d}$	$4.57 \pm 0.43^{C,b}$
Pinto bean	$5.39 \pm 0.11^{B,a}$	$9.36 \pm 0.67^{D,c}$	$4.64 \pm 0.31^{C,a}$	$8.12 \pm 0.48^{C,b}$	$5.20 \pm 0.41^{C,a}$
Borlotti bean	$2.81 \pm 0.17^{A,b}$	$3.99 \pm 0.09^{B,c}$	$1.85 \pm 0.04^{A,a}$	$6.70 \pm 0.65^{B,d}$	$2.98 \pm 0.05^{B,b}$
Black bean	$5.42 \pm 0.38^{B,c}$	$8.88 \pm 0.29^{D,d}$	$4.59 \pm 0.30^{C,b}$	$8.21 \pm 0.17^{C,d}$	$2.29 \pm 0.12^{B,a}$
Soy bean	$2.66 \pm 0.25^{A,c}$	$2.17 \pm 0.17^{A,b}$	$1.31 \pm 0.06^{A,a}$	$1.83 \pm 0.21^{A,b}$	$0.91 \pm 0.06^{A,a}$
ABTS (µmol TEq g^{-1} DW)					
Kidney bean	$0.85 \pm 0.09^{C,a}$	$1.89 \pm 0.08^{C,c}$	$0.97 \pm 0.06^{B,a}$	$2.38 \pm 0.14^{D,d}$	$1.37 \pm 0.06^{D,b}$
Pinto bean	$1.46 \pm 0.06^{D,a}$	$2.34 \pm 0.07^{D,c}$	$1.43 \pm 0.10^{C,a}$	$1.98 \pm 0.02^{C,b}$	$1.56 \pm 0.09^{E,a}$
Borlotti bean	$0.47 \pm 0.02^{A,b}$	$0.79 \pm 0.04^{B,c}$	$0.33 \pm 0.02^{A,a}$	$1.05 \pm 0.04^{B,d}$	$0.41 \pm 0.02^{B,b}$
Black bean	$1.67 \pm 0.06^{E,c}$	$2.22 \pm 0.02^{D,e}$	$1.31 \pm 0.04^{C,b}$	$1.83 \pm 0.01^{C,d}$	$0.63 \pm 0.01^{C,a}$
Soy bean	$0.61 \pm 0.03^{B,c,d}$	$0.57 \pm 0.02^{A,c}$	$0.37 \pm 0.02^{A,b}$	$0.66 \pm 0.05^{A,d}$	$0.27 \pm 0.01^{A,a}$
FRAP (µmol TEq g^{-1} DW)					
Kidney bean	$4.83 \pm 0.39^{A,a}$	$10.72 \pm 0.44^{C,c}$	$5.19 \pm 0.28^{B,a}$	$14.19 \pm 1.29^{C,d}$	$7.37 \pm 0.31^{D,b}$
Pinto bean	$8.03 \pm 0.15^{B,a}$	$14.09 \pm 0.16^{D,b}$	$7.33 \pm 0.57^{C,a}$	$12.60 \pm 0.74^{B,C,b}$	$7.91 \pm 0.62^{D,a}$
Borlotti bean	$4.47 \pm 0.63^{A,ab}$	$8.43 \pm 0.17^{B,c}$	$3.82 \pm 0.10^{A,a}$	$11.65 \pm 0.80^{B,d}$	$5.13 \pm 0.10^{C,b}$
Black bean	$10.37 \pm 0.70^{C,c}$	$15.70 \pm 0.24^{E,d}$	$7.92 \pm 0.20^{C,b}$	$14.62 \pm 0.65^{C,d}$	$4.24 \pm 0.09^{B,a}$
Soy bean	$5.72 \pm 0.14^{A,c}$	$5.81 \pm 0.19^{A,c}$	$3.52 \pm 0.15^{A,b}$	$6.44 \pm 0.40^{A,d}$	$2.18 \pm 0.12^{A,a}$

Data presented as mean and SD from three replicates, n=3. The data marked by the same letters were not significantly different ($p>0.05$). Different upercase letters for comparison within each column and lowercase letters for comparison within each row, indicating significant differences ($p<0.05$) between varieties and different processing, respectively. DPPH - 2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activity; ABTS - 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid radical-scavenging activity; FRAP - Ferric reducing antioxidant power; µmol TEq g^{-1} DW – µmol Trolox equivalents per gram of dry weight.

3.3.3. Phytochemical composition

Quantitative and qualitative differences in phenolic composition of raw and processed samples were detected by HPLC and are presented in Tables 3A and 3B.

Gallic acid (GA) was found to be the predominant phenolic acid and was present in all varieties studied. In *P. vulgaris*, GA content was the highest and followed in decreasing order of abundance by caffeic acid (CafA), chlorogenic acid (ChlA), ferulic acid (FerA), caffeic acid (CafA) and *p*-coumaric acid (*p*-CoumA). In the different raw samples, GA concentration was found to decrease in the following order: black > pinto > soy > borlotti and kidney bean. In soy bean extracts, only gallic acid was detected. In all samples, an increase in GA content was found after processing, whereas in black beans the opposite was observed. Overall, processing whether by boiling or by pressure cooking, had a positive effect on the increase of the phenolic acid content in the extracts from the different varieties of beans compared to the raw samples, including soy bean.

With regard to flavonoids, as shown in Table 3B, catechin was detected in higher amount in soy followed by black bean but was present in all varieties both before and after cooking. Except for pressure-cooked black bean, processing increased catechin in all other varieties. Kaempferol-3-*O*-glucoside (k-3-G) was found in all *P. vulgaris* and overall processing increased its content. Regarding the isoflavones genistein and daidzein, they were only detected in soy bean and were increased by processing, particularly by pressure cooking. In black and kidney bean extracts, myricetin, quercetin, and kaempferol with different glycosylation were also detected. Extracts of pinto and borlotti bean only presented derivatives of kaempferol. Higher total phenolic (TPC) and total flavonoid (TFC) content detected after cooking by the colorimetric assays are corroborated by the results obtained by HPLC analyses. Also visible on the HPLC data, content of phenolic acids and flavonoids dramatically decreased when the cooking water is drained. The highest oDC was observed in black bean, which reflects the high levels of the oDC species such as GA, ChlA and C, M, Q, K and derivatives. In soy bean, the high oDC was correlated only to the high amount of GA and genistein.

Our results are in agreement with those reported by Ranilla *et al*., (2009), using Brazilian *Phaseolus vulgaris*, where an increase in kaempferol and quercetin derivatives, *p*-coumaric and ferulic acids were found after cooking, independently of temperature. Also, draining had the effect of decreasing the levels of the different phenolics, independently of the heat treatment.

In the study performed by Price *et al*., (1998) no significant change in levels of quercetin and kaempferol derivates was found after thermal cooked green beans. In contrast, Díaz-Batalla *et al*., (2006) reported a reduction of quercetin and kaempferol content in pressure-cooked Mexican beans in comparison with the initial content. Also, in contrast with our results, Kumar *et al*., (2012) observed a decrease in isoflavones (genistein, daidzein and glycitein) in boiled and pressure-cooked soy.

Table 3A: Effect of boiling and pressure cooking on phenolic acid profile of methanolic extracts from the different beans.

	GA	CaftA	ChlA	CafA	*p*-CoumA	FerA
Kidney bean						
Raw	18.58 ± 6.07	7.62 ± 0.78	12.92 ± 2.22	13.61 ± 0.05	1.93 ± 0.03	9.57 ± 0.82
Boiled	64.52 ± 4.25	16.41 ± 1.64	19.72 ± 1.30	25.86 ± 1.54	2.79 ± 0.08	7.85 ± 0.3
Boil. drained	30.66 ± 1.71	10.15 ± 0.09	11.00 ± 0.25	14.89 ± 1.16	1.94 ± 0.04	4.04 ± 0.03
Press. cooked	68.95 ± 7.50	21.77 ± 0.27	25.37 ± 1.42	25.37 ± 1.42	2.81 ± 0.06	8.04 ± 0.36
Press. cooked drained	11.75 ± 1.68	12.91 ± 0.46	10.89 ± 0.90	19.13 ± 1.79	1.45 ± 0.06	3.34 ± 0.11
Pinto bean						
Raw	56.52 ± 5.46	29.41 ± 3.73	34.22 ± 2.17	45.54 ± 3.18	2.81 ± 0.14	13.20 ± 1.38
Boiled	79.41 ± 5.16	25.30 ± 2.36	32.48 ± 1.35	44.92 ± 3.09	5.33 ± 0.23	23.61 ± 0.9
Boil. drained	44.13 ± 2.31	17.39 ± 0.15	17.50 ± 0.78	27.99 ± 1.18	3.69 ± 0.19	16.33 ± 0.56
Press. cooked	56.53 ± 3.47	25.38 ± 0.10	27.34 ± 0.26	41.06 ± 0.54	4.20 ± 0.00	17.58 ± 0.45
Press. cooked drained	28.25 ± 1.49	11.35 ± 1.22	15.62 ± 0.10	21.75 ± 2.30	2.92 ± 0.38	14.20 ± 1.32
Borlotti bean						
Raw	32.88 ± 3.8	15.82 ± 0.84	17.61 ± 2.72	23.08 ± 3.79	1.82 ± 0.05	7.25 ± 0.67
Boiled	49.02 ± 3.96	20.90 ± 2.49	44.24 ± 2.37	32.53 ± 1.00	3.93 ± 0.14	14.19 ± 0.14
Boil. drained	17.77 ± 1.37	6.10 ± 0.64	12.90 ± 1.18	9.11 ± 0.75	1.70 ± 0.07	5.78 ± 0.21
Press. cooked	63.37 ± 4.95	54.57 ± 3.72	54.57 ± 3.72	41.99 ± 7.43	4.95 ± 0.25	16.30 ± 0.22
Press. cooked drained	22.38 ± 0.09	16.19 ± 0.35	16.19 ± 0.35	11.79 ± 0.27	2.03 ± 0.09	6.90 ± 0.23
Black bean						
Raw	67.88 ± 3.22		33.38 ± 2.78	54.96 ± 4.50	1.90 ± 0.26	9.35 ± 0.26
Boiled	44.78 ± 2.97	22.33 ± 0.18	32.57 ± 1.32	66.82 ± 5.14	3.48 ± 0.51	18.28 ± 1.51
Boil. drained	25.41 ± 0.25	11.35 ± 0.38	19.73 ± 0.07	34.66 ± 2.12	1.77 ± 0.08	10.18 ± 0.08
Press. cooked	36.02 ± 3.05	26.39 ± 1.75	31.13 ± 2.99	72.73 ± 9.40	2.56 ± 0.01	13.84 ± 1.89
Press. cooked drained	8.91 ± 0.32	5.09 ± 0.62	7.31 ± 1.17	9.55 ± 1.31	0.76 ± 0.02	3.06 ± 0.46
Soy bean						
Raw	54.96 ± 2.58	-	-	-	-	-
Boiled	84.48 ± 2.61	-	-	-	-	-
Boil. drained	39.49 ± 4.48	-	-	-	-	-
Press. cooked	79.81 ± 0.73	-	-	-	-	-
Press. cooked drained	27.32 ± 0.41	-	-	-	-	-

Data presented as mean and SD from four replicates. Results are expressed in µg/g of dry weight (DW). – not detected, GA: Galic Acid, CaftA: Caftaric Acid, ChlA: Chlorogenic Acid, p-CoumA: p-Coumaric Acid, FerA: Ferulic Acid.

Table 3B: Effect of boiling and pressure cooking on flavonoid profile of methanolic extracts from the different beans.

	C	M-3-G	Q-3-G	Q-3-MG	K-3-G	M	K-3-MG	Q	K	G	D
Kidney bean											
Raw	140.54±1.08	-	-	-	0.90±0.07	-	-	-	-	-	-
Boiled	209.92±15.19	-	-	29.11±1.48	0.78±0.03	10.69±0.23	-	-	-	-	-
Boil. drained	93.40±11.26	-	-	11.19±0.08	0.40±0.08	4.50±0.01	-	-	-	-	-
Press. cooked	283.91±23.28	-	-	36.33±2.94	1.20±0.03	13.53±0.78	-	-	-	-	-
Press. cooked drained	104.17±4.79	-	-	8.82±0.2	0.55±0.13	4.64±0.17	-	-	-	-	-
Pinto bean											
Raw	188.70±12.32	-	-	-	3.57±0.13	-	-	-	-	-	-
Boiled	301.17±23.69	-	-	-	3.52±0.24	-	-	-	-	-	-
Boil. drained	284.82±26.53	-	-	-	3.17±0.32	-	-	-	-	-	-
Press. cooked	238.70±14.92	-	-	-	2.35±0.01	-	-	-	-	-	-
Press. cooked drained	162.92±16.02	-	-	-	1.90±0.10	-	-	-	-	-	-
Borlotti bean											
Raw	98.81±1.57	-	-	-	3.50±0.22	-	-	-	-	-	-
Boiled	118.01±0.67	-	-	-	8.59±0.51	-	-	-	-	-	-
Boil. drained	60.96±5.47	-	-	-	0.57±0.04	-	-	-	-	-	-
Press. cooked	143.30±5.44	-	-	-	9.91±0.06	-	-	-	-	-	-
Press. cooked drained	88.72±5.44	-	-	-	0.82±0.03	-	-	-	-	-	-
Black bean											
Raw	372.24±37.83	44.87±4.50	94.73±8.93	-	8.35±0.57	5.98±0.18	4.53±0.20	30.88±2.11	2.51±0.29	-	-
Boiled	485.21±0.03	66.82±5.14	128.01±3.23	54.05±1.54	11.60±0.44	32.87±.85	4.46±1.34	144.32±15.17	7.73±0.89	-	-
Boil. drained	162.95±7.16	34.45±2.12	73.88±1.22	20.02±0.01	6.50±0.13	6.24±0.74	2.48±0.30	26.69±1.75	2.13±0.07	-	-
Press. cooked	287.87±4.87	67.11±1.83	104.49±7.27	40.00±3.23	9.94±0.01	31.94±3.36	3.08±0.23	110.98±9.87	8.32±1.47	-	-
Press. cooked drained	61.23±6.09	13.15±0.94	29.18±1.55	13.21±1.15	2.94±0.18	4.31±0.99	4.31±0.99	11.81±1.55	0.90±0.06	-	-
Soy bean											
Raw	1132.52±108.1	-	-	-	-	-	-	-	-	260.41±18.70	76.09±1.65
Boiled	1498.1±148.72	-	-	-	-	-	-	-	-	847.05±90.68	238.82±24.90
Boil. drained	518.51±50.00	-	-	-	-	-	-	-	-	176.08±14.52	56.65±1.21
Press. cooked	1495.48±50.37	-	-	-	-	-	-	-	-	1166.15±3.83	1064.56±68.11
Press. cooked drained	336.47±15.88	-	-	-	-	-	-	-	-	164.60±6.74	60.84±3.12

Data presented as mean and SD from four replicates. Results are expressed in µg/g of dry weight (DW). – not detected, C: Catechin, M-3-G: Myrcetin-3-*O*-glucoside, Q-3-G: Quercetin-3-*O*-glucoside, Q-3-MG: Quercetin-3-*O*-(6-O-manolyl)glucoside, K-3-G: Kaempferol-3-*O*-glucoside, M: Myrcetin, K-3-MG: Kaempferol-3-*O*-(6-O-manolyl)glucoside), Q: Quercetin, K: Kaempferol, G: Genistein, D: Daidzein.

3.4. Conclusion

In the present study, we evaluated the effect of two cooking methods (boiling and pressure cooking) on total phenolic content and individual composition as well as antioxidant activity of hydromethanolic extracts of several common bean varieties and soy. Total phenolic compounds of raw samples showed highest values in soy, although the antioxidant activity was higher in black bean and pinto bean methanolic extracts which were higher in flavonoids. Processing by the two cooking methods (boiling and pressure cooking) increased phenolic content and antioxidant activity in extracts of the four *Phaseolus* varieties in contrast with the effects on soy. In spite of the modest increase in antioxidant activity, genistein and daidzein were substantially enriched in extract of boiled and particularly of pressure-cooked soy compared to raw seeds. Discarding the cooking water resulted in loss of significant amounts of phytochemicals and decreased antioxidant potential.

Acknowledgements

- The author Catarina Teixeira-Guedes acknowledges the financial support provided FCT-Portuguese Foundation for Science and Technology (SFRH/BD/52544/2014), under the Doctoral Program "Agricultural Production Chains-from fork to farm" (PD/00122/2012). This work was supported by the European Investment Funds FEDER/COMPETE/POCI– Operational Competitiveness and Internationalization Programme [POCI-01-0145-FEDER-006958] and Portuguese Foundation for Science and Technology (FCT) [UID/AGR/04033/2013]; Project I&D Interact - Integrative Research in Environment, Agro-Chain and Technology [NORTE-01-0145-FEDER-000017], co-financed by European Regional Development Fund (FEDER) through NORTE 2020 (Programa Operacional Regional do Norte 2014/2020).

3.5. References

Açar, O. C., Gokmen, V., Pellegrini, N., & Fogliano, V. (2009). Direct evaluation of the total antioxidant capacity of raw and roasted pulses, nuts and seeds. *European Food Research and Technology*, *229*(6), 961–969. https://doi.org/10.1007/s00217-009-1131-z

Bolanos De La Torre, A.A.S., Henderson, T., Nigam, P.S., & Owusu-Apenten, R. K.

(2015). A universally calibrated microplate ferric reducing antioxidant power (FRAP) assay for foods and applications to Manuka honey. *Food Chemistry*, *174*, 119–123. https://doi.org/10.1016/j.fooDChem.2014.11.009

Campos-Vega, R., Loarca-Piña, G., & Oomah, B. D. (2010). Minor components of pulses and their potential impact on human health. *Food Research International*, *43*(2), 461–482. https://doi.org/10.1016/j.foodres.2009.09.004

Chávez-Mendoza, C., & Sánchez, E. (2017). Bioactive compounds from mexican varieties of the common bean (*Phaseolus vulgaris*): Implications for health. *Molecules*, *22*(8), 1–32. https://doi.org/10.3390/molecules22081360

Chon, S. U. (2013). Total polyphenols and bioactivity of seeds and sprouts in several legumes. *Current Pharmaceutical Design*, *19*(34), 6112–6124. https://doi.org/10.2174/1381612811319340005

Deol, J.K., & Bains, K. (2010). Effect of household cooking methods on nutritional and anti nutritional factors in green cowpea (*Vigna unguiculata*) pods. *Journal of Food Science and Technology*, *47*(5), 579–581. https://doi.org/10.1007/s13197-010-0112-3

Díaz-Batalla, L., Widholm, J.M., Fahey, G.C., Castaño-Tostado, E., & Paredes-López, O. (2006). Chemical components with health implications in wild and cultivated Mexican common bean seeds (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry*, *54*(6), 2045–2052. https://doi.org/10.1021/jf051706l

Domínguez-Perles, R., Teixeira, A. I., Rosa, E., & Barros, A.I. (2014). Assessment of (poly)phenols in grape (*Vitis vinifera* L.) stems by using food/pharma industry compatible solvents and Response Surface Methodology. *Food Chemistry*, *164*, 339–346. https://doi.org/10.1016/j.fooDChem.2014.05.020

Eshraq, B., Mona, A., Sayed, A., & Emam, A. (2016). Effect of Soaking, Cooking and Germination on Chemical Constituents and Bioactive Compounds as well as their Cytotoxic Activities of Black Bean Extracts. *Natural Products Chemistry & Research*, *04*(06). https://doi.org/10.4172/2329-6836.1000237

Ganesan, K., & Xu, B. (2017). Polyphenol-rich dry common beans (*Phaseolus vulgaris* L.) and their health benefits. *International Journal of Molecular Sciences*, *18*(11). https://doi.org/10.3390/ijms18112331

Granito, M., Yannellis, B., & Torres, A. (2007). Chemical composition, antioxidant capacity and functionality of raw and processed *Phaseolus lunatus*. *Journal of the*

Science of Food and Agriculture, *87*, 2801–2809. https://doi.org/10.1002/jsfa.2926

Hayat, I., Ahmad, A., Masud, T., Ahmed, A., & Bashir, S. (2014). Nutritional and health perspectives of beans (*Phaseolus vulgaris* L.): An overview. *Critical Reviews in Food Science and Nutrition*, *54*(5), 580–592. https://doi.org/10.1080/10408398.2011.596639

Itoh, T., Umekawa, H., & Furuichi, Y. (2005). Potential ability of hot water adzuki (*Vigna angularis*) extracts to inhibit the adhesion, invasion, and metastasis of murine B16 melanoma cells. *Bioscience, Biotechnology, and Biochemistry*, *69*(3), 448–454. https://doi.org/10.1271/bbb.69.448

Jiratanan, T., & Liut, R. H. (2004). Antioxidant activity of processed table beets (*Beta vulgaris var*, conditiva) and green beans (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry*, *52*(9), 2659–2670. https://doi.org/10.1021/jf034861d

Kalogeropoulos, N., Chiou, A., Ioannou, M., Karathanos, V.T., Hassapidou, M., & Andrikopoulos, N.K. (2010). Nutritional evaluation and bioactive microconstituents (phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the Mediterranean countries. *Food Chemistry*, *121*(3), 682–690. https://doi.org/10.1016/j.fooDChem.2010.01.005

Kumar, V., Chauhan, G.S., Rani, A., Raghvanshi, M., & Jatav, R. (2012). Effect of boiling treatments on biochemical constituents of vegetable-type soy. *Journal of Food Processing and Preservation*, *36*(5), 393–400. https://doi.org/10.1111/j.1745-4549.2011.00595.x

Lin, L., Harnly, J.M., Pastor-corrales, M.S., & Luthria, D.L. (2008). The polyphenolic profiles of common bean (*Phaseolus vulgaris* L .). *Food Chemistry*, *107*, 399–410. https://doi.org/10.1016/j.fooDChem.2007.08.038

Madhujith, T., Amarowicz, R., & Shahidi, F. (2004). Phenolic antioxidants in beans and their effects on inhibition of radical-induced DNA damage. *Journal of the American Oil Chemists' Society*, *81*(7), 691–696. https://doi.org/10.1007/s11746-004-963-y

Marathe, S.A., Rajalakshmi, V., Jamdar, S.N., & Sharma, A. (2011). Comparative study on antioxidant activity of different varieties of commonly consumed legumes in India. *Food and Chemical Toxicology*, *49*(9), 2005–2012. https://doi.org/10.1016/j.fct.2011.04.039

Mkanda, A.V, Minnaar, A., & de Kock, H.L. (2007). Relating consumer preferences to

sensory and physicochemical properties of dry beans (*Phaseolus vulgaris*). *Journal of the Science OfFood and Agriculture*, *87*, 2868–2879. https://doi.org/10.1002/jsfa3046

Price, K.R., Colquhoun, I.J., Barnes, K.A., & Rhodes, M.J.C. (1998). Composition and content of flavonol glycosides in green beans and their fate during processing. *Journal of Agricultural and Food Chemistry*, *46*(12), 4898–4903. https://doi.org/10.1021/jf980687v

Ranilla, L.G., Genovese, M.I., & Lajolo, F.M. (2009). Effect of different cooking conditions on phenolic compounds and antioxidant capacity of some selected Brazilian bean (*Phaseolus vulgaris* L.) cultivars. *Journal of Agricultural and Food Chemistry*, *57*(13), 5734–5742. https://doi.org/10.1021/jf900527v

Rehman, Z., Salariya, A.M., & Zafar, S.I. (2001). Effect of processing on available carbohydrate content and starch digestibility of kidney beans (*Phaseolus vulgaris* L.). *Food Chemistry*, *73*(3), 351–355. https://doi.org/10.1016/S0308-8146(00)00311-3

Rocha-Guzmán, N.E., González-Laredo, R.F., Ibarra-Pérez, F.J., Nava-Berúmen, C.A., & Gallegos-Infante, J.A. (2007). Effect of pressure cooking on the antioxidant activity of extracts from three common bean (*Phaseolus vulgaris* L.) cultivars. *Food Chemistry*, *100*(1), 31–35. https://doi.org/10.1016/j.fooDChem.2005.09.005

Rosato, V., Guercio, V., Bosetti, C., Negri, E., Serraino, D., Giacosa, A., Montella, M., La Vecchia, C., & Tavani, A. (2016). Mediterranean diet and colorectal cancer risk: a pooled analysis of three Italian case–control studies. *British Journal of Cancer*, *115*(7), 862–865. https://doi.org/10.1038/bjc.2016.245

Sarmento, A., Barros, L., Fernandes, A., Carvalho, A. M., & Ferreira, I. C. F. R. (2015). Valorization of traditional foods: Nutritional and bioactive properties of *Cicer arietinum* L. and *Lathyrus sativus* L. pulses. *Journal of the Science of Food and Agriculture*, *95*(1), 179–185. https://doi.org/10.1002/jsfa.6702

Siah, S.D., Konczak, I., Agboola, S., Wood, J.A., & Blanchard, C.L. (2012). In vitro investigations of the potential health benefits of Australian-grown faba beans (*Vicia faba* L.): chemopreventative capacity and inhibitory effects on the angiotensin-converting enzyme, α-glucosidase and lipase. *British Journal of Nutrition*, *108*(S1), S123–S134. https://doi.org/10.1017/S0007114512000803

Siah, S., Wood, J.A., Agboola, S., Konczak, I., & Blanchard, C.L. (2014). Effects of

soaking, boiling and autoclaving on the phenolic contents and antioxidant activities of faba beans (*Vicia faba* L.) differing in seed coat colours. *Food Chemistry*, *142*, 461–468. https://doi.org/10.1016/j.fooDChem.2013.07.068

Soufi, O., Romero, C., & Louaileche, H. (2014). *Ortho*-diphenol profile and antioxidant activity of Algerian black olive cultivars: Effect of dry salting process. *Food Chemistry*, *157*, 504–510. https://doi.org/10.1016/j.fooDChem.2014.02.075

Waqas, M.K., Akhtar, N., Mustafa, R., Jamshaid, M., Khan, H.M.S., & Murtaza, G. (2015). Dermatological and cosmeceutical benefits of *Glycine max* (soy) and its active components. *Acta Poloniae Pharmaceutica - Drug Research*, *72*(1), 3–11.

CHAPTER 4

Anti-colorectal cancer effect of phenolic extracts from cooked cowpea, black bean and soy

This chapter encloses the following manuscript:

Teixeira-Guedes, C. I., Carvalho, A. C. & Pereira-Wilson, C. (2018). Anticancer effects by cell cycle modulation of polyphenolic-rich extracts from cooked cowpea, black bean, and soy on colorectal cancer cell lines. Submitted

Anticancer effects by cell cycle modulation of polyphenolic-rich extracts from cooked cowpea, black bean, and soy on colorectal cancer cell lines

Catarina I. Teixeira-Guedes[ab]*, Ana C. Carvalho[ab], Cristina Pereira-Wilson[abc]

[a]*Department of Biology, University of Minho, Braga, Portugal*

[b]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Minho, Braga, Portugal*

[c]*Centre of Biological Engineering, University of Minho, Braga, Portugal*

Abstract

Colorectal cancer is one of the leading cause of cancer-related deaths worldwide. It has been suggested that legume consumption can help in the prevention of colorectal cancer. The aim of this study was to investigate the anticancer potential of extracts from cooked cowpea, black bean and soy at molecular level. The present study showed that cooking positively affects the contents in phenolic compounds and antioxidant activity of extracts of all three legumes. Both HCT116 p53wt and HCT116 p53null cells were suppressed by treatment with all extracts in a dose-dependent manner. HCT116 p53wt cells were more sensitive that HCT116 p53null cells, particular when treated with extracts of cooked black bean. Overall, incubation with bean extracts resulted in cell cycle arrest in G0-G1 phase, confirmed by down-regulation of cyclin E, pRB and up-regulation of p27, p21, and p16 (all cycle inhibitors). Cell cycle modulation also seems to be correlated with decreased activation of the PI3K/AKT signaling pathway and by p53.

Keywords: cell cycle arrest, colorectal cancer, cowpea, black bean, soy, cooking,

4.1. Introduction

Colorectal cancer (CRC) is one of the major causes of cancer-related mobility and mortality worldwide being the second- and the third-most common cancer in women and men respectively. The global colorectal cancer burden is expected to increase by 60 % in the next 15 years to more than 2.2 million new cases and 1.1 million deaths per year (World Cancer Research Fund, 2017).

The incidence of CRC is not uniformly distributed throughout the world, it is mainly a disease of developed western countries (Haggar & Boushey, 2009). The geographical patterns evidence the influence of lifestyle risk factors, including diet, physical activity, smoking, diabetes and obesity (Huxley *et al*., 2009; Luna-Vital *et al*., 2016; World Cancer Research Fund, 2017). In particular, diet strongly influences the etiology of this disease, and substantial evidence suggests an increased risk by high intakes of red meat and fat and a decrease by high intakes of fruits, vegetables, legumes, fiber, folate, and calcium (World Cancer Research Fund, 2017). It has also been demonstrated that dietary strategies can potentially avoid colorectal cancer death by up to 50%. Epidemiological studies have indicated that legume consumption may reduce the incidence of CRC, based on the observation that countries where dry bean consumption is high, the prevalence of CRC is lower (Dan *et al*., 2016).

Experiments in animal models also showed a significantly lower size and number of colon cancer pre-lesions (hyperplasia and dysplasia), adenomas and adenocarcinoma in azoxymethane-treated Ob/Ob mice fed with whole beans, bean residue and bean phenolic extract in comparison to control-fed mice (Bobe *et al*., 2008). A study performed by Feregrino-Perez *et al*., (2014) further demonstrated that the non-digestible fraction from common cooked beans inhibited azoxymethane (AOM)-induced CRC, influencing the expression of genes involved in the apoptosis and cell cycle arrest. Also described antiproliferative, pro-apoptotic effects by cell cycle arrest modulation in HT29 CRC cells treated with the fermented non-digestible fraction of common bean (Cruz-Bravo *et al*., 2014).

As previously mentioned, high consumption of legumes can help in the prevention and reduction of mortality from CRC, this anti-colorectal cancer effect may be due to the intake of dietary fiber, resistant starch, oligosaccharides, proteins, minerals and vitamins and a large range bioactive compounds such as phenolic compounds (Kalogeropoulos *et al*., 2010; Luna-Vital *et al*., 2016; Siah *et al*., 2014). Phenolic

compounds present in legumes contribute with their health-promoting properties, largely attributed to their antioxidant properties (Açar *et al*., 2009; Siah *et al*., 2012), particularly the protective effects against radical-induced DNA damage (Madhujith *et al*., 2004).

Many investigators have reported substantial amount of phenolic compounds and antioxidant activity in raw beans (Açar *et al*., 2009; Marathe *et al*., 2011), but limited reports have focused on the impact of food preparation on phenolic composition, antioxidant activity and anticancer effects. The objective of this study was, therefore, to investigate the effects of two cooking methods (boiling and pressure cooking) on the anticancer potential and mechanisms of action of phenolic enriched extracts of cowpea (*Vigna unguiculata* L.), black common bean *(Phaseolus vulgaris* L.) and soy (*Glycine max* L.) on CRC using two isogenic human colorectal carcinoma cell lines HCT116 expressing p53 (p53wt) and complete knockout for p53 (p53null).

4.2. Material and methods

4.2.1. Plant Material

*Vigna unguiculata (*cowpea), *Phaseolus vulgaris* (black bean) and *Glycine max* (soy) were used in this work. All legumes were purchased as dry seeds from a Portuguese market.

4.2.2. Processing

Three samples of each legume were subjected to atmospheric boiling (100°C) and pressure cooking (115 °C). Before processing, 100 ml of distillated water was added to bean seeds (10 g) and left 12 h for soaking. In the process of boiling, beans were cooked under atmospheric pressure for 50 min. For pressure cooking, beans were autoclaved at 115 °C for 20 min.

4.2.3. Preparation of crude phenolic extract

All samples, regardless of the treatment used, and including raw beans, were freeze-dried and ground to a powder. Extraction was carried out in methanol:water (80:20, v/v) at a solid to solvent ratio of 1:10. The extractions were ultrasounds assisted (Ultrasonic cleaning baths UCI-50, Raypa, Spain) during 30 min followed by 24 h in orbital shaker (Orbital shaker 501, Bibby Stuart, United Kingdom) in the dark. The extracts were then vacuum filtrated through Whatman No. 1 paper and concentrated using a rotatory

evaporator (BÜCHI 461 water bath REIII, Germany) under reduced pressure at 40 °C. After while they were freeze-dried to obtain a fine lyophilized (Freeze Dryer Christ alpha 2-4 with an LDC-1M for system control, Martin Christ, Germany). For biological activities, lyophilized extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

4.2.4. Characterization of total phenolic content and antioxidant activity

The total phenolic content (TPC) and the antioxidant activity of extracts were determined by colorimetric assays using a spectrophotometer. All protocols were previously optimized to 96-well plates (Domínguez-Perles *et al*., 2014; Teixeira-Guedes *et al*., 2018). To each well, 20 µL of samples or standard were added followed by 100 µL of Folin–Ciocalteau (Sigma-Aldrich) (10 % in H_2O) and 80 µL of sodium carbonate (Sigma-Aldrich) (7.5 % in H_2O). The reaction was incubated in the dark at 42 °C for 30 min and the absorbance measured at 750 nm in a microplate reader (Infinite M200 microplate reader, Tecan, Austria). Gallic acid (Sigma-Aldrich) was used as standard for the calibration curve. Results were expressed as mg of gallic acid equivalents per g of extract (mg GAEq/g Ex).

Determination of antioxidant activity was performed using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) and ferric reducing antioxidant power (FRAP) assays using Trolox (Sigma-Aldrich) as standard.

DPPH work solution was prepared in methanol/H_2O (70:30, v/v) and diluted until achieve an absorbance of 1.000 ± 0.1 at 520 nm. To 10 µL of sample or Trolox standard 190 µL of the DPPH solution were added. The mixture was placed in the dark at room temperature for 30 min, and absorbance was measured at 520 nm in a microplate reader. Inhibition of free radical DPPH in percent (%) was calculated using the formula: $\%\ \text{inhibition} = \frac{\text{AbsBlank} - \text{AbsSample}}{\text{AbsBlank}} \text{x}\ 100$. Antioxidant activity of samples were calculated by interpolation from the calibration standard curve of Trolox. Results were expressed in µM Trolox equivalent per g of extract (µM TEq/g Ex).

The ferric reducing antioxidant power (FRAP) working solution was prepared by mixing 10-volumes of acetate buffer (300 mM, pH 3.6) with 1-volume of TPTZ (Sigma-Aldrich) (10 mM dissolved with 40 mM of HCl) and 1-volume of ferric chloride (Sigma-Aldrich) (20 mM in water). To a 96-well microplate, 20 µL of sample were added, followed by 280 µL of FRAP working solution. The reaction was incubated

at 37 ºC in the dark for 30 min and then read at 593 nm. The ability of the extract to chelate the ferrous ion was obtained through the interpolation from the calibration standard curve of Trolox. Results were expressed in µM TEq/g Ex.

4.2.5. Characterization of phenolic profile using HPLC

The phenolic compound profiling was carried according to Lin *et al*., (2008). In this experiment was used a Thermo Finnigan Surveyor HPLC system (Thermo-Fisher Scientific, Inc. Waltham, USA) with a diode array detector (DAD). Separation was performed on a ACE 5 C18 column (5 µm, 250 x 4.6 mm i.d.) (Advanced Chromatography Technologies Ltd., Scotland) at a flow rate of 1 ml/min. The mobile phase A consisted of water-formic acid (99.9:0.1; v/v) and B acetonitrile-formic acid (99.9:0.1; v/v). The diluted extracts were injected and eluted using the gradient reported by Lin *et al*., (2008). DAD was set at 280, 310 and 520 nm while a UV/VIS spectrum from 10-650 nm, was continuously collected. The chromatograms were analyzed using the qualitative software of Xcalibur (Thermo Fisher Scientific, Inc. Waltham, USA) and results expressed based on calibration curves obtained by standards.

4.2.6. Cell culture

Two HCT116 isogenic human colorectal carcinoma cell lines were used. These cells harbor a mutation in the codon 13 of the Ras proto-oncogene, HCT116 is wild-type for p53 (p53wt) and the other line is a complete knockout for p53 (p53null) (cells were kindly provided by Vogelstein, Johns Hopkins Oncology Center, Baltimore, USA). The cell lines were maintained in T25 cm^2 polystyrene flacks with RPMI-1640 medium (Sigma-Aldrich) supplemented with 6 % of bovine fetal serum (FBS; EU standard, Lonza, Verviers, Belgium), 1 % antibiotic-antimitotic solution (Sigma-Aldrich), 10 mM Hepes, 0.1 mM sodium pyruvate (Sigma-Aldrich) and 2 g/L sodium bicarbonate (Sigma-Aldrich). Cells were kept at 37 °C in a humidified atmosphere with 5 % CO_2 in a Binder C150 incubator (Binder, Germany). For all experiments HCT116 p53wt and p53null cells were seeded at a density of 0.6 x 10^5 cell/ml.

4.2.7. Viability assay

The effect of extracts on cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Sigma-Aldrich). The MTT substrate was prepared and added to the cells in culture at a final concertation of 0.5 mg/ml and incubated for 2 h. The formazan formed (proportional to the number of viable cells) was dissolved in ethanol/DMSO (1:1) and the absorption was measured at 570 nm using microplate reader.

4.2.8. Cell cycle analysis by flow cytometry

Cell were incubated 24 h with IC50 concentration of the extracts and harvested 24 h later. Cells were then fixed with cold ethanol (70 %) for 30 min in ice, washed with PBS, and the supernatant discarded after centrifugation. The samples were then treated with ribonuclease and stained with propidium iodide PI (50 µg/ml stock solution). Stained cells (20 000 single cell events) were analyzed and sorted based on area and pulse width of the PI signal. Analysis was performed in an Coulter Epics XL Flow Cytometer (Beckman Coulter Inc. USA) with excitation at 488 nm and emission at 605-635 nm. Cell-cycle analyses of DNA histograms were evaluated using FlowJo Software (Tree Star Inc, USA). The percentages of cells with different DNA contents were calculated from histograms generated with gating (portions of cells in different phases of the cell cycle).

4.2.9. Protein extraction and Western-blot

After the incubation periods (24 h with the IC50 concentration), cells were collected and lysed for 15 min, at 4 ºC with RIPA buffer supplemented with 20 mM of NaF (Sigma-Aldrich), 1 mM of phenylmethylsufonyl fluoride (PMSF) (Sigma-Aldrich), 20 mM $Na_2V_3O_4$ (Sigma-Aldrich) and a protease inhibitor cocktail (Roche, Germany). Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc. USA) was used to quantify protein using BSA as standard. Protein samples were separated by SDS-PAGE gel electrophoresis and then semi-dry transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Membranes were then blocked with 5 % BSA in phosphate buffered saline (PBS) with Tween-20 (PBS-T), incubated with the primary antibodies (anti-cyclin E, anti-phospho-Retinoblastoma, anti-Retinoblastoma, anti-phospho-AKT, anti-AKT and anti-β-Actin) followed by the secondary antibody

conjugated with IgG horseradish peroxidase. All antibodies were acquired from Cell Signaling Technology (Portugal) Immunodetection was performed using enhanced chemiluminescence (ECL) (Bio-Rad Laboratories) under chemiluminescence detection system Chemi Doc XRS (Bio-Rad Laboratories). Band area intensity was quantified using the Quantity One Software from Bio-Rad. β-actin was used as loading control.

4.2.10. Quantitative real-time PCR (qPCR)

Previously to total RNA extraction, cells were incubated for 24 h with IC50 concentration. Total RNA was extracted from cells with SV Total RNA Isolation System (Promega) following the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo-Scientific). Sample purity was considered adequate when the absorbance ratio of A260/A280 was approximately 2 and the ratio of A260/A230 was greater than 2. cDNA synthesis was performed using 1 μg of template RNA, as recommended by the iScript cDNA Synthesis manufacturer (Bio-Rad Laboratories).

Quantitative real-time PCR was conducted using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. Reactions were prepared in 20 μl volumes containing 10 μl of Supermix, 10 pmol of forward (Fwd) and reverse (Rev) primers, and 50 ng of template cDNA. The following primers were used: p53 (Fwd: 5' GGCCCACTTCACCGTACTAA; Rev: 5' GTGGTTTCAAGGCCAGATGT), p27 (Fwd: 5'TAATTGGGGCTCCGGCTAACT; Rev: 5' TGCAGGTCGCTTCCTTATTCC), p21 (Fwd: 5' TGTCCGTCAGAACCCATGC; Rev: 5' AAAGTCGAAGTTCCATCGCTC), p16 (Fwd: 5' GTGGACCTGGCTGAGGAG; Rev: 5' CTTTCAATCGGGGATGTCTG), c-MYC (Fwd: 5' AAAGGCCCCCAAGGTAGTTA; Rev: 5' GCACAAGAGTTCCGTAGCTG), 36B4 (Fwd: 5' CAGCAAGTGGGAAGGTGTAAT; Rev: 5' CCCATTCTATCATCAACGGGTACA).

PCR reactions were run in triplicate in the CFX96 real-time PCR detection system (Bio-Rad Laboratories) and the conditions used were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30s. Relative gene expression was calculated by a mathematical method based on the PCR efficiencies (calculated from standard curves) and the Ct deviation of an unknown sample versus a control, and expressed in comparison to a reference gene (Pfaffl, 2001).

4.2.11. Statistical analysis

Results are present as mean ± SD from three independent experiments. Statistical analyses were performed using IBM SPSS statistics 21.0 software (SPSS Inc.). Differences between samples were tested using analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests, $p<0.05$ was considered as being statistically significant.

4.3. Results and discussion

4.3.1. Effects of processing on polyphenolic content and antioxidant activity

The total phenolic content (TPC) and antioxidant capacity of cowpea, black bean and soy extracts are presented in Table 1. Comparing between raw samples, TPC was found to be significantly higher in black bean than soy and cowpea. Processing by both cooking methods significantly increased the TPC in cowpea and black bean, but not in soy. Boiling and pressure cooking increased the TPC by approximately 20 % and 35 %, respectively, in both cowpea and black bean, when compared with raw samples.

The antioxidant activity assessed by the DPPH and FRAP assays (Table 1) showed the same pattern, with black bean extract having higher activity followed by cowpea and soy both in raw or cooked beans. The different cooking conditions increased the antioxidant activity of extracts from cowpea and black bean, while in soy there was a decrease in DPPH antioxidant activity in both boiled and pressure-cooked samples.

Our finding suggested that in cowpea and black bean, cooking could cause the destruction of seed structure enabling the release of phenolic compounds, increasing the extraction efficiency and consequently the increase of antioxidant activity. However, we cannot exclude the possibility of the heat processing can also induce the release of other components, such as soluble sugars, proteins and soluble fibers, contributing to the extraction yield, which may have happened with soy.

These results support earlier findings obtained by Ranilla *et al*., (2009) where TPC and antioxidant activity increased in cooked Brazilian bean cultivars, independently of cooking temperatures. In chickpea, a significant increase of tocopherols, phenolic compounds and antioxidant activity in cooked samples were also found when compared to raw (Sarmento *et al*., 2015). The study performed by Açar *et al*., (2009) in lentils, chickpea, black, borlotti, kidney and soy beans, highlighted the increase of the

antioxidant activity of all roasted legumes compared with raw ones. The opposite, however, was reported by Siah *et al*., (2012) in extracts from roasted fava bean.

Table 1: Effect of cooking and pressure cooking on the total phenolic content (TPC) and antioxidant activity of methanolic extracts from the different beans expressed per gram of extract.

	Raw	**Boiled**	**Pres. Cooked**
Cowpea			
TPC (mg GAEq g^{-1} Ex.)	$8.01\pm0.48^{a;A}$	$9.56\pm0.78^{b;A}$	$10.87\pm0.26^{b;A}$
DPPH (µmol TEq g^{-1} Ex.)	$38.09\pm2.55^{a;B}$	$37.93\pm3.61^{a;B}$	$39.57\pm1.09^{a;B}$
FRAP (µmol TEq g^{-1} Ex.)	$67.42\pm2.07^{a;A}$	$71.47\pm5.71^{ab;B}$	$77.77\pm2.26^{b;B}$
Black bean			
TPC (mg GAEq g^{-1} Ex.)	$14.74\pm0.85^{a;C}$	$17.98\pm0.78^{b;C}$	$20.11\pm0.82^{c;C}$
DPPH (µmol TEq g^{-1} Ex.)	$88.85\pm6.20^{a;C}$	$113.79\pm3.74^{b;C}$	$129.58\pm2.66^{c;C}$
FRAP (µmol TEq g^{-1} Ex.)	$169.79\pm11.48^{a;B}$	$201.25\pm3.07^{b;C}$	$230.93\pm10.33^{c;C}$
Soya bean			
TPC (mg GAEq g^{-1} Ex.)	$12.81\pm0.32^{ab;B}$	$12.09\pm0.70^{a;B}$	$14.01\pm0.59^{b;B}$
DPPH (µmol TEq g^{-1} Ex.)	$25.01\pm2.32^{b;A}$	$19.57\pm1.50^{a;A}$	$15.71\pm1.79^{a;A}$
FRAP (µmol TEq g^{-1} Ex.)	$53.71\pm1.29^{a;A}$	$52.33\pm1.68^{a;A}$	$55.76\pm3.37^{a;A}$

Data presented as mean and SD from three independent replicates, n=3. The data marked by the same letters were not significantly different ($p>0.05$). Different uppercase letters (for comparison within each column) and lowercase (for comparison within each row) letters were used to indicate significant differences at ($p<0.05$) between varieties and different processing, respectively. TPC – Total phenolic content. DPPH – 2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activity. FRAP - Ferric reducing antioxidant power.

Quantitative and qualitative differences detected by HPLC in phenolic composition, expressed in g of extracts of raw and cooked beans are presented in Tables 2. Ferulic acid was the predominant phenolic acid found in extracts from raw cowpea and it increased after boiling. An increase of gallic, *p*-coumaric and ferulic acids content was observed in cooked cowpea samples. Regarding cowpea flavonoids, catechin, myricetin and quercetin derivatives were detected, and an overall increase was observed in cooked samples.

The predominant phenolic acids present in black bean were gallic and caffeic acids. It was observed a decrease of gallic and chlorogenic acids content in extracts from cooked samples. An overall increase was found on caftaric, caffeic, p-coumaric and ferulic acids in processed black bean samples whether by boiling or by pressure cooking. Catechin was the predominant flavonoid found in black bean and its content decreased after cooking. The same was observed for kaempferol-3-*O*-(6-*O*-manolyl)glucoside content. Of the other flavonoids, a general increase was observed after cooking, with the exception of quercetin-3-*O*-glucoside in boiled samples. In soy,

the only phenolic acid present was gallic acid and its content increased after cooking. Genistein and daidzein, the only flavonoids found in soy extracts, increased with both cooking methods. These isoflavones were not present in cowpea or black beans extracts.

Globally, processing whether by boiling or by pressure cooking, had a positive effect on phenolic acids and flavonoid content in extracts from the different bean species. In cowpea extracts, boiling and pressure cooking increased by 41 % and 27 % the phenolic content, respectively. Black bean displayed an increase of the phenolic content of approximately 17 % and 13 % for boiled and pressure-cooked samples, respectively. The highest increase was observed in soy samples, where an increase of 187 % for boiling and 440 % for pressure cooking, comparing to raw samples.

Our results are in agreement with those reported by Ranilla *et al*., (2009) in Brazilian *Phaseolus vulgaris*, where an increase in phenolic acids and flavonoid content were found after cooking, independently of temperature.

Table 2: Effect of cooking and pressure cooking on the phenolic composition of methanolic extracts from cowpea, black bean and soy.

	Concentration mg g^{-1} of extract	Cowpea			Black bean			Soy		
		Raw	Boiled	Pres. Cooked	Raw	Boiled	Pres. Cooked	Raw	Boiled	Pres. Cooked
Phenolic acids	**Gallic acid**	0.14±0.01	0.34±0.06	0.27±0.02	1.11±0.05	0.5740.04	0.57±0.05	0.52±0.024	0.76±0.02	0.69±0.06
	Caftaric acid	-	-	-	-	0.27±0.00	0.42±0.03	7.61±0.54	8.07±0.99	10.64±0.1
	Chlorogenic acid	0.15±0.08	0.15±0.01	0.17±0.01	0.55±0.05	0.42±0.02	0.49±0.05	-	-	-
	Caffeic acid	-	-	-	0.90±0.07	0.86±0.07	1.15±0.15	-	-	-
	***p*-Coumaric acid**	0.15±0.01	0.19±0.00	0.20±0.01	0.03±0.00	0.04±0.01	0.04±0.00	-	-	-
	Ferulic acid	0.27±0.00	0.31±0.01	0.26±0.03	0.15±0.00	0.23±0.02	0.22±0.02	-	-	-
Flavonoids	**Catechin**	2.72±0.14	3.08±0.12	2.70±0.14	6.10±0.62	6.22±0.00	4.55±0.08	10.65±1.02	13.50±1.34	12.84±0.43
	Myricetin-3-*O*-glucoside	0.36±0.03	0.40±0.02	0.40±0.03	0.74±0.07	0.96±0.04	1.06±0.03	-	-	-
	Quercetin-3-*O*-xylosylglucoside	0.94±0.17	2.50±0.45	2.32±0.11	-	-	-	-	-	-
	Quercetin-3-*O*-glucoside	1.22±0.06	1.41±0.05	1.22±0.08	1.55±0.15	1.16±0.04	1.77±0.23	-	-	-
	Quercetin-3-*O*-(6-*O*-manolyl)glucoside	-	-	-	-	0.69±0.02	0.63±0.05	-	-	-
	Kaempferol-3-O-glucoside	-	-	-	0.14±0.01	0.15±0.01	0.16±0.00	-	-	-
	Myricetin	-	-	-	0.10±0.00	0.42±0.01	0.50±0.05	-	-	-
	Kaempferol-3-*O*-(6-*O*-manolyl)glucoside	-	-	-	0.07±0.00	0.06±0.00	0.05±0.00	-	-	-
	Quercetin	-	-	-	0.51±0.04	1.85±0.19	1.75±0.16	-	-	-
	Kaempferol	-	-	-	0.04±0.01	0.10±0.01	0.13±0.01	-	-	-
	Genistein	-	-	-	-	-	-	2.45±0.18	7.63±0.82	10.01±0.03
	Daidzein	-	-	-	-	-	-	0.71±0.02	2.15±0.22	9.14±0.58

Data presented as mean and SD from two injections from two independent replicates. Results were expressed in mg per gram of extract (mg g^{-1} Ex.).

4.3.2. Effects of extracts on cell proliferation

The effects of polyphenolic rich extracts obtained from raw, boiled and pressure-cooked beans on cell viability/proliferation were investigated using MTT assay and are presented in Figure 1. The proliferation of both HCT116 p53wt and HCT116 p53null was found to be suppressed by treatment with the extracts in a dose-dependent manner. HCT116 p53wt cells were more sensitive than HCT116 p53null, in particular to extracts of cooked black bean.

Raw cowpea and black bean extracts decreased cell viability by approximately 25 and 40 %, respectively, in both cell lines. Extracts from cooked (boiled and pressure cooked) cowpea and black bean samples showed higher effects in HCT116 p53wt cells, however, the decrease in cell viability in HCT116 p53null was also significant. Extract from raw and cooked soy displayed a similar effect in both cell lines.

In a previous study conducted by Ombra *et al*., (2016) a decrease of phenolic content and antioxidant activity was observed in extracts from cooked common bean varieties when compared with raw. However, extracts from cooked samples were equally capable of inhibiting the proliferation of the colorectal adenocarcinoma cells (Caco-2), breast cancer cells (MCF-7) and lung cancer cells (A549). Similar effect was reported by Siah *et al*., (2012) in extracts from raw and roasted faba bean (*Vicia faba*). In that experiment, both extracts similarly inhibited the proliferation of bladder cancer cells (BL13), stomach adenocarcinoma cells (AGS), liver carcinoma cells (HepG2) and colorectal adenocarcinoma cells (HT29).

In our experiment, the decrease of cell viability seems to be correlated to the phenolic content/composition and antioxidant activity particularly in extracts from processed cowpea and black bean, where an increase on TPC and antioxidant activity were observed concomitantly with decreased of cell proliferation. However, the effects of soy extracts do not seem to be correlated with the antioxidant capacity.

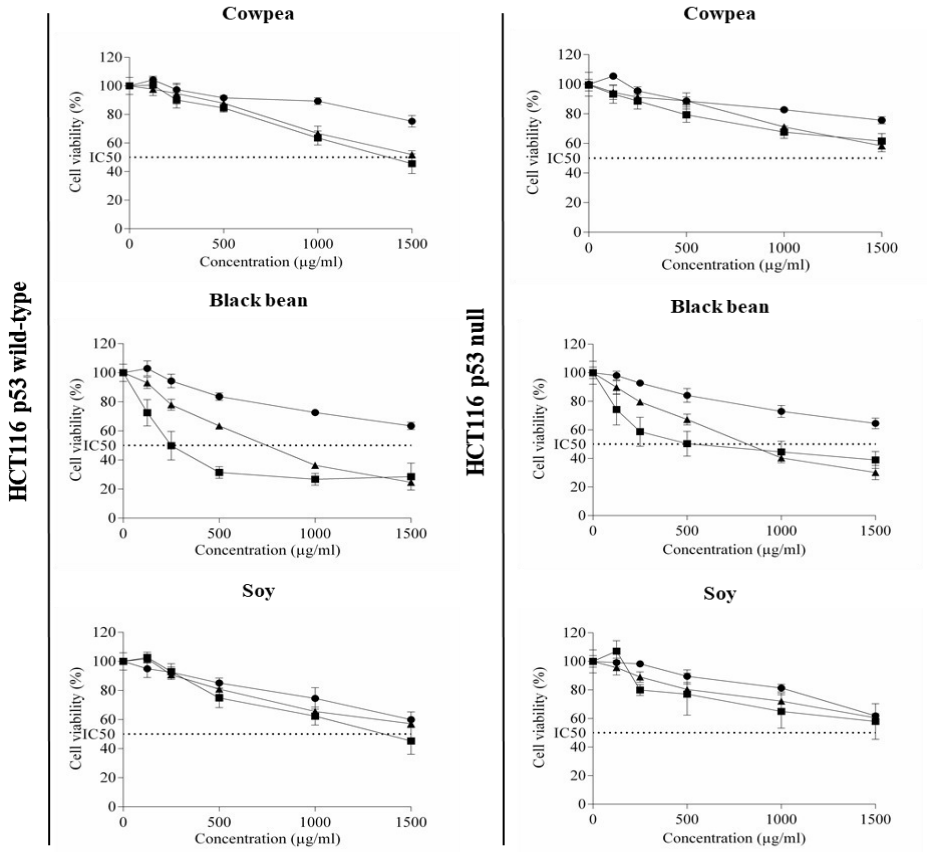

Figure 1: Effect of crude extracts obtained from the raw (●), boiled (■) and pres. cooked (▲) cowpea, black bean and soya bean on viability/proliferation of HCT116 p53wt and HCT116 p53null, human colon cancer cells. Values are expressed as means and standard deviations from at least three independent experiments.

4.3.3. Effects of bean extracts on cell cycle

To determine the effects of bean extracts on cell cycle progression, flow cytometry was performed. Figure 2 shows the relative percentage of HCT116 p53wt and HCT116 p53null in each phase of the cell cycle following treatment.

Raw cowpea extracts displayed no significant effect in both cell lines. Cowpea extract obtained after boiling or pressure cooking showed to increase in both cell lines the cell population in G0-G1 phase in comparison with the control, although not significantly for pressure-cooked cowpea. Extract from boiled cowpea induced an increase of 46 % and 24 % of cells in G0-G1 phase in HCT116 p53wt and HCT116 p53null, respectively. The G0-G1 phase arrest was accompanied by a decrease in S and G2/M cells.

Incubation with boiled black bean extracts displayed an increase of 35 % in HCT116 p53wt cells and 20 % for HCT116 p53null. The extracts of pressure-cooked black bean

were not as effective inducing an increase in G0-G1 cells and only 20 and 12 % in HCT116 p53wt and HCT116 p53null, respectively.

Soy was shown to be the least efficient inducer of cell cycle arrest, showing a significant effect only on HCT116 p53wt by pressure-cooked extract, despite the fact that a massive increase of isoflavones in processed samples was observed. No effects were observed in raw or cooked soy extracts on p53null cells.

Cancer is frequently considered a cell cycle disease due to alterations in different families of the cell-cycle regulators, playing an important role in tumor development (Otto & Sicinski, 2017). The inhibition of cell cycle progression in cancer cells is, therefore, an important strategy to control cancer progression. With the present experiment, we demonstrate that extracts from cowpea, black bean and soy were able to induce cell cycle arrest in G0-G1 phase, particularly extracts from processed beans. In our knowledge, this finding has not been reported in in vitro experiments after incubations with polyphenolic beans extracts.

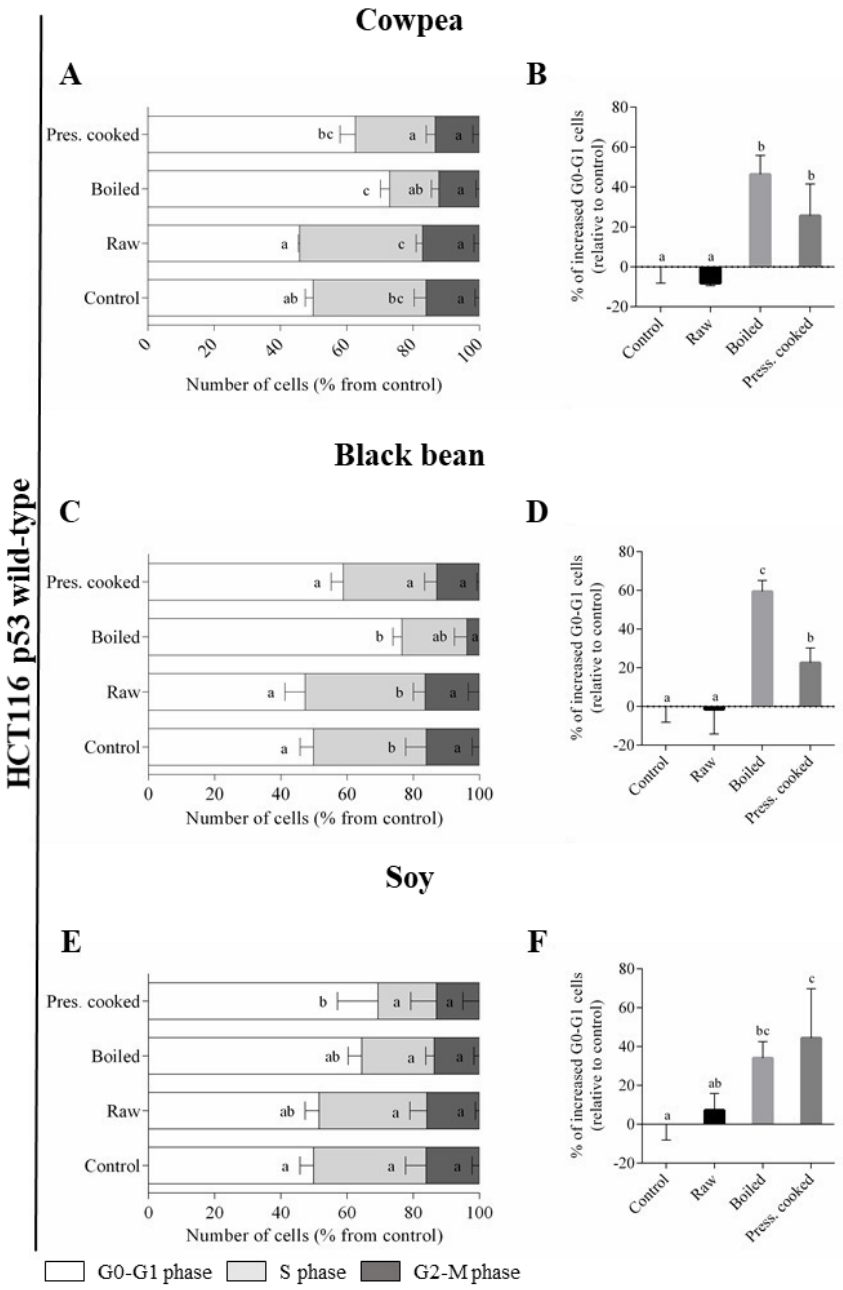

Figure 2: Cell cycle analysis by flow cytometry of human colon cancer cells HCT116 p53 wild-type treated with IC50 of the raw, cooked and pressure-cooked cowpea, black bean and soya bean extracts (A, C and E). Percentage (%) of increase or decrease in G0-G1 cells relative to control (B, D and F). Values are expressed as means and standard deviations of three independent experiments as percentage. Different letters were used to indicate significant differences at ($p<0.05$) between groups of each cell cycle phase.

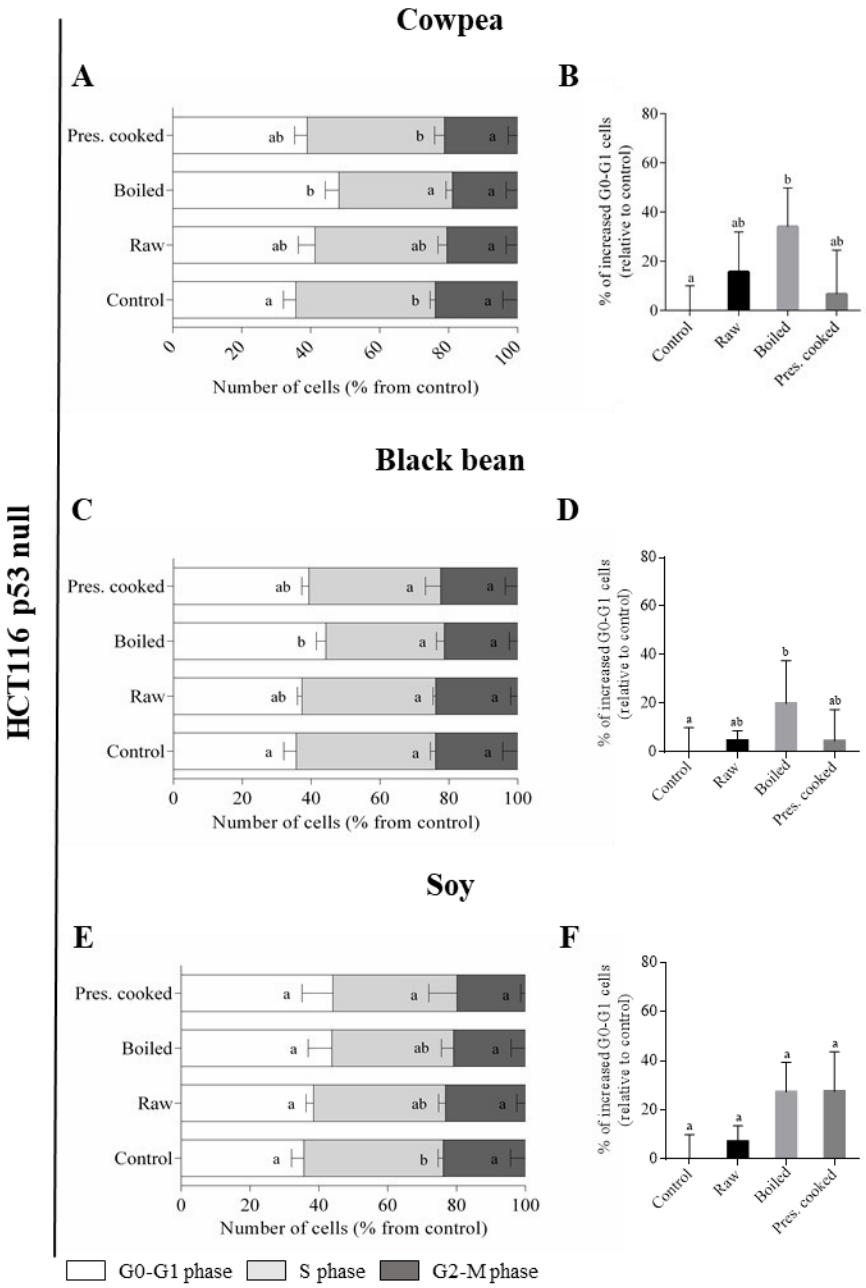

Figure 3: Cell cycle analysis by flow cytometry of human colon cancer cells HCT116 p53 null treated with IC50 of the raw, cooked and pressure-cooked cowpea, black bean and soya bean extracts (A, C and E). Percentage (%) of increase or decrease in G0-G1 cells relative to control (B, D and F). Values are expressed as means and standard deviations of three independent experiments as percentage. Different letters were used to indicate significant differences at ($p<0.05$) between groups of each cell cycle phase.

4.3.4. Effect of extracts on the expression of cell cycle-related mRNAs and proteins

To further investigate the mechanisms involved in the effects of bean extracts, cell cycle-related protein and mRNA expression levels were analyzed using Western-blot and qPCR, respectively.

The cell cycle progression is regulated by several signaling pathways (Figure 4). The entry into the cell cycle is generally induced in response to mitogenic signals that lead to the activation of G0-G1 transition signaling pathways, such as, RAS dependent pathway (MAPK/ERK and PI3K/AKT), leading to the induction of various cell cycle proteins, including D-type cyclins. The activation of the cyclin D-CDK4/6 complex is antagonized by the INK4 family (p16) in response to senescence inducing signals or growth inhibitors. Cyclin D-CDK4/6 complexes induce phosphorylation (P) of the retinoblastoma protein (RB). RB hyperphosphorylation liberates E2F transcription factors, that they induce the expression of cyclin E and promote the entrance into the S phase. Cyclin E-CDK2 complexes contribute to G1-S progression of the cell cycle are antagonized by the inhibitors p27 and p21, which in turn are regulated by growth-inhibitory signals and/or p53 activation. Active cyclin E-CDK2 complexes also phosphorylate RB, as well as many other targets, leading to the entry of cells into the S phase (Otto & Sicinski, 2017).

HCT116 cells have an activating KRAS mutation (Ahmed *et al*., 2013). RAS family is a group of membrane-bound GTPase proteins that regulate numerous cellular processes by activating signaling pathways, such as, the MEK/ERK mitogen-activated protein kinase (MAPK) and PI3K-AKT pathways by a cascade of phosphorylations (Polosukhina *et al*., 2017).

The effects of the different bean extracts on the cell cycle regulatory proteins are presented in Figures 5 and 6. Considering the cell cycle arrest in G0-G1 phase observed by flow cytometry, we focused our study on the expression of some proteins involved in the regulation of the G1-S cell cycle progression.

Although we did not observe a significant effect on MEK/ERK activation (pERK levels) in both cell lines incubated with the different extracts (data not shown), a decreased activation of PI3K-AKT pathways (lower pAKT/AKT levels) was observed, particularly in HCT116 p53wt. The down-regulation of cyclin E and pRB in both cell lines was observed when cells were treated with all cowpea and black bean extracts in both cell lines. In soy extracts, these effects were less pronounced.

qPCR results of modulation of gene expression of p53, p27, p21 and p16, responsible for the regulation of D-cyclin and E-cyclin proteins were reinforced. As shown in Table 3,

in HCT116 p53wt, both cowpea and soy extracts induced an increase of mRNA levels of p16 while soy also increased the mRNA of p21. Remarkably, in HCT116 p53wt black bean extracts were capable of modulating all studied pathways, inducing an increase of mRNA levels of p53, p27, p21 and p16.

Overall, in HCT116 p53null, all extracts were able to modulate p27, p21 and p16. Comparing HCT116 p53wt and HCT116 p53null, cells without p53 presented a higher p16 mRNA induction compared to p53wt, which may explain the G0-G1 arrest in absence of p53.

By flow cytometry, in both cells lines, the effects were particularly pronounced in cells treated with extracts from cooked cowpea and black bean. The cell cycle arrest was confirmed by Western-blot with the decrease of phosphorylated AKT and RB, decreased levels of cyclin E, consistent with decreased cell cycle progression signaling and arrest in G0-G1. Furthermore, extracts were also capable of inducing mRNA expression of proteins responsible for the modulation of cyclins D and E.

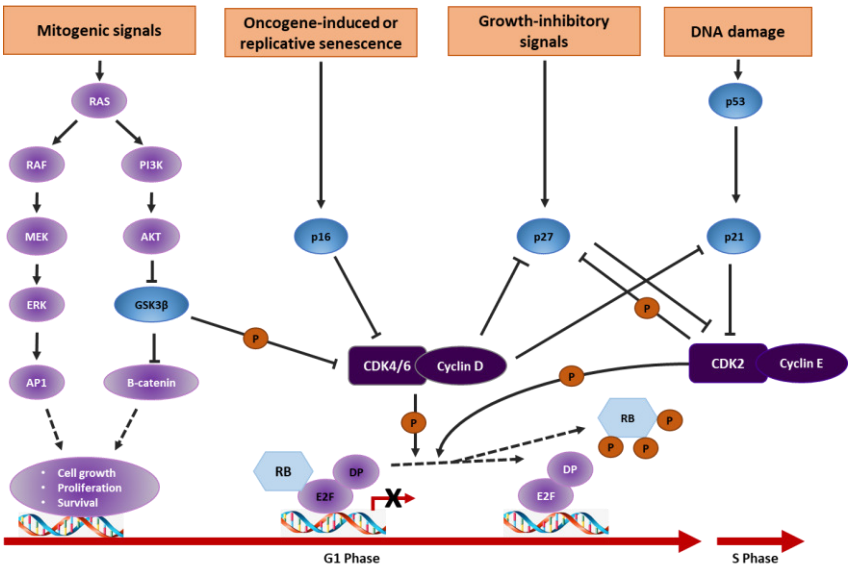

Figure 4: Regulation of G1-S cell cycle progression by multiple cellular pathways. The entry into the cell cycle is generally induced in response to mitogenic signals that leads to the activation of signaling pathways such as the RAS pathway. These pathways may influence the expression of various transcription factors, such as MYC, activator protein-1or β-catenin and may lead to the induction of various cell cycle proteins, including D-type cyclins. The activation of the cyclin D-CDK4/6 complex is antagonized p16 in response to senescence inducing signals or growth inhibitors. Cyclin D-CDK4/6 complexes induce phosphorylation (P) of the retinoblastoma protein (RB). RB hyperphosphorylation liberates E2F transcription factors, which activate transcription of genes inducing the entrance into the S phase, including cyclin E. The cyclin E-CDK2 complexes are antagonized by p27 and p21, which in turn are down-regulated by growth-inhibitory signals and p53 activation induced by DNA damage. Active cyclin E-CDK2 complexes also

phosphorylate RB, as well as many other targets, leading to the entry of cells in the S phase. Figure adapted from (Otto & Sicinski, 2017).

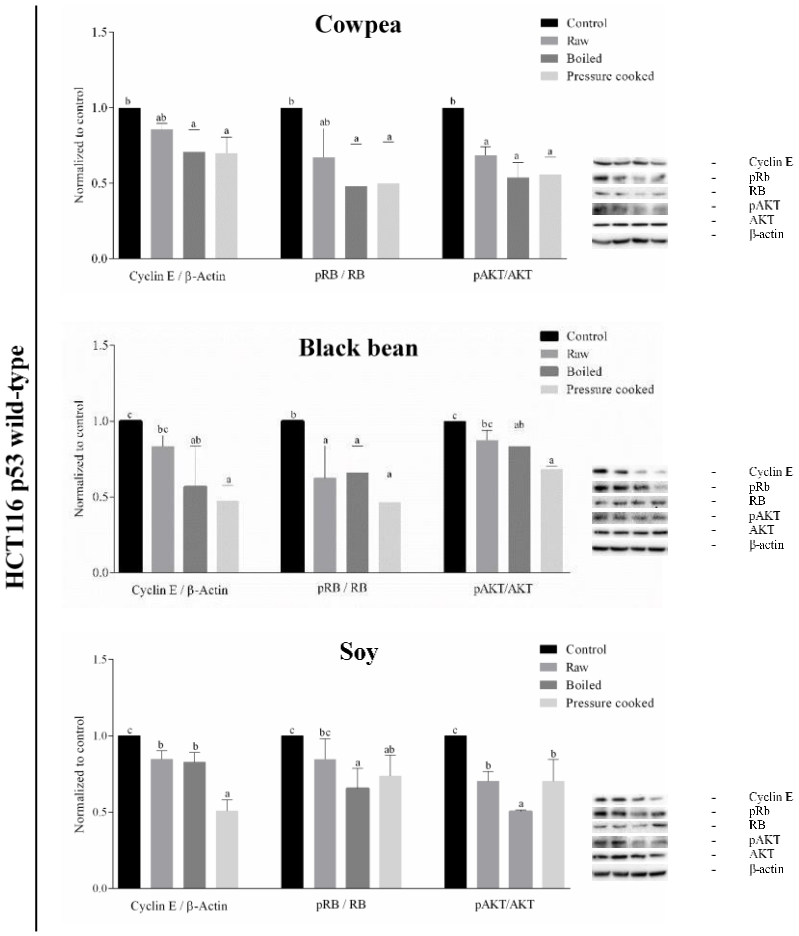

Figure 5: Expression of cyclin E, pRB/RB, pAKT/AKT and β-actin proteins on HCT116 p53wild type, treated with IC50 of the raw, cooked and pressure-cooked cowpea, black bean and soy. Protein expression levels among the various conditions were compared on the basis of the ratio of expression of a protein in each condition to that in non-treated one. Values are expressed as means and standard deviations of three independent experiments. Different letters were used to indicate significant differences at ($p<0.05$) between groups.

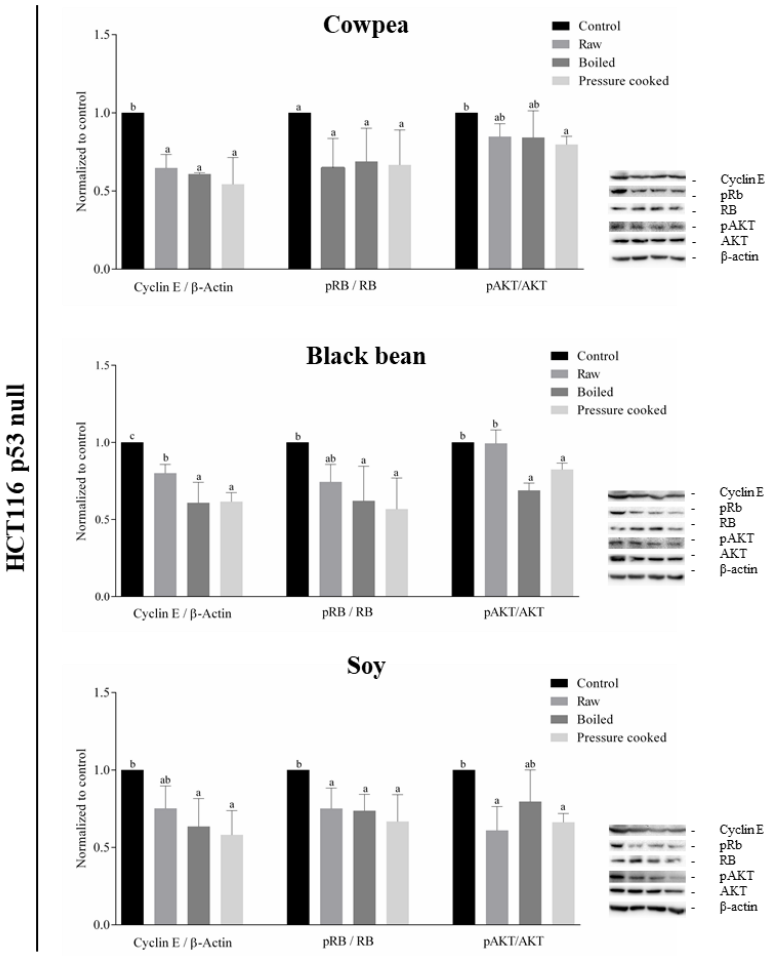

Figure 6: Expression of cyclin E, pRB/RB, pAKT/AKT and β-actin proteins on HCT116 p53 null, treated with IC50 of the raw, cooked and pressure-cooked cowpea, black bean and soy. Protein expression levels among the various conditions were compared on the basis of the ratio of expression of a protein in each condition to that in non-treated one. Values are expressed as means and standard deviations of three independent experiments. Different letters were used to indicate significant differences at ($p<0.05$) between groups.

Table 3: Effect on relative gene expression of some cell cycle regulate genes on HCT116 p53wt and p53 null cells, treated with IC50 of the raw, cooked and pressure-cooked cowpea, black bean and soy. Results were normalized to the 36B4 relative expression. Expression levels among the various conditions were compared on the basis of expression of each condition to that in non-treated one.

			p53	p27	p21	p16
HCT116 p53 wild-type		Control	1.00±0.02^{a}	1.00±0.02^{a}	1.00±0.04^{a}	1.00±0.02^{a}
	Cowpea	Raw	1,31±0.20^{abc}	1.46±0,13^{abcd}	1.12±0.03^{ab}	1.87±0.14^{bc}
		Boiled	1,22±0.08^{abc}	1.34±0.17^{abc}	1.38±0.15^{abc}	1,76±0.72^{bc}
		Pres. Cooked	1.08±0.05^{ab}	1.02±0.08^{a}	1.01±0.13^{a}	1.49±0.26^{ab}
	Black bean	Raw	1.58±0.51^{bc}	1.85±0.20^{cd}	1.43±0.15^{abc}	1.54±0.67^{ab}
		Boiled	1.59±0.45^{bc}	1.71±0.57^{bcd}	1.87±0.61^{c}	2.14±0.12^{c}
		Pres. Cooked	1.72±0.60^{c}	2.02±0.80^{d}	2.02±0.96^{c}	2.19±0.23^{c}
	Soy	Raw	0.96±0.21^{a}	1.17±0.30^{ab}	1.13±0.19^{ab}	2.01±0.29^{bc}
		Boiled	1.07±0.26^{ab}	1.28±0.31^{abc}	1.56±0.45^{abc}	1.91±0.13^{bc}
		Pres. Cooked	1.49±0.50^{abc}	1.59±0.45^{abcd}	1.79±0.34^{bc}	2.95±0.25^{d}
HCT116 p53 null		Control		1.00±0.08^{a}	1.00±0.07^{a}	1.00±0.00^{a}
	Cowpea	Raw		1.45±0.13^{bcd}	1.17±0.22^{ab}	2.87±0.35^{b}
		Boiled		1.06±0.20^{a}	1.50±0.29^{bcd}	2.69±0.36^{b}
		Pres. Cooked		1.67±0.38^{d}	1.93±0.62^{de}	3.39±0.59^{bc}
	Black bean	Raw		1.50±0.03^{cd}	1.74±0.13^{cde}	4.22±0.36^{de}
		Boiled		2.12±0.42^{e}	2.07±0.39^{e}	4.64±0.99^{e}
		Pres. Cooked		1.25±0.08^{abc}	1.57±0.13^{bcd}	3.93±0.09^{cd}
	Soy	Raw		0.98±0.04^{a}	1.34±0.05^{abc}	3.61±0.10^{cd}
		Boiled		1.42±0.18^{bcd}	1.92±0.08^{de}	3.67±0.23^{cd}
		Pres. Cooked		1.16±0.06^{ab}	1.62±0.15^{cd}	3.63±0.38^{cd}

Values are expressed as means and standard deviations of three independent experiments. Different letters were used to indicate significant differences at ($p<0.05$) between groups.

4.4. Conclusions

The phenolic extracts obtained from the raw and processed beans evaluated in the present study showed potential anticancer properties, as well as, antioxidant activities and antiproliferative effects by modulation the cell cycle progression. Overall, cooking increased phenolic content and antioxidant activity in cowpea and black bean whereas in soy extracts not. Regarding soy, cooking did not increase antioxidant activity although genistein and daidzein content were increased. Extracts from different beans exhibited chemopreventive effects demonstrated by the decrease of cell viability/proliferation and through induction of cell cycle arrest in G0-G1 phase. Bean extracts suppressed proliferation of different types of cancer cells with and without p53 in a dose-dependent manner, particularly after cooking. G0-G1 cell cycle arrest was confirmed by protein cyclin E and pRB down-regulation. Therefore, we conclude that bean extracts may induce

G0/G1 arrest via AKT signaling pathway and that the modulation of AKT, cyclin D, and cyclin E via p27, p21, and p16.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

We are especially grateful for the help provided by Professor Ana Isabel Barros from CITAB-Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal for the phytochemical characterization of the extracts.

The author Catarina Teixeira-Guedes acknowledges the financial support provided FCT-Portuguese Foundation for Science and Technology (SFRH/BD/52544/2014), under the Doctoral Program "Agricultural Production Chains – from fork to farm" (PD/00122/2012).

This work was supported by the European Investment Funds FEDER/COMPETE/POCI– Operational Competitiveness and Internationalization Programme [POCI-01-0145-FEDER-006958] and Portuguese Foundation for Science and Technology (FCT) [UID/AGR/04033/2013]; Project I&D Interact - Integrative Research in Environment, Agro-Chain and Technology [NORTE-01-0145-FEDER-000017], co-financed by European Regional Development Fund (FEDER) through NORTE 2020 (Programa Operacional Regional do Norte 2014/2020).

4.5. References

Açar, O.C., Gokmen, V., Pellegrini, N., & Fogliano, V. (2009). Direct evaluation of the total antioxidant capacity of raw and roasted pulses, nuts and seeds. *European Food Research and Technology*, *229*(6), 961–969. https://doi.org/10.1007/s00217-009-1131-z

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknæs, M., Hektoen, M., & Lothe, R. A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis*, *2*(0424). https://doi.org/10.1038/oncsis.2013.35

Bobe, G., Barret, K. G., Mentor-Marcel, R., Saffiotti, U., Young, M. R., Colburn, N. H., & Lanza, E. (2008). Dietary cooked navy beans and their fractions attenuate colon

carcinogenesis in azoxymethane-induced Ob/Ob mice. *Nutrition and Cancer*, *60*(3), 373–381. https://doi.org/10.1016/j.pestbp.2011.02.012.Investigations

Cruz-Bravo, R.K., Guevara-González, R.G., Ramos-Gómez, M., Oomah, B.D., Wiersma, P., Campos-Vega, R., & Loarca-Piña, G. (2014). The fermented non-digestible fraction of common bean (*Phaseolus vulgaris* L.) triggers cell cycle arrest and apoptosis in human colon adenocarcinoma cells. *Genes and Nutrition*, *9*(1). https://doi.org/10.1007/s12263-013-0359-1

Dan, X., Ng, T.B., Wong, J.H., Chan, Y.S., Chi, R., Cheung, F., & Chan, W.Y. (2016). A hemagglutinin isolated from Northeast China black beans induced mitochondrial dysfunction and apoptosis in colorectal cancer cells. *BBA - Molecular Cell Research*, *1863*(9), 2201–2211. https://doi.org/10.1016/j.bbamcr.2016.05.019

Domínguez-Perles, R., Teixeira, A. I.,Rosa, E., & Barros, A.I. (2014). Assessment of (poly)phenols in grape (*Vitis vinifera* L.) stems by using food/pharma industry compatible solvents and Response Surface Methodology. *Food Chemistry*, *164*, 339–346. https://doi.org/10.1016/j.fooDChem.2014.05.020

Feregrino-Perez, A.A. ngelic., Piñol-Felis, C., Gomez-Arbones, X., Guevara-González, R. G., Campos-Vega, R., Acosta-Gallegos, J., & Loarca-Piña, G. (2014). A non-digestible fraction of the common bean (*Phaseolus vulgaris* L.) induces cell cycle arrest and apoptosis during early carcinogenesis. *Plant Foods for Human Nutrition (Dordrecht, Netherlands)*, *69*(3), 248–254. https://doi.org/10.1007/s11130-014-0428-7

Teixeira-Guedes, C., Oppolzer, D., Barros, A., & Pereira-Wilson, C. (2018). Impact of cooking method on phenolic composition and antioxidant potential of four varieties of *Phaseolus vulgaris* L. and *Glycine max* L. *LWT - Food Science and Technology* (Minor recvision for acceptence)

Haggar, F., & Boushey, R. (2009). Colorectal cancer epidemiology : Incidence , mortality , survival , and risk factors. *Clinics in Colon and Rectal Surgery*, *6*(212), 191–197. https://doi.org/10.1055/s-0029-1242458.

Huxley, R.R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C.L., & Woodward, M. (2009). The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *International Journal of Cancer*, *125*(1), 171–180. https://doi.org/10.1002/ijc.24343

Kalogeropoulos, N., Chiou, A., Ioannou, M., Karathanos, V.T., Hassapidou, M., & Andrikopoulos, N.K. (2010). Nutritional evaluation and bioactive microconstituents

(phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the Mediterranean countries. *Food Chemistry*, *121*(3), 682–690. https://doi.org/10.1016/j.fooDChem.2010.01.005

Lin, L., Harnly, J.M., Pastor-corrales, M.S., & Luthria, D.L. (2008). The polyphenolic profiles of common bean (*Phaseolus vulgaris* L.). *Food Chemistry*, *107*, 399–410. https://doi.org/10.1016/j.fooDChem.2007.08.038

Luna-Vital, D.A., Liang, K., González de Mejía, E., & Loarca-Piña, G. (2016). Dietary peptides from the non-digestible fraction of: *Phaseolus vulgaris* L. decrease angiotensin II-dependent proliferation in HCT116 human colorectal cancer cells through the blockade of the renin-angiotensin system. *Food and Function*, *7*(5). https://doi.org/10.1039/c6fo00093b

Madhujith, T., Amarowicz, R., & Shahidi, F. (2004). Phenolic antioxidants in beans and their effects on inhibition of radical-induced DNA damage. *Journal of the American Oil Chemists' Society*, *81*(7), 691–696. https://doi.org/10.1007/s11746-004-963-y

Marathe, S. A., Rajalakshmi, V., Jamdar, S. N., & Sharma, A. (2011). Comparative study on antioxidant activity of different varieties of commonly consumed legumes in India. *Food and Chemical Toxicology*, *49*(9), 2005–2012. https://doi.org/10.1016/j.fct.2011.04.039

Ombra, M.N., D 'Acierno, A., Nazzaro, F., Riccardi, R., Spigno, P., Zaccardelli, M., & Fratianni, F. (2016). Phenolic composition and antioxidant and antiproliferative activities of the extracts of twelve common bean (*Phaseolus vulgaris* L.) endemic ecotypes of southern Italy before and after cooking. *Oxidative Medicine and Cellular Longevity*, *2016*, 1–11. https://doi.org/10.1155/2016/1398298

Otto, T., & Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. *Nature Reviews Cancer*, *17*(2), 93–115. https://doi.org/10.1038/nrc.2016.138

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), 2003–2007. https://doi.org/10.1093/nar/29.9.e45

Polosukhina, D., Love, H.D., Correa, H., Su, Z., Dahlman, K.B., Pao, W., … Clark, P.E. (2017). Functional KRAS mutations and a potential role for PI3K/AKT activation in Wilms tumors. *Molecular Oncology*, *11*(4), 405–421. https://doi.org/10.1002/18780261.12044

Ranilla, L.G., Genovese, M.I., & Lajolo, F.M. (2009). Effect of different cooking conditions on phenolic compounds and antioxidant capacity of some selected

Brazilian bean (*Phaseolus vulgaris* L.) cultivars. *Journal of Agricultural and Food Chemistry*, *57*(13), 5734–5742. https://doi.org/10.1021/jf900527v

Sarmento, A., Barros, L., Fernandes, A., Carvalho, A. M., & Ferreira, I. C. F. R. (2015). Valorization of traditional foods: Nutritional and bioactive properties of *Cicer arietinum* L. and *Lathyrus sativus* L. pulses. *Journal of the Science of Food and Agriculture*, *95*(1), 179–185. https://doi.org/10.1002/jsfa.6702

Siah, S.D., Konczak, I., Agboola, S., Wood, J.A., & Blanchard, C.L. (2012). In vitro investigations of the potential health benefits of Australian-grown faba beans (*Vicia faba* L.): chemopreventative capacity and inhibitory effects on the angiotensin-converting enzyme, α-glucosidase and lipase. *British Journal of Nutrition*, *108*(S1), S123–S134. https://doi.org/10.1017/S0007114512000803

Siah, S., Wood, J.A., Agboola, S., Konczak, I., & Blanchard, C.L. (2014). Effects of soaking, boiling and autoclaving on the phenolic contents and antioxidant activities of faba beans (*Vicia faba* L.) differing in seed coat colours. *Food Chemistry*, *142*, 461–468. https://doi.org/10.1016/j.fooDChem.2013.07.068

World Cancer Research Fund. (2017). Diet, nutrition, physical activity and colorectal cancer. Retrieved from https://www.wcrf.org/sites/default/files/Colorectal-Cancer-2017-Report.pdf

---

CHAPTER 5

Effect of cowpea sprouting in the phenolic composition, antioxidant activity and anti-colorectal cancer properties

This chapter enclose the following manuscript:

Teixeira-Guedes, C.I., Oppolzer, D., Barros, A.I. & Pereira-Wilson, C. (2018). Sprouting of cowpea increases its phenolic content, antioxidant activity and anti-colorectal cancer properties. Submitted

Sprouting of cowpea increases its phenolic content, antioxidant activity and anti-colorectal cancer properties

Catarina I. Teixeira-Guedes[ab]*, David Oppolzer[c], Ana I. Barros[c], Cristina Pereira-Wilson[abd]

[a]*Department of Biology, University of Minho, Braga, Portugal*

[b]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Minho, Braga, Portugal*

[c]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

[d]*Centre of Biological Engineering, University of Minho, Braga, Portugal*

Abstract

Diets rich in fruits, vegetables, legumes and whole grains are associated with a decreased risk of developing chronic diseases including cancer. Sprouting of legumes increases the digestibility and nutrient bioavailability, thereby increasing the nutritional value. The aim of the present work was to study the effects of cowpea (*Vigna unguiculata*) sprouting on polyphenolic profile and antioxidant activity over time. In order to demonstrate the anticancer potential of sprouts, antiproliferative and cell cycle modulation effects were evaluated in a panel of five colorectal cancer cell lines. Five-day germination of cowpea significantly increased the total phenolic (2.6-fold), flavonoid (1.2-fold) and *ortho*-diphenol content (1.7-fold) in a time-dependent manner, compared to cowpea seeds. Antioxidant activity was also increased by sprouting. Quercetin glucosides were the major phenolic compound found in sprouts. Significant decrease on cell proliferation and cell cycle arrest were induced in the several cell lines by cowpea sprouts phenolic extract when compared to extract of raw seeds. Therefore, sprouting of cowpea can significantly improve the nutritional quality of this Mediterranean diet ingredient, with regards to its phytochemical composition, antioxidant activity and anticancer potential. Sprouting is an easy and an inexpensive way to increase the health-promoting properties of cowpea, renewing and adding value to a traditional food and add value as functional food.

Keywords: cowpea, sprouting, phenolic compounds, antioxidant activity, antiproliferative effects, colorectal cancer

5.1. Introduction

Legumes are cultivated worldwide and represent an important component of the human diet, where they contribute as a significant protein source (Duranti, 2006; Kalogeropoulos *et al*., 2010). In addition to protein, legumes also contain complex carbohydrates, dietary fiber and micronutrients, including minerals, vitamins and phenolic compounds, and are low in fat (Kalogeropoulos *et al*., 2010; Luna-Vital *et al*., 2016; Siah *et al*., 2012). Evidence suggests that high consumption of legumes can help in the prevention and/or reduction of chronic diseases such as type II diabetes, cardiovascular disease, obesity and prevent cancer development (Campos-Vega and Loarca-Piña, 2010; Duranti, 2006; Hosseinpour-Niazi *et al*., 2015; Luna Vital *et al*., 2014).

Cowpea (*Vigna unguiculata* L. Walp) is originated in African where contributes to the diet of millions of people (Gonçalves *et al*., 2016; Karapanos *et al*., 2017; Phillips *et al*., 2003). Its cultivation has spread worldwide, including Europe, where it is traditionally produced in the Mediterranean Basin (Gonçalves *et al*., 2016). Compared to other legumes, cowpea is very versatile and drought tolerant, adapting well to different types of soil and arid conditions, producing significant yields under low-input farming systems (Gonçalves *et al*., 2016; Karapanos *et al*., 2017).

The process of sprouting or controlled germination applied to bean seeds is becoming increasingly popular in recent years. Sprouting is a simple and inexpensive method to improve palatability, digestibility and nutrient availability increasing the level of free amino acids, carbohydrates, dietary fiber, bioactive compounds, and decreasing antinutritional components, such as enzyme inhibitors, trypsin and tannins (Devi *et al*., 2015; Fernandez-Orozco *et al*., 2006; López-Amorós *et al*., 2006; López *et al*., 2013). Legumes sprouts are increasingly well accepted by consumers and has the potential to renew interest in legumes and to add value to a traditional food such as cowpea.

Phenolic compounds are micronutrients found in raw beans, which have gained increasing interest for their health benefits, namely antioxidant activity (Guo *et al*., 2012; Okada and Okada, 2007; Zou and Chang Sam, 2014), protective against radical-induced DNA damage (Madhujith *et al*., 2004; Nderitu *et al*., 2013), antimutagenic (Cruz-Bravo *et al*., 2014; Siah *et al*., 2012) and anticancer properties (Lima *et al*., 2016; Ombra *et al*., 2016; Siah *et al*., 2012). However, knowloge of the effect of sprouting of cowpea seeds on phenolic acids and flavonoid profile, as well as, on antioxidant activity and anticancer

activity is insufficient. The aim of this research was, thehefore, to study the effects of sprouting on polyphenolic content, composition and antioxidant activity overtime. The antiproliferative potential, against several colorectal cancer cell lines and the effects on cell cycle modulation were also evaluated. An improved information on the phytochemical composition and antioxidant activity and anticancer effects could help to valorize cowpea as a potential nutraceutical and functional foods and suggest to the consumers.

5.2. Materials and methods

5.2.1. Dry cowpea

Dry cowpea seed were ground into a powder in a coffee mill machine. The flour was passed through a 0.5 mm sieve and kept at -20 °C until extraction.

5.2.2. Germination procedure

Thirty grams of seeds were sterilized with 0.07 % of sodium hypochlorite solution for 30 min, then the seeds were washed with distilled water until neutral pH. After that, seeds were soaked with bottled water, at a ratio of 1:5 (w/v), during 12 h prior germination. At the end of the soaking time, the water was discarded. Soaked seeds were germinated in petri dishes in a germination chamber at 23 ºC and 92 % of humidity for 1, 2, 3, 4 and 5 days (Guajardo-Flores *et al*., 2013). All samples were freeze-dried and milled into powder, passed through a 0.5 mm sieve and kept at -20 °C until extraction.

5.2.3. Preparation of phenolic rich extracts

Extraction was carried out in methanol:water (80:20, v/v) at a solid to solvent ratio of 1:10 (w:v) (Chávez-santoscoy *et al*., 2016). The extractions were ultrasound assisted during 10 min and maintained for 1 h in an orbital shaker at room temperature in the dark. The extraction procedure was repeated 3 times. The extracts were vacuum filtered through Whatman No. 1 paper.

5.2.4. Total phenolic, flavonoid and ortho-diphenol content

The total phenolic content (TFC) was assessed by the Folin–Ciocalteau method. Total flavonoid content (TFC) by the aluminium chloride complexation method and *ortho*-diphenol content (oDC) by sodium molybdate complexation method. TPC and oDC were expressed as mg of gallic acid equivalents per 100 g of dry weight (mg GAEq/100g DW) and TFC was expressed as mg of catechin equivalents per 100 g of dry weight (mg

CEq/100g DW) (Domínguez-Perles *et al*., 2014; Teixeira-Guedes *et al*., 2018). All methods were optimized for microplate reader.

5.2.5. Antioxidant capacity assays

DPPH radical scavenging and ABTS radical scavenging were carried out as previously reported by (Domínguez-Perles *et al*., 2014) and optimized for microplates (Teixeira-Guedes *et al*., 2018). The ferric reducing antioxidant power (FRAP) was performed as described by (Bolanos De La Torre *et al*., 2015) and optimized for microplates by (Teixeira-Guedes *et al*., 2018). In all different methods, the results were expressed in μM Trolox equivalent per 100 g of dry weight (μM TEq/100g DW).

5.2.6. High performance liquid chromatography

In this work a Thermo Finnigan Surveyor HPLC system was used with a diode array detector (DAD). Separation was performed in a ACE 5 C18 column (maintained at 25 ºC) at a flow rate of 1 ml/min. The injection volume was 20 μL. The mobile phase A consisted in water-formic acid (99.9:0.1; v/v) and phase B acetonitrile-formic acid (99.9:0.1; v/v). The extract was injected and eluted using the gradient described by Lin *et al*., (2008). DAD was set at 280, 310 and 520 nm while a UV/VIS spectrum from 200-600 nm was continuously collected. The chromatograms were analyzed using the qualitative software of Xcalibur (Thermo Fisher Scientific, Inc., Waltham, USA). Samples were analyzed in duplicate.

5.2.7. Cell culture

The cell lines were maintained in T25 cm^2 polystyrene flacks with RPMI-1640 medium for HCT116, HCT15 and CO115, and DMEM for RKO and HT29. All mediums were obtained from Sigma-Aldrich and supplemented with 10 % bovine fetal serum (FBS; EU standard, Lonza, Verviers, Belgium), and antibiotic-antimitotic solution (1%) (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in a Sanyo COM-17 AIC incubator in a humidified atmosphere with 5 % CO_2 at 37 °C. For all experiments, HCT116, RKO cells were seeded at a density of 1 x 10^5 cell/ml. HCT15 were seeded at a density of 0,75 x 10^5 cell/ml and CO115 and HT29 at 1,2 x 10^5 cell/ml.

5.2.8. Assessment of cell viability

The evaluation of cell viability was performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. The MTT substrate was added to the cells at a final concertation of 0.5 mg/ml and incubated for 2 h. The quantity of formazan was measured at 570 nm using a microplate reader.

5.2.9. Cell cycle analysis by flow cytometry

Cells were incubated for 24 h with IC_{50} concentration of extracts and harvest and fixed with cold ethanol (70%) for 30 min in ice. Cells were then treated with ribonuclease and stained with propidium iodide (PI) (50 µg/ml stock solution). The analysis was performed in a Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Miami, FL, USA) with excitation at 488 nm and emission at 605-635 nm. Cell-cycle analyses were performed using FlowJo Software (Tree Star Inc, Ashland, Oregon, U.S.A.). Stained cells (20 000 single cell events) were analyzed and sorted based on area and pulse width of the PI signal. The percentages of cells with different DNA contents were calculated from histograms generated with gating (portions of cells in different phases of the cell cycle).

5.2.10. Statistical analysis

Results were presented as mean ± SD from three independent experiments. Statistical analyses were performed using IBM SPSS statistics 21.0 software (SPSS Inc., Chicago, IL, USA). Differences between samples were tested using analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and value of $p<0.05$ was considered as being statistically significant. The inhibitory concentration (IC_{50}) values were calculated from the corresponding dose-inhibition response with the best-fit shape curve, results were treated using GraphPad Prism 6 (GraphPad software).

5.3. Results and discussion

5.3.1. Total phenolic, flavonoid and ortho-diphenol content

The TPC of extracts from dry cowpea, soaked and sprouting are presented in Figure 1A and relative changes to dry seeds on Figure 1B. Soaking decreased by 15 % the TPC in cowpea, although, not significantly ($p>0.05$). TPC increased significantly in a time-dependent manner after soaking up to 270.45 ± 10.70 mg GAEq/100g DW five days after sprouting, corresponding to 2.6-fold higher than the original cowpea seeds extracts.

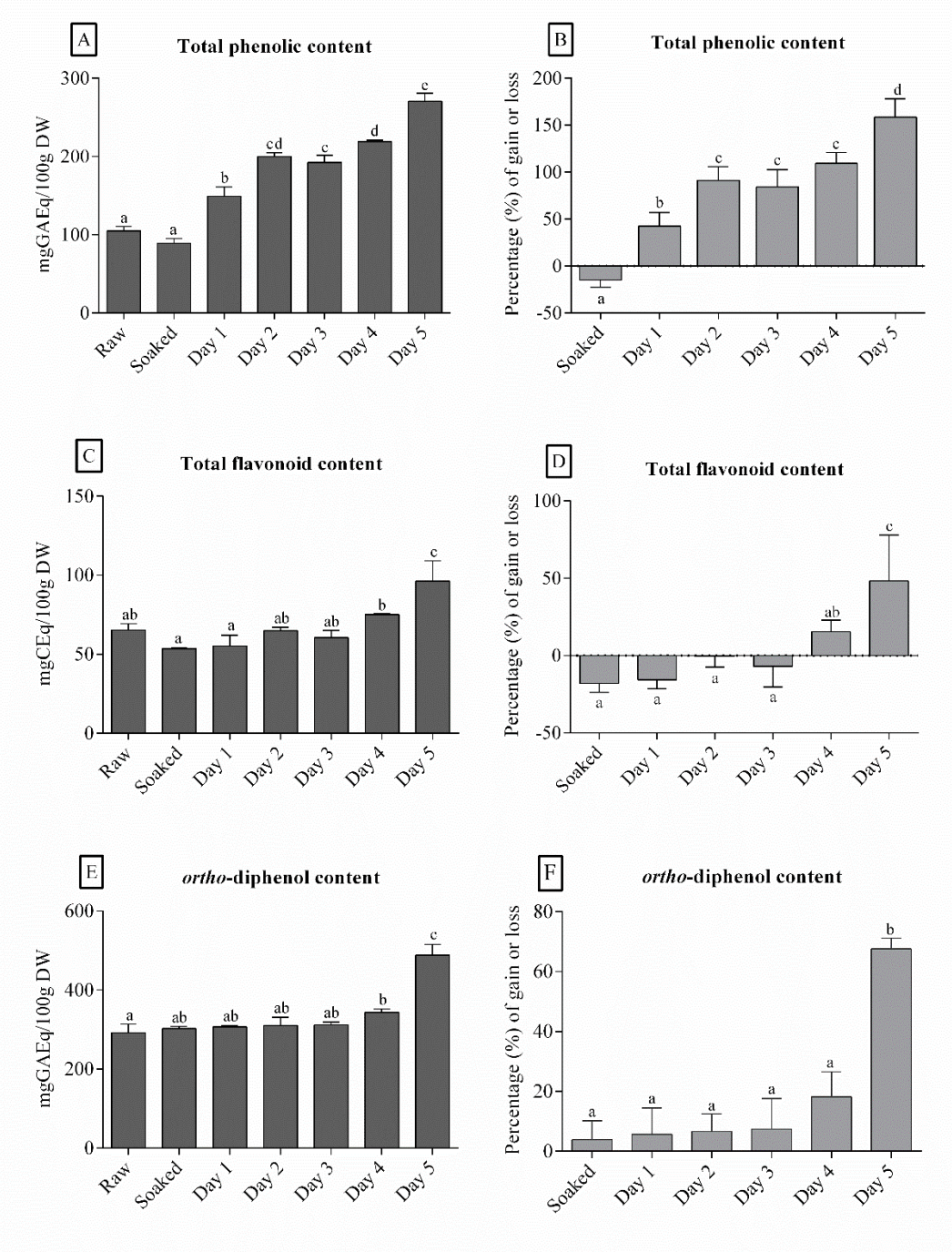

Figure 1. Effect of soaking and sprouting on (A) - total phenolic contents (TPC), (C) - total flavonoid content (TFC) and (E) - *ortho*-diphenol content (oDC). TPC and oDC are expressed in mg of gallic acid equivalent per 100 g of dry weight (mg GAEq/100g DW). TFC is expressed in mg of catechin equivalent per 100g of dry weight (mgCEq/100g DW). Data are presented as mean and SD from three different replicates (n=3). Different letters indicate statistically significant differences ($p<0.05$). Figure B, D and F reflects the percentage of gain or loss on (B) - total phenolic content, (D) - total flavonoid content, (F) - *ortho*-diphenol content. Values were calculated based on the initial contents in raw beans.

This finding is in accordance with a previous study performed in mung bean (*Vigna radiata*), where a significant increase of TPC in 5-day sprouts was observed (Guo *et al*., 2012). Similar impact of germination was observed in 5-day chickpea (*Cicer arietinum*) sprouts (Tarzi *et al*., 2012). On the other hand, a decrease of TPC was described in 3-day black bean sprouts (Chon, 2013) and in 7-day cowpea sprouts (Soufi *et al*., 2014). The change in the phenolic content in the germination process has been considered as a normal phenomenon during the plant development (Khang *et al*., 2016) and may be due to the activation of endogenous enzymes, such as, hydrolases and polyphenoloxidases (Guzmán-Ortiz *et al*., 2017). Phenolics compounds are secondary metabolites produced during the plant development, protecting the plant from biotic and abiotic stresses, such as, diseases, insects and environmental stresses (Guo *et al*., 2012). The present research supports the theory that phenolic content positively increases with germination.

The TFC of cowpea seeds and sprouts, as well as, relative changes are presented in Figure 1C and 1D. Soaking induced a decrease of approximately 18 %, which may indicate that these compounds leached to the water and were lost when the soaking water was removed. The TFC was recovered after 4 days and on day 5, occurred an increase of approximately 48 % in comparison to dry beans. Flavonoids are the major family of phenolic compounds present in fruits, vegetables and legumes, having antioxidant properties and biological activities linked to the reduction of major chronic diseases. The oDC of cowpea seeds and sprouts are presented in Figure 1E and relative changes to dry seed in Figure 1F. oDC significantly increased during sprouting time (1.7-fold higher than seeds). Soaking did not have a negative impact on oDC.

5.3.2. Phenolic profile in seeds and cowpea sprouts assessed by HPLC

Analysis of the phenolic profile was performed by HPLC with DAD detection. Results of phenolic profile of extracts from dry seeds and 5-day sprouts are shown in Table 1. Quantitative and qualitative differences between the phenolic composition of seeds and sprouts were observed. In dry samples, the concentration of phenolic compound was found to be highest in flavonoids, such as, catechin, epicatechin, followed by phenolic acid, namely gallic, chlorogenic and ferulic acid. On 5-day sprouts, an increase of catechin, gallic, chlorogenic and ferulic acid was observed, while epicatechin was not detected in sprouts. Caftaric and caffeic acids were found in sprouts but not in dry seeds extracts. Sprouting enriched the extracts in flavonoids such quercetin-3-O-xylosylglucoside, quercetin-3-O-glucoside, quercetin-3-O-(6"-O-manolyl)glucoside, kaempferol-3-O-

glucoside and kaempferol-3-O-xylosylglucoside, most of which were not detected in dry seeds extracts. In dry seeds extracts, flavonoids were approximately 537 µg/100g DW and significantly increased in cowpea sprouts after 5 days of germination to 1680 µg/100g DW (3-fold higher than the original concentration). Quercetin glucosides, present only in sprouts extracts, represented approximately 75 % of flavonoids and 60 % of total identified phenolic compounds. Sprouting resulted therefore in

Quercetin is known for its anti-cancer activities (Xavier *et al*., 2009), as well as, anti-inflammatory, antihypertensive, anti-diabetic, anti-obesity, anti-hypercholesterolemic, (Anand David *et al*., 2016). Previous increase in quercetin glucosides in sprouts have been reported by López-Amorós *et al*., (2006) in germinated black beans (*Phaseolus vulgaris*). In a study performed by Guzmán-Ortiz *et al*., (2017) in 6-day soy bean sprouts similar concentration of quercetin was observed.

The most significant increase was found for quercetin glucosides. Quercetin is an *ortho*-diphenol with a high antioxidant activity which is in agreement with the increased observed in the oDC and the antioxidant activity in sprouts.

A study performed by Eshraq *et al*., (2016) also reported an increase of several phenolic acids such as gallic and ferulic acid in black bean extracts with 1, 2 and 3 days of germination. Similarly, Guzmán-Ortiz *et al*., (2017) shown an increase of gallic, chlorogenic and ferulic acids in 6-day soy bean sprouts.

Table 2. Phenolic composition in dry seeds and 5-day old cowpea sprouts assessed by HPLC.

	Phenolic compounds (mg/100g DW)	**Raw**	**Germinated**
Phenolic acids	Gallic acid	9.94 ± 0.097	16.22 ± 2.25
	Caftaric acid	nd	5.04 ± 0.30
	Chlorogenic acid	4.74 ± 0.11	6.13 ± 0.35
	Caffeic acid	nd	9.46 ± 0.16
	Ferulic acid	1.00 ± 0.01	1.60 ± 0.07
Flavonoids	Catechin	28.79 ± 0.93	41.79 ± 0.29
	Epicatechin	24.77±0.75	nd
	Quercetin-3-O-glucoside	nd	15.66 ± 0.77
	Quercetin-3-O-(6"-O-manolyl)glucoside	nd	9.63 ± 0.15
	Quercetin-3-O-xylosylglucoside	nd	97.38 ± 4.60
	Kaempferol-3-O-glucoside	nd	1.87 ± 0.03
	Kaempferol-3-O-xylosylglucoside	nd	1.70 ± 0.02

nd: not detected

5.3.3. Antioxidant activity

The effect of soaking and sprouting on the antioxidant activity was evaluated using three different methods, DPPH radical scavenging, ABTS radical scavenging and FRAP. Results are presented in Figure 2A, 2C and 2E and the percentage of gain or loss relative to dry seeds are shown in Figure 2B, 2D and 2F. Regardless the method used, soaking decreased antioxidant activity and sprouting increased it. Antioxidant activity increased with germination time. The percentages of gain in 5 days relative to the dry samples using the three different methods were approximately 46 %, 51 % and 16 % as assessed by DPPH, ABTS, and FRAP, respectively.

Antioxidant activity is closely reflects phenolic content. After continually increasing, 5-day sprouts contained the maximum concentration of phenolics, of which flavonoids and *ortho*-diphenol, which is in agreement with strongest antioxidant activity.

These results are in agreement with a report perform by López-Amorós *et al*., (2006) in 6-day peas, beans and lentils sprouts, where a significant increase in the antioxidant activity together with an increase of several phenolic compounds were also observed over time.

It is known that phenolic structures play a crucial role in the antioxidant activity, particularly, in the number and location of hydroxyl groups in phenolic structures. The *ortho*-diphenols have been recognized as the group of phenolics with highest antioxidant activity (Soufi *et al*., 2014) of which catechin, epicatechin, quercetin and kaempferol belong.

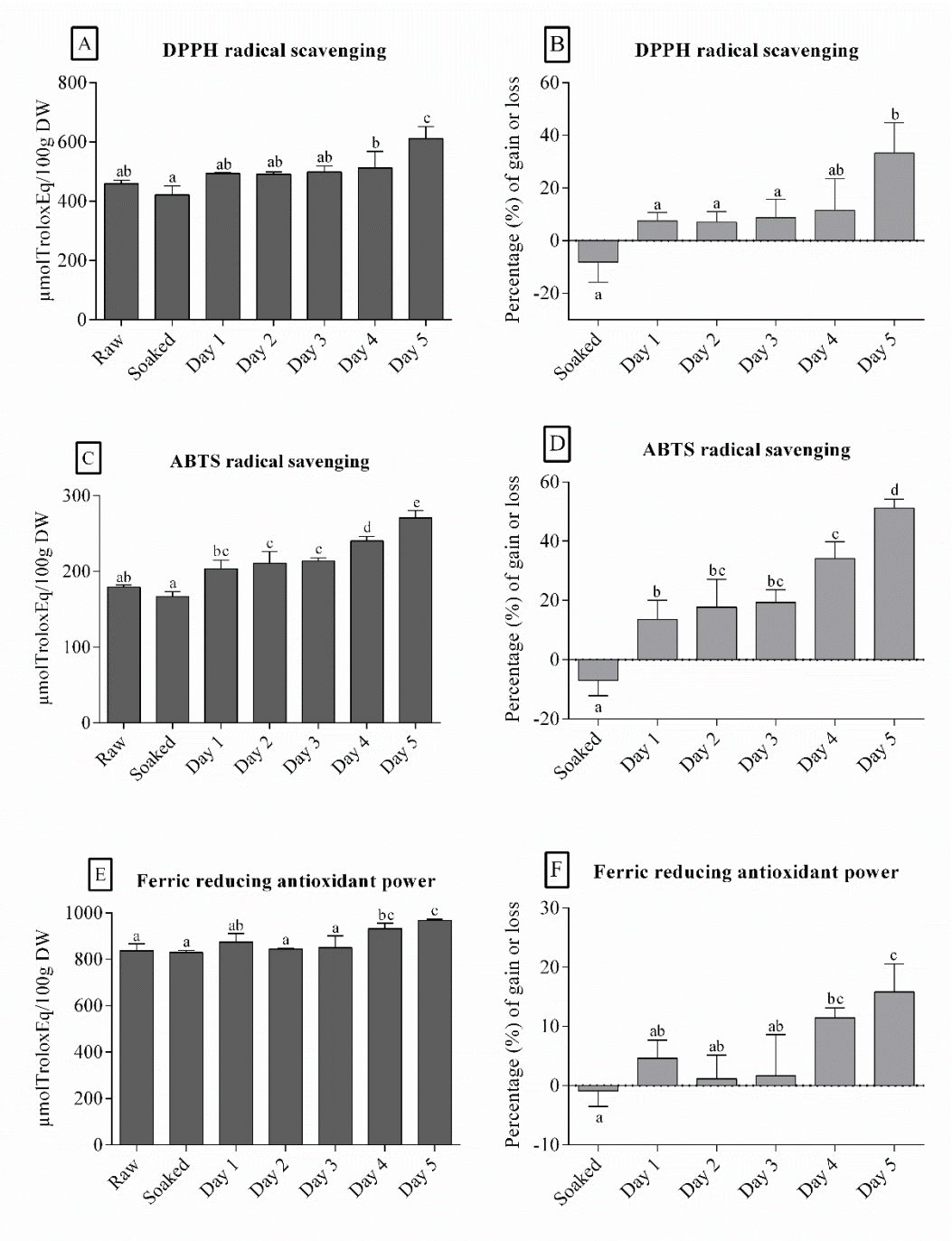

Figure 2. Effect of soaking and sprouting on (A) – DPPH radical scavenging, (C) – ABTS radical scavenging, (E) – ferric reducing antioxidant power. Results are expressed in µmol of Trolox equivalent per 100 g of dry weight (µmol TEq/100g DW). Different letters indicate statistically significant differences ($p<0.05$). Percentage of gain or loss on (B) - DPPH radical scavenging, (D) - ABTS radical scavenging, (E) - Ferric reducing antioxidant power. Values were calculated based on the initial contents in raw beans.

5.3.4. Effects of extracts on cell proliferation

The effects of phenolic extracts of cowpea seeds and or 5-day sprouts on cell viability were tested on five different colorectal carcinoma-derived cell lines (CO115, HCT116, HCT15, RKO and HT29) were used. The effect on cell proliferation after 48 h incubation with extracts was performed using MTT assay and are present in Figure 3. Extracts were applied at a concentration range of 250-2000 µg/ml, exhibiting a dose-response suppression in HCT116, HCT15, CO115 and RKO cancer cell lines. However, a negligible effect was observed in HT29 cells incubated with raw extract.

Sprouts extract were significantly more efficient than seeds extract on the inhibition of cell proliferation/viability in all cell lines representative of multiple molecular profiles of CRC. HT29 were less sensitive than cell the other cell lines to sprouts extract. RKO was the cell line where the difference in sensitivity both extracts was smaller, although sprouts extract still presented higher effects on cell viability.

Effects on cell viability seem to be in line with the phenolic content and antioxidant activity since effects were more pronounced in extracts from 5-day cowpea sprouts, where an increase on TPC, TFC, oDC and antioxidant activity was observed in comparison to raw cowpea seeds. The extract of cowpea sprouts was mainly enriched in quercetin, that had previously show to decrease cell viability in CO115 and HCT15 cells (Xavier *et al*., 2009), HT29 cells (Atashpour *et al*., 2015; Yang *et al*., 2016) and leukemic cells (Srivastava *et al*., 2016). This may explain why sprouts extract were more active than seeds extract.

IC_{50} value is the concentration required to inhibit 50 % of cell proliferation, lower IC_{50} indicates higher antiproliferative effect. Regarding cowpea sprouts extracts, the lowest value of was for CO115 and increased in the following order HCT116, HCT15, RKO and HT29 (Table 3). At 48 h of incubation with raw cowpea extract it was not achieved the IC_{50} value for any cell line tested, indicating its smaller anticancer potential.

The extract from cowpea sprouts was shown to be more efficient in decreasing cell viability when compared to raw cowpea extract. The different degrees of sensitivity to extracts seems to be related to the mutation profilr of the colorectal cancer cell lines used in this study, which explains the difference of IC_{50} obtained.

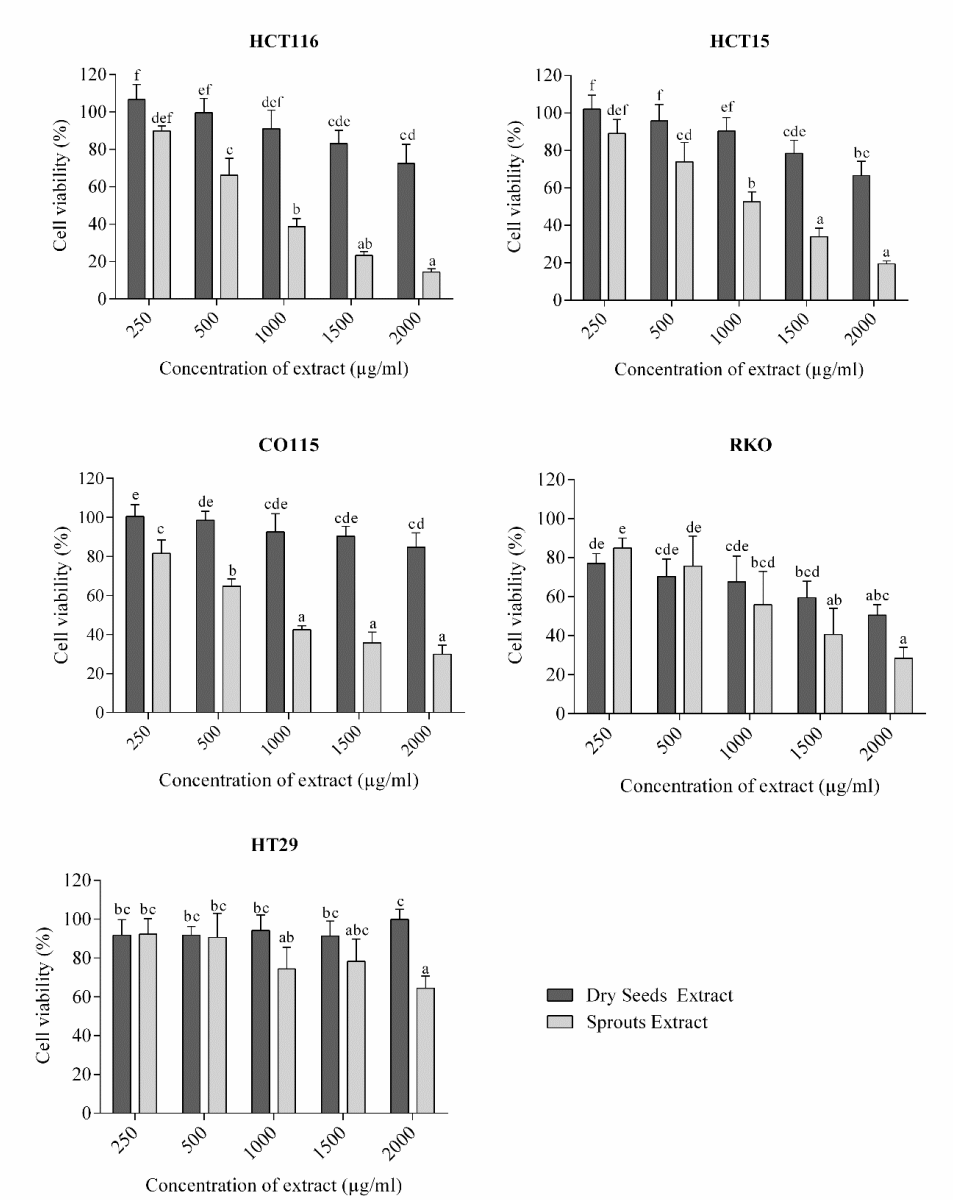

Figure 3. Effect on cell proliferation of 48 h incubation with extracts of dry seeds and 5-day cowpea sprouts in five different colorectal cancer cell lines. Values are expressed as mean and standard deviation of at least three independent experiments. Different letters indicate statistically significant differences ($p<0.05$). 100% of cell viability was taken to be that shown by the cells at 0 µg/ml of extract incubation with.

Table 3. Effect of raw and 5-day cowpea extracts on the proliferation of human colorectal cancer cell lines.

IC_{50} (µg/ml)	**HCT116**	**HCT15**	**CO115**	**RKO**	**HT29**
Raw extract	>2000	>2000	>2000	>2000	>2000
Sprouts extract	730.5±75.8	945.2 ± 97.3	612.6 ± 67.41	1257.3 ± 91.6	1998.9 ± 92.5

IC_{50} half maximal inhibitory concentration. Results are expressed as mean and standard deviation of the µg/ml needs to achieved suppression of cell growth by 50%.

5.3.5. Effects of extracts on cell cycle

In order to further characterize the potential mechanisms by which cowpea extracts are exerting their biological activity, analysis of cell cycle distribution by flow cytometry was performed. Figure 4 shows the relative percentage of HCT116, HCT15, CO115, RKO and HT29 cells in each phase of the cell cycle following treatment of 24 h with the IC_{50} obtained for each cell line.

As seen in Figure 4, raw bean extract did not produce cell cycle arrest in HCT116, HCT15 and HT29. Both cowpea extracts (raw and 5-day sprouts) induced cell cycle arrest at G0-G1 phase in CO115 cells and in G2-M phase in RKO cells. The treatment with the extract of 5-day cowpea sprouts gave rise to an increased fraction of cells in the G2-M phase in HCT116 and S phase in HCT15. In the HT29 cells, the sprouts extract induced an increase in the number of cells in S and G2-M phase however not significantly ($p>0.05$).

The different mutation profiles of the cell lines used in this study show the arrest in different phases of the cell cycle is dependent of the molecular signature of the tumor cells. To our knowledge, effects of cowpea extract or other bean extracts has not been reported *in vitro*. However, this effect has already been demonstrated with quercetin, the major compound present in cowpea sprouts extract. The incubation of HT-29 cells with quercetin led to significant cell cycle arrest in the S phase (Yang *et al*., 2016), and in the G2-M phase (Atashpour *et al*., 2015; Yang *et al*., 2016) in two different studies. Quercetin also induced S phase arrest following 16 h of Nalm6 leukemic cells (Srivastava *et al*., 2016).

Cell cycle arrest differ between cell lines with regard to the phase where the arrest takes place but is reflected on the proliferation. In HT29, where the effects of cowpea sprouts on proliferation were less pronounced, was also the only cell line where effects on cell cycle arrest were not significant.

Cancer is frequently considered a cell cycle disease, due to alterations in different families of the cell cycle regulators, which plays important roles in tumor development. Inhibition of cell cycle progression in cancer cells has been considered one of the most effective strategies to control tumor growth (Otto and Sicinski, 2017). Our data clearly showed that extract from cowpea sprouts can modulate cell growth by cell cycle arrest.

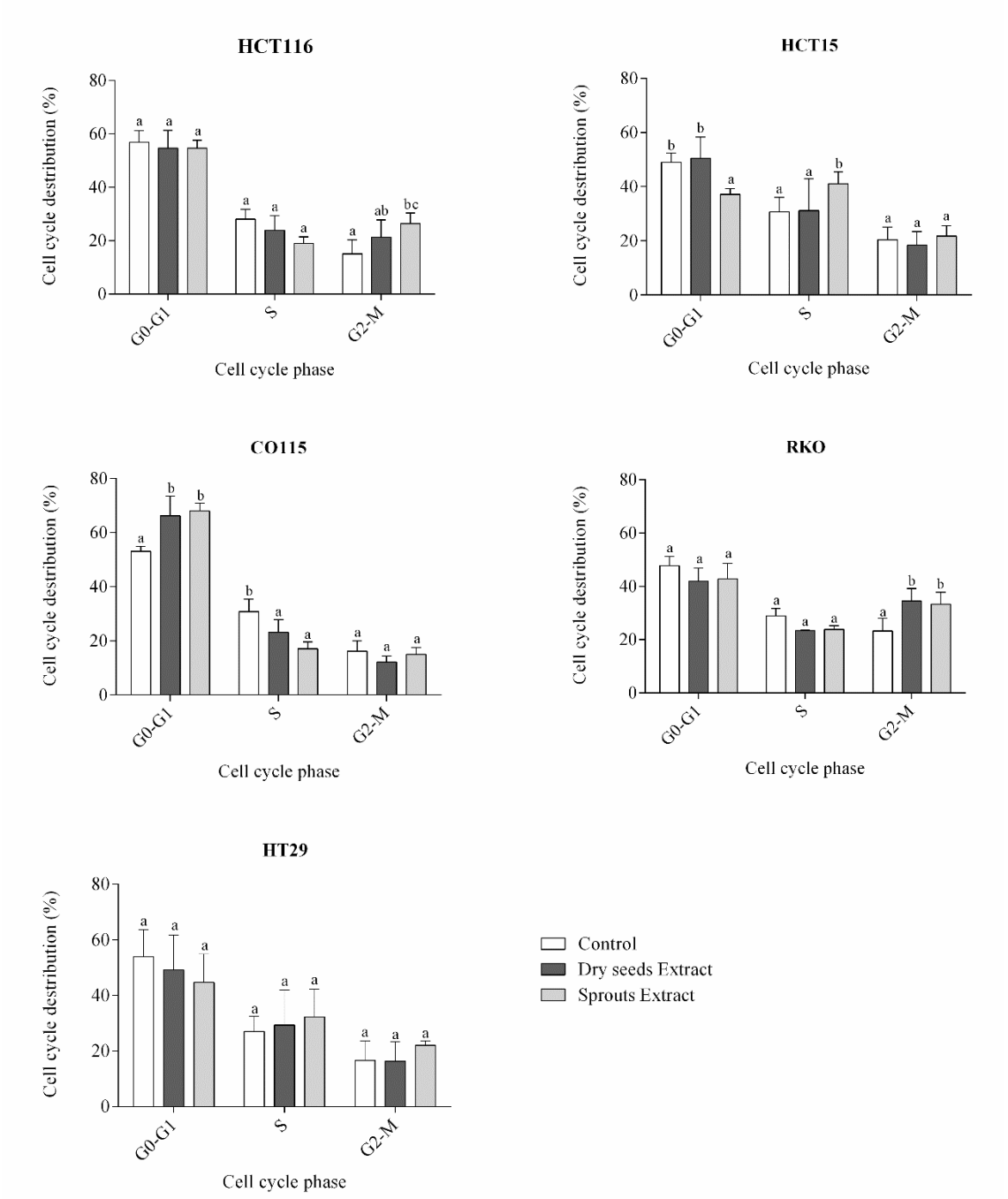

Figure 4. Effect of 24 h incubation with IC_{50} of dry seed and 5-day cowpea sprouts extracts on cell cycle modulation in five human colorectal cancer cells. Values are expressed as means and standard deviations of three independent experiments as percentage. Different letters indicate statistically significant differences ($p<0.05$) in each cell cycle phase.

5.4. Conclusion

A recent trend of healthy lifestyles and lower meat consumption is leading to the increase in popularity of legumes and legumes related foods. The present study shows that germination of cowpea seeds significantly increases the total phenolic (TPC), flavonoid (TFC) and *ortho*-diphenol content (oDC), as well as, antioxidant activity, particularly after 5 days of germination. This work demonstrates the potential of this traditional food to became more attractive to people. The extracts of germinated cowpea were particularly enriched in quercetin. The crude polyphenolic extracts obtained from cowpea sprouts exhibited anti-colorectal cancer potential, including antiproliferative effects and cell cycle arrest. The present study encourages the utilization of cowpea sprouts in the human diet, alone or incorporate in new food products, in order to obtain desirable health benefits.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

- The author Catarina Teixeira-Guedes acknowledges the financial support provided by FCT-Portuguese Foundation for Science and Technology (SFRH/BD/52544/2014), under the Doctoral Program "Agricultural Production Chains – from fork to farm" (PD/00122/2012).

This work was supported by:

- European Investment Funds by FEDER/COMPETE/POCI– Operational Competitiveness and Internacionalization Programme, under Project POCI-01-0145-FEDER-006958 and National Funds by TFCT - Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013.

- Project I&D Interact - Integrative Research in Environment, Agro-Chain and Technology, n.º of operation NORTE-01-0145-FEDER-000017, on the investigation line entitled: Innovation for Sustainable Agro-food Chains - ISAC, co-financed by European Regional Development Fund (FEDER) through NORTE 2020 (Programa Operacional Regional do Norte 2014/2020).

5.5. References

Anand David, A., Arulmoli, R., Parasuraman, S. (2016). Overviews of biological importance of quercetin: A bioactive flavonoid. *Pharmacogn. Rev*. 10, 84. https://doi.org/10.4103/0973-7847.194044

Atashpour, S., Fouladdel, S., Movahhed, T.K., Barzegar, E., Ghahremani, M.H., Ostad, S.N., Azizi, E. (2015). Quercetin induces cell cycle arrest and apoptosis in CD133(+) cancer stem cells of human colorectal HT29 cancer cell line and enhances anticancer effects of doxorubicin. *Iran. J. Basic Med. Sci*. 18, 635–43. https://doi.org/10.22038/IJBMS.2015.4643

Bolanos De La Torre, A.A.S., Henderson, T., Nigam, P.S., Owusu-Apenten, R.K. (2015). A universally calibrated microplate ferric reducing antioxidant power (FRAP) assay for foods and applications to Manuka honey. *Food Chem*. 174, 119–123. https://doi.org/10.1016/j.fooDChem.2014.11.009

Campos-Vega, R., Loarca-Piña, G. (2010). Minor components of pulses and their potential impact on human health. *Food Res. Int*. 43, 461–482. https://doi.org/10.1016/j.foodres.2009.09.004

Chávez-santoscoy, R.A., Lazo-vélez, M.A., Serna-sáldivar, S.O. (2016). Delivery of flavonoids and saponins from black bean (*Phaseolus vulgaris*) Seed Coats Incorporated into Whole Wheat Bread. *Int. J. Mol. Sci*. 17. https://doi.org/10.3390/ijms17020222

Chon, S.U., 2013. Total polypheno bioactivity of seeds and sprouts in several legumes. *Curr. Pharm. Des*. 19, 6112–6124. https://doi.org/10.2174/1381612811319340005

Cruz-Bravo, R.K., Guevara-González, R.G., Ramos-Gómez, M., Oomah, B.D., Wiersma, P., Campos-Vega, R., Loarca-Piña, G. (2014). The fermented non-digestible fraction of common bean (*Phaseolus vulgaris* L.) triggers cell cycle arrest and apoptosis in human colon adenocarcinoma cells. *Genes Nutr*. 9. https://doi.org/10.1007/s12263-013-0359-1

Devi, C.B., Kushwaha, A., Kumar, A. (2015). Sprouting characteristics and associated changes in nutritional composition of cowpea (*Vigna unguiculata*). *J. Food Sci. Technol*. 52, 6821–6827. https://doi.org/10.1007/s13197-015-1832-1

Domínguez-Perles, R., Teixeira, A.I., Rosa, E., Barros, A.I. (2014). Assessment of (poly)phenols in grape (*Vitis vinifera* L.) stems by using food/pharma industry compatible solvents and response surface methodology. *Food Chem*. 164, 339–346. https://doi.org/10.1016/j.fooDChem.2014.05.020

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia*, 77, 67–82. https://doi.org/10.1016/j.fitote.2005.11.008

Eshraq, B., Mona, A., Sayed, A., Emam, A. (2016). Effect of soaking, cooking and germination on chemical constituents and bioactive compounds as well as their cytotoxic activities of black bean extracts. *Nat. Prod. Chem. Res*. 04. https://doi.org/10.4172/2329-6836.1000237

Fernandez-Orozco, R., Piskula, M.K., Zielinski, H., Kozlowska, H., Frias, J., Vidal-Valverde, C. (2006). Germination as a process to improve the antioxidant capacity of *Lupinus angustifolius* L. var. Zapaton. *Eur. Food Res*. Technol. 223, 495–502. https://doi.org/10.1007/s00217-005-0229-1

Gonçalves, A., Goufo, P., Barros, A., Domínguez-Perles, R., Trindade, H., Rosa, E.A.S., Ferreira, L., Rodrigues, M. (2016). Cowpea (*Vigna unguiculata* L. Walp), a renewed multipurpose crop for a more sustainable agri-food system: Nutritional advantages and constraints. *J. Sci. Food Agric*. 96, 2941–2951. https://doi.org/10.1002/jsfa.7644

Guajardo-Flores, D., Serna-Saldívar, S.O., Gutiérrez-Uribe, J.A. (2013). Evaluation of the antioxidant and antiproliferative activities of extracted saponins and flavonols from germinated black beans (*Phaseolus vulgaris* L.). *Food Chem*. 141, 1497–1503. https://doi.org/10.1016/j.fooDChem.2013.04.010

Teixeira-Guedes, C., Oppolzer, D., Barros, A., Pereira-Wilson, C. (2018). Impact of cooking method on phenolic composition and antioxidant potential of four varieties of *Phaseolus vulgaris* L. and *Glycine max* L. *LWT - Food Science and Technology,* Minor revisions for acceptance

Guo, X., Li, T., Tang, K., Liu, R.H. (2012). Effect of germination on phytochemical profiles and antioxidant activity of mung bean sprouts (*Vigna radiata*). *J. Agric. Food Chem.* 60, 11050–11055. https://doi.org/10.1021/jf304443u

Guzmán-Ortiz, F.A., San Martín-Martínez, E., Valverde, M.E., Rodríguez-Aza, Y., Berríos, J.D.J., Mora-Escobedo, R. (2017). Profile analysis and correlation across phenolic compounds, isoflavones and antioxidant capacity during germination of soys (*Glycine max* L.). CyTA - *J. Food* 15, 516–524. https://doi.org/10.1080/19476337.2017.1302995

Hosseinpour-Niazi, S., Mirmiran, P., Hedayati, M., Azizi, F. (2015). Substitution of red meat with legumes in the therapeutic lifestyle change diet based on dietary advice improves cardiometabolic risk factors in overweight type 2 diabetes patients: A cross-over randomized clinical trial. *Eur. J. Clin. Nutr*. 69, 592–597.

https://doi.org/10.1038/ejcn.2014.228

Kalogeropoulos, N., Chiou, A., Ioannou, M., Karathanos, V.T., Hassapidou, M., Andrikopoulos, N.K. (2010). Nutritional evaluation and bioactive microconstituents (phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the Mediterranean countries. *Food Chem*. 121, 682–690. https://doi.org/10.1016/j.fooDChem.2010.01.005

Karapanos, I., Papandreou, A., Skouloudi, M., Makrogianni, D., Fernández, J.A., Rosa, E., Ntatsi, G., Bebeli, P.J., Savvas, D. (2017). Cowpea fresh pods – a new legume for the market: assessment of their quality and dietary characteristics of 37 cowpea accessions grown in southern Europe. *J. Sci. Food Agric*. 97, 4343–4352. https://doi.org/10.1002/jsfa.8418

Khang, D., Dung, T., Elzaawely, A., Xuan, T. (2016). Phenolic profiles and antioxidant activity of germinated legumes. *Foods* 5, 27. https://doi.org/10.3390/foods5020027

Lima, A.I.G., Mota, J., Monteiro, S.A.V.S., Ferreira, R.M.S.B. (2016). Legume seeds and colorectal cancer revisited: Protease inhibitors reduce MMP-9 activity and colon cancer cell migration. *Food Chem*. 197, 30–38. https://doi.org/10.1016/j.fooDChem.2015.10.063

Lin, L., Harnly, J.M., Pastor-corrales, M.S., Luthria, D.L. (2008). The polyphenolic profiles of common bean (*Phaseolus vulgaris* L .). *Food Chemistry,* 107, 399–410. https://doi.org/10.1016/j.fooDChem.2007.08.038

López-Amorós, M.L., Hernández, T., Estrella, I. (2006). Effect of germination on legume phenolic compounds and their antioxidant activity. *J. Food Compos. Anal*. 19, 277–283. https://doi.org/10.1016/j.jfca.2004.06.012

López, A., El-Naggar, T., Dueñas, M., Ortega, T., Estrella, I., Hernández, T., Gómez-Serranillos, M.P., Palomino, O.M., Carretero, M.E. (2013). Effect of cooking and germination on phenolic composition and biological properties of dark beans (*Phaseolus vulgaris* L.). *Food Chem*. 138, 547–555. https://doi.org/10.1016/j.fooDChem.2012.10.107

Luna-Vital, D.A., Liang, K., González de Mejía, E., Loarca-Piña, G. (2016). Dietary peptides from the non-digestible fraction of: *Phaseolus vulgaris* L. decrease angiotensin II-dependent proliferation in HCT116 human colorectal cancer cells through the blockade of the renin-angiotensin system. *Food Funct*. 7. https://doi.org/10.1039/c6fo00093b

Luna Vital, D., González De Mejía, E., Dia, G., Loarca-Piña, D. (2014). Peptides in

common bean fractions inhibit human colorectal cancer cells. *Food Chem*. 157, 347–355. https://doi.org/10.1016/j.fooDChem.2014.02.050

Madhujith, T., Amarowicz, R., Shahidi, F. (2004). Phenolic antioxidants in beans and their effects on inhibition of radical-induced DNA damage. *J. Am. Oil Chem*. Soc. 81, 691–696. https://doi.org/10.1007/s11746-004-963-y

Nderitu, A.M., Dykes, L., Awika, J.M., Minnaar, A., Duodu, K.G. (2013). Phenolic composition and inhibitory effect against oxidative DNA damage of cooked cowpeas as affected by simulated in vitro gastrointestinal digestion. *Food Chem*. 141, 1763–1771. https://doi.org/10.1016/j.fooDChem.2013.05.001

Okada, M., Okada, Y. (2007). Effects of methanolic extracts from broad beans on cellular growth and antioxidant enzyme activity 2, 251–257.

Ombra, M.N., D 'Acierno, A., Nazzaro, F., Riccardi, R., Spigno, P., Zaccardelli, M., Pane, C., Maione, M., Fratianni, F. (2016). Phenolic composition and antioxidant and antiproliferative activities of the extracts of twelve common bean (*Phaseolus vulgaris* L.) endemic ecotypes of southern Italy before and after cooking. *Oxid. Med. Cell. Longev*. 2016, 1–11. https://doi.org/10.1155/2016/1398298

Otto, T., Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. *Nat. Rev. Cancer* 17, 93–115. https://doi.org/10.1038/nrc.2016.138

Phillips, R.D., McWatters, K.H., Chinnan, M.S., Hung, Y.C., Beuchat, L.R., Sefa-Dedeh, S., Sakyi-Dawson, E., Ngoddy, P., Nnanyelugo, D., Enwere, J., Komey, N.S., Liu, K., Mensa-Wilmot, Y., Nnanna, I.A., Okeke, C., Prinyawiwatkul, W., Saalia, F.K. (2003). Utilization of cowpeas for human food. *F. Crop. Res*. 82, 193–213. https://doi.org/10.1016/S0378-4290(03)00038-8

Siah, S.D., Konczak, I., Agboola, S., Wood, J. a., Blanchard, C.L. (2012). In vitro investigations of the potential health benefits of Australian-grown faba beans (*Vicia faba* L.): chemopreventative capacity and inhibitory effects on the angiotensin-converting enzyme, α-glucosidase and lipase. *Br. J. Nutr*. 108, S123–S134. https://doi.org/10.1017/S0007114512000803

Soufi, O., Romero, C., Louaileche, H. (2014). *Ortho*-diphenol profile and antioxidant activity of Algerian black olive cultivars: Effect of dry salting process. *Food Chem*. 157, 504–510. https://doi.org/10.1016/j.fooDChem.2014.02.075

Srivastava, S., Somasagara, R.R., Hegde, M., Nishana, M., Tadi, S.K., Srivastava, M., Choudhary, B., Raghavan, S.C. (2016). Quercetin, a natural flavonoid interacts with DNA, arrests cell cycle and causes tumor regression by activating mitochondrial

pathway of apoptosis. *Sci. Rep*. 6, 1–13. https://doi.org/10.1038/srep24049

Tarzi, B.G., Gharachorloo, M., Baharinia, M., Mortazavi, A., (2012). The effect of Germination on phenolic content and antioxidant activity of chickpea. *Iran. J. Pharm. Res*. 11, 1137–1143.

Xavier, C.P.R., Lima, C.F., Preto, A., Seruca, R., Fernandes-Ferreira, M., Pereira-Wilson, C. (2009). Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells. *Cancer Lett*. 281, 162–170. https://doi.org/10.1016/j.canlet.2009.02.041

Yang, L., Liu, Y., Wang, M., Qian, Y., Dong, X., Gu, H., Wang, H., Guo, S., Hisamitsu, T. (2016). Quercetin-induced apoptosis of HT29 colon cancer cells via inhibition of the Akt-CSN6-Myc signaling axis. *Mol. Med. Rep*. 14, 4559–4566. https://doi.org/10.3892/mmr.2016.5818

Zou, Y., Chang Sam, K. (2014). Antioxidant and antiproliferative properties of extract and fractions from small red bean (*Phaseolus vulgaris* L .). *J Food Nutr*, 1, 1–11.

CHAPTER 6

Effects of cowpea sprout extract in cell proliferation, apoptosis and enhancement of 5-FU sensitivity using several colorectal cancer cell lines

This chapter enclose the following manuscript:

Teixeira-Guedes, C.I. & Pereira-Wilson, C. (2018). Phenolic rich extracts from cowpea sprouts decrease cell proliferation and enhance of 5-fluorouracil effects in human colorectal cancer cell lines. Submitted

Phenolic rich extracts from cowpea sprouts decrease cell proliferation and enhance of 5-fluorouracil effects in human colorectal cancer cell lines

Catarina I. Teixeira-Guedes[ab]*, Cristina Pereira-Wilson[abc]

[a]*Department of Biology, University of Minho, Braga, Portugal*

[b]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Minho, Braga, Portugal*

[c]*Centre of Biological Engineering, University of Minho, Braga, Portugal*

Abstract

It is known that diets high in legumes can help in the prevention and/or reduction of colorectal cancer. These effects have been attributed in part with their high phenolic content. The aim of this study was to investigate the effect of sprouting in phenolic content, antioxidant potential, anti-colorectal cancer potential and explore the effect of potentiating the 5-fluorouracil efficacy. Sprouting increased 1.7-fold the total phenolic content and 2.8-fold the antioxidant activity when compared to raw cowpea seeds. The analysis of phenolic profile showed an enrichment in quercetin of cowpea sprouts. Extracts from raw or sprouts displayed a dose-response effect against all cell lines used. Effects on cell viability were more pronounced for HCT116 and HCT15 for sprout extract. A significant induction of apoptosis was observed in HCT116, HCT15, RKO and HT29 cells when incubated with cowpea sprout extract. The combination of extracts of cowpea sprouts with 5-fluorouracil induced a decrease of cell viability especially to the cells more resident to 5-fluorouracil treatment (RKO and HT29). Overall, spouting increased the health-promoting properties of cowpea, with regard to phenolics and antioxidant activity. In addition, also increased the anti-colorectal cancer potential and potentiate the 5-fluorouracil efficacy in the initially most cells resistant.

Keywords: Cowpea, sprouts, colorectal cancer, 5-fluoroacil

6.1. Introduction

Colorectal cancer (CRC) is one of the major causes of cancer-related mobility and mortality worldwide. It is the third most commonly occurring cancer in men (approximately 10 % of total cancer cases) and the second in women (9 % of total cancer cases) (Kuipers *et al*., 2015; World Cancer Research Fund, 2017). The highest estimated rates in Australia, New Zealand, Canada, USA and parts of Europe and the lowest in Western Africa, China, India and South America, and it is projected to increase in the future.

Both genetic and environmental factors play an important role in the etiology of CRC. Only, 5 to 10% of all CRC case are due to inherited genes defects, a hereditary colorectal cancer syndrome, being the most common Lynch syndrome and the familial adenomatous polyposis (FAP). The risk is increased by chronic colitis due to inflammatory bowel disease (Capelle *et al*., 2010; Vasen, Tomlinson *et al*., 2015). The development of CRC seems to be largely influenced by lifestyle risk factors namely, alcohol intake, increased body weight, type 2 diabetes mellitus, and dietary habits including high intake of red meat and processed meat (Doubeni *et al*., 2012; Marley & Nan, 2016; World Cancer Research Fund International, 2015). By contrast, consumption of milk, whole grains, fruits, vegetables, legumes resulting in a high intake of calcium, fiber, vitamins, such us, folate and vitamin D and decreasing the risk. These diets are also high in phenolic compounds (Ceter, Jemal, Smith, & Ward, 2009; Haggar & Boushey, 2009; World Cancer Research Fund, 2017).

The most common alterations seen in colorectal cancer include those genes affecting proliferative and apoptotic signaling as well as resistance to chemotherapy, these genes are KRAS, BRAF, PI3KA, TP53 and the MMR system (Bracht, Nicholls, Liu, & Bodmer, 2010; Grady & Pritchard, 2014; Kuipers *et al*., 2015).

Current treatment of CRC is based on surgery and chemotherapy. The most commonly used chemotherapeutic agent in advance CRC is 5-fluorouracil (5-FU) alone or in combination with oxaliplatin (Rejhová *et al*., 2018). However, 5-FU is associated with several adverse effects and 10 to15% of advanced CRC cases show limited response to the drug (Pardini *et al*., 2011; Rejhová *et al*., 2018).

Recent evidence also suggests that diets high in legumes can help in the prevention and/or reduction of chronic diseases including colorectal cancer (Hosseinpour-Niaz *et al*., 2015; Luna-Vital *et al*., 2016). The protective effect of legume consumption has been associated with the presence dietary fiber (soluble or insoluble) and phenolic compounds

(phenolic acids, flavonoids, tannins and anthocyanins) (Cruz-Bravo *et al*., 2014). Cowpea, a drought resistant legume, is an excellent food ingredient containing high amounts of protein, carbohydrates (including dietary fiber and resistant starches) and it is low in fat. It is also a rich source of vitamins, minerals and phenolic compounds (Ayog *et al*., 2016; Gonçalves *et al*., 2016; Pereira *et al*., 2014).

The cowpea consumption has been decreasing due to the growing influence of western lifestyle. In spite of this, awareness of the impact of diet in human health has however also increased creating the opportunity for the development of new added value to traditional foods.

To our knowledge, studies on the effect of sprouting of cowpea seeds on the phytochemical composition and health-promotion potential with regard to colorectal cancer effects are limited. The objectives of this work were to 1) study the effects of germination on polyphenols and the antioxidant activity of extracts compared to those obtained from raw seeds; 2) characterize their anti-colorectal cancer effects using a panel of human colorectal cancer cell lines displaying molecular profile representatives of most common CRC molecular signatures; 3) investigate the possible therapeutic enhancement of 5-FU efficiency (in those cells) when used in combination.

6.2. Material and methods

6.2.1. Chemical and equipment

The reagents of acetic acid, formic acid, acetonitrile, methanol and ethanol were purchased from Merck (Germany). Sodium hydrochloride, sodium carbonate, sodium citrate, triton X-100, gallic acid, Folin–Ciocalteau, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetremethychroman-2-carboxylic acid (Trolox), gallic acid, dimethyl sulfite (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT), paraformaldehyde, Hoechst dyes, cell culture medium (RPMI-1640 and DMEM), fetal bovine serum (FBS), antibiotic-antimycotic solution, quercetin and staurosporine were purchased from Sigma-Aldrich (USA). Filters of 11 µm pore size from Whatman No. 1 paper (UK), cell culture polystyrene flasks surface area 25 cm^2 from TPP (Germany), 96-well plates from Frilabo (Portugal) and poly-L-lysine coated slide from VWR (Portugal) were used. The equipment used were a germination chamber (Idiamart, Germany), orbital shaker 501 (Bibby Stuart, UK), rotatory evaporator (BÜCHI 461 water bath REIII, Germany), freeze dryer (Christ alpha 2-4 Martin Christ, Germany), microplate reader (Infinite M200 microplate reader, Tecan, Austria), high performance liquid

chromatography (HPLC) system with diode array detector (DAD) (Thermo Fisher Scientific, Inc., USA), ACE 5 C18 column (Advanced Chromatography Technologies Ltd, Scotland), CO_2 incubator (Binder C150, Germany), cytospin 4 cyto centrifuge (Thermo-Scientific, Germany) and fluorescence microscope (Leica DM5000B, Germany). TUNEL assay was performed using an *in situ* cell death detection kit, fluorescein (Roche, Germany).

6.2.2. Germination protocol

Vigna unguiculata (cowpea) was used in this work. Seeds were purchased dried from a Portuguese market. Seeds were sterilized with 0.07 % of sodium hypochlorite solution for 30 min, then washed with distilled water until neutral pH. Prior to germination, seeds were soaked for 12 h with bottled water, at a ratio of 1:5 (w/v). The soaked seeds were germinated in Petri dishes in a germination chamber at 23 ºC and 90 % of humidity for 5 days. Dry seed and sprouts were freeze-dried and milled into powder, passed through a 0.5 mm sieve and kept at -20°C until extraction.

6.2.3. Phenolic extraction

The phenolic extraction was carried out in methanol:water (80:20, v/v) at a solid to solvent ratio of 1:10. The extractions were ultrasounds assisted for 10 minutes and maintained for 1 hours in orbital shaker at room temperature in dark. The extracts were vacuum filtrated through Whatman No. 1 paper. The extraction procedure was repeated 3 times. Methanol was removed using a rotatory evaporated and then freeze-dried. The powder was stored in dark vials at -20 °C before using. To apply in cell culture, DMSO was used as solvent.

6.2.3. Total phenolic content

The total phenolic content (TFC) was determined by the Folin–Ciocalteau method (Domínguez-Perles *et al*., 2014; Teixeira-Guedes, Oppolze *et al*., 2018). In each well, 20 µL of sample or standard were added, followed by 100 µL of Folin-Ciocalteau:H20 (1:10, v:v) and 80 µL of sodium carbonate (7.5 %). The plate was incubated for 30 min at 42 ºC in the dark and the absorbance was measured at 750 nm in a microplate reader. Results were calculated using a calibration curve prepared with gallic acid and expressed as mg of gallic acid equivalents per g of extract (mg GAEq/g Ex).

6.2.4. DPPH radical scavenging

DPPH radical scavenging was carried out as previously reported by Domínguez-Perles *et al*., (2014) and Teixeira-Guedes *et al*., (2018) and optimized for microplates. A solution of 8.87 mM of DPPH in methanol/H2O (70:30, v/v) was prepared and then diluted to achieve an absorbance closer to 1.000 at 520 nm. To each well, 10 µL of sample or standard were added, followed by 190 µL of DPPH solution. The mixture was incubated at room temperature in the dark for 15 min and then the absorbance was measured at 520 nm in a microplate reader. The percentage (%) of DPPH radical scavenging was calculated with the following formula: % inhibition = (Abs Blank-Abs Sample) / (Abs Blank) x 100, where Abs Blank is the control reaction and Abs Sample is the absorbance of the test extract or standard. Results were calculated using a calibration curve prepared with Trolox and expressed as µM Trolox equivalent per g of extract (µM TEq/g EX).

6.2.5. High performance liquid chromatography

The phenolic characterization was carried according to Lin *et al*., (2008). In this work was used a HPLC system with a diode array detector (DAD). The injection volume was 20 µL and the separation was performed in an ACE 5 C18 column (maintained at 25 ºC) at a flow rate of 1ml/min. The mobile phase A consisted in water-formic acid (99.9:0.1; v/v) and phase B acetonitrile-formic acid (99.9:0.1; v/v). The chromatograms were analyzed using the qualitative software of Xcalibur (Thermo-Fisher Scientific, Inc., Waltham, USA).

6.2.6. Cell culture

Four human colorectal cancer cell lines were used in this experiment, HCT116, HCT15, RKO and HT29. The cell lines were maintained in T25 cm^2 polystyrene flacks with RPMI-1640 medium for HCT116, HCT15 and DMEM for RKO and HT29. All mediums were supplemented with 10 % of fetal bovine serum, 1 % antibiotic-antimitotic solution. Cells were maintained in an incubator at 37 °C in a humidified atmosphere with 5 % CO_2. For all experiments, HCT116, RKO cells were seeded at a density of 1 x 10^5 cell/ml, HCT15 at 0,75 x 10^5 cell/ml and HT29 at 1,2 x 10^5 cell/ml.

6.2.7. Assessment of effect of extracts on cell viability by MTT reduction test

The effects on cell viability of incubation with the polyphenolic rich extracts from raw and cowpea sprouts were performed using MTT reduction assay. Briefly, cells were plated for 24 h and then incubated with extracts at concentration ranging from 0.25 to 2.00 mg/ml

for 72 h. At the end of the incubation time, MTT substrate was added to the cells at a final concertation of 0.5 mg/ml and incubated for 2 h. The formazan formed was dissolved in ethanol:DMSO (1:1) and then measured at 570 nm using a microplate reader. The color of each condition is proportional to the number of viable cells in comparison with control.

6.2.8. Assessment of extracts on cell apoptosis by TUNEL assay

Cells were plated for 24 h and then treated with 2.00 mg/ml for 72 h. Staurosporine (2.5 µM) was used as positive control for apoptosis induction. At the end of incubation time, both floating and attached cells were collected and fixed for 15 minutes with 4 % of paraformaldehyde at room temperature. Cells were then attached into poly-L-lysine coated slide using a cytospin, washed with PBS and permeabilized with 0.1 % of triton X-100 in 0.1 % of sodium citrate on ice for 2 min. TUNEL assay was performed using the *in situ* cell death detection kit following the manufacturer instructions. For nuclei staining, cells were incubated with Hoechst. The percentage of apoptotic cells was calculated by the ratio of TUNEL positive cells by the total number of cells stained with Hoechst. At least 500 cells per condition were counting using in a fluorescence microscope.

6.2.9. Assessment of combined effect of 5-FU with tested extracts on cell viability

To investigate the effect of combination of 5-FU with extracts on cell viability, MTT reduction assay was used. All cell lines were plated for 24 h and then treated with 5-FU, extracts and quercetin alone or in combination (co-incubation). The doses of 5-FU used were 2.5 µM for HCT116 and HCT15 and 10 µM for RKO and HT29. The concentration of extracts used was 0.25 mg/ml and quercetin was 5 µM (approximately the concentration of quercetin in the sprout extract).

6.2.10. Statistical analysis

All results are present as mean ± SD from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 6 software (San Diego, CA, USA). Differences between samples were tested using analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests, $p<0.05$ was considered as being statistically significant.

6.3. Results

6.3.1. Total phenolic content, antioxidant activity and phenolic profile of extracts

The TPC and antioxidant activity of extracts from raw and cowpea sprouts are presented in Table 1. TPC in extracts increased significantly after sprouting, corresponding to approximately 1.7-fold higher than the original cowpea seeds extracts.

Using the DPPH radical scavenging method, sprouting increased antioxidant capacity around 2.8-fold, from 413,22 µmol TEq/g of extracts of raw to 1136.30 µmol TEq/g in extracts of cowpea sprouts.

The analysis of the phenolic profile is shown in Table 2. Quantitative and qualitative differences between the phenolic composition of raw and sprouts extracts were observed. Globally, a significant increase on the identified phenolic compounds was observed in sprouts, 2.1-fold higher than in raw extracts. A 1.7-fold increase in phenolic acids and a 2.2-fold increase in flavonoids were observed. In raw samples, the composition of phenolic compound was found to be highest in flavonoids such as catechin, epicatechin, followed by the gallic, chlorogenic and ferulic acid. Caftaric and caffeic acids were found in sprouts but not in dry seed extracts. Sprouting enriched the extracts in flavonoids such as quercetin glucosides and kaempferol glucosides, both of which were not detected in raw extracts. Quercetin glucosides in sprouts extracts represented approximately 58 % of flavonoids and 47 % of total identified phenolic compounds.

Table 1. Effect of germination on total phenolic content (TPC) and antioxidant activity assessed by DPPH on raw and cowpea sprouts.

	Total phenolic content	**DPPH radical scavenging**
Raw Extract (RE)	256.95 ± 1.23	413,22 ± 24.95
Sprouts Extract (SE)	438.52 ± 5.48	1136.30 ± 108.75

Data presented as mean and SD from three replicates, n=3. Total phenolic content – mg gallic acid equivalents per gram of extract (mg GAEq g^{-1} Ex). DPPH radical scavenging – µmol Trolox equivalents per gram of extract (µmol TEq g^{-1} Ex).

Table 2. Phytochemical profile of crude methanolic extracts from raw and 5-day sprouts of cowpea.

	Phenolic compounds (mg g^{-1} Ex.)	Raw Extract (RE)	Sprouts Extract (SE)
Phenolic acids	Gallic acid	0.92 ± 0.09	1.02 ± 0.02
	Caftaric acid	nd	0.32 ± 0.02
	Chlorogenic acid	0.42 ± 0.01	0.39 ± 0.02
	Caffeic acid	nd	0.59 ± 0.01
	Ferulic acid	0.09 ± 0.00	0.10 ± 0.00
Flavonoids	Catechin	2.57 ± 0.08	2.62 ± 0.02
	Epicatechin	2.21 ± 0.07	nd
	Quercetin glucosides	nd	7.71 ± 0.39
	Kaempferol glucosides	nd	0.23 ± 0.01

Data presented as mean and SD from two HPLC injections. Result are expressed in mg per gram of extract (mg g^{-1} Ex.). nd: not detected.

6.3.2. Effect of cowpea extracts on colorectal cancer cell proliferation

To test the effects of extracts from cowpea seeds and sprouts on cell viability, four different carcinoma-derived cell lines with different mutation status (HCT116, HCT15, RKO and HT29) were used (Table 3). The effect on cell proliferation after 72 h of incubation with extracts was measured using the MTT assay and results are presented in Figure 1. Cowpea extracts from raw seeds and sprouts were applied in a concentration range of 0.25 - 2.00 mg/ml, exhibiting a dose-response suppression in all colorectal cancer cell lines used. Extract from raw seeds not achieved IC50 for any cell line. Effects on cell viability were more pronounced for HCT116 and HCT15 for sprout extract. The lowest value of IC_{50} was for HCT116 (0.70 ± 0.08) followed by HCT15 (0.95 ± 0.08) (Table 4). The IC_{50} values for sprout extracts in RKO and HT29 were higher than 2.00 mg/ml.

Table 3. Colon cancer cell lines classified by mutation status of critical genes involved in colorectal cancer.

Cell line	KRAS	BRAF	PI3KCA	TP53
HCT116	G13D	WT	H1047R	WT
HCT15	G13D	WT	E545K/D549N	S241F
RKO	WT	V600E	H1047R	WT
HT29	WT	V600E	P449T	R273H

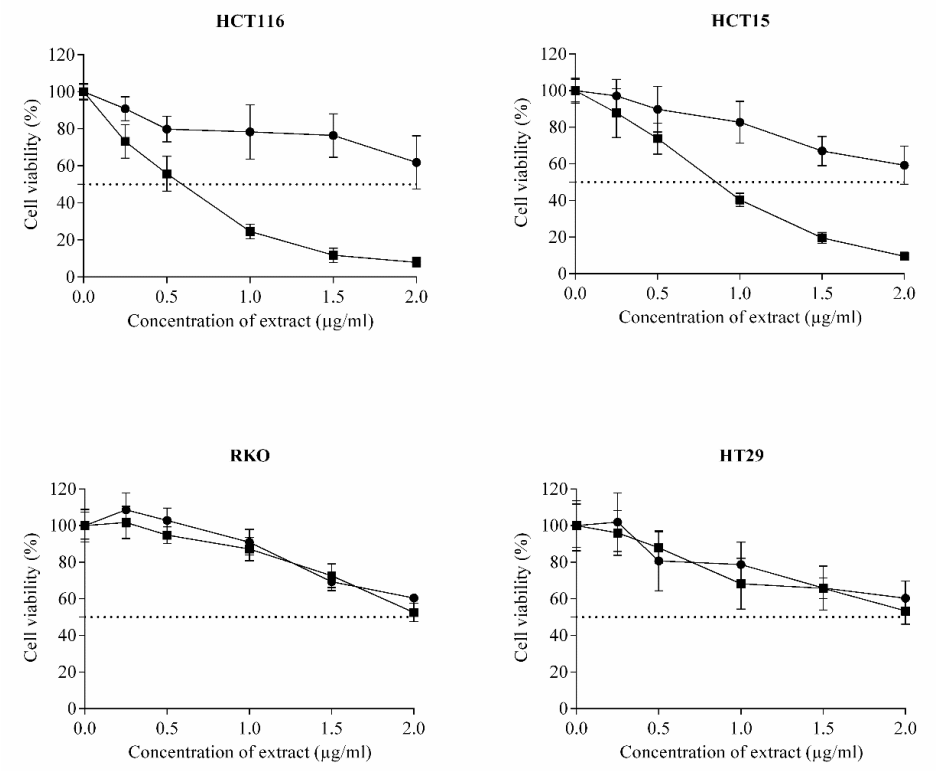

Figure 1. Effect on cell viability of raw extract (RE) and sprouts extract (SE) of cowpea in five different colorectal cancer cell lines. Values are expressed as mean and standard deviation of at least three independent experiments.

Table 4. Half-maximal inhibitory concentration (IC_{50}) of raw and cowpea sprouts extracts on proliferation in four human colorectal cancer cell lines.

IC_{50}	Raw Extract (RE)	Sprouts extract (SE)
HCT116	> 2.00	0.70 ± 0.08
HCT15	> 2.00	0.95 ± 0.08
RKO	> 2.00	> 2.00
HT29	> 2.00	> 2.00

Results are expressed as mean and standard deviation of the mg/ml needs to achieved suppression of cell growth by 50 %.

6.3.3. Effects on apoptosis induction

The TUNEL assay was carried out with the different cell lines to identify apoptotic cells after 72h of incubation with 2.00 mg/ml of extracts from raw seeds and sprouts (Figure 2). A significant induction of apoptosis was observed in HCT116 (53.76 %), HCT15 (46.62 %), RKO (34.64 %) and HT29 (30.17 %) cells when incubated with cowpea sprouts extract. No significant induction of apoptosis was observed in any cell line after 72h of incubation with raw cowpea extract excepting for HCT116.

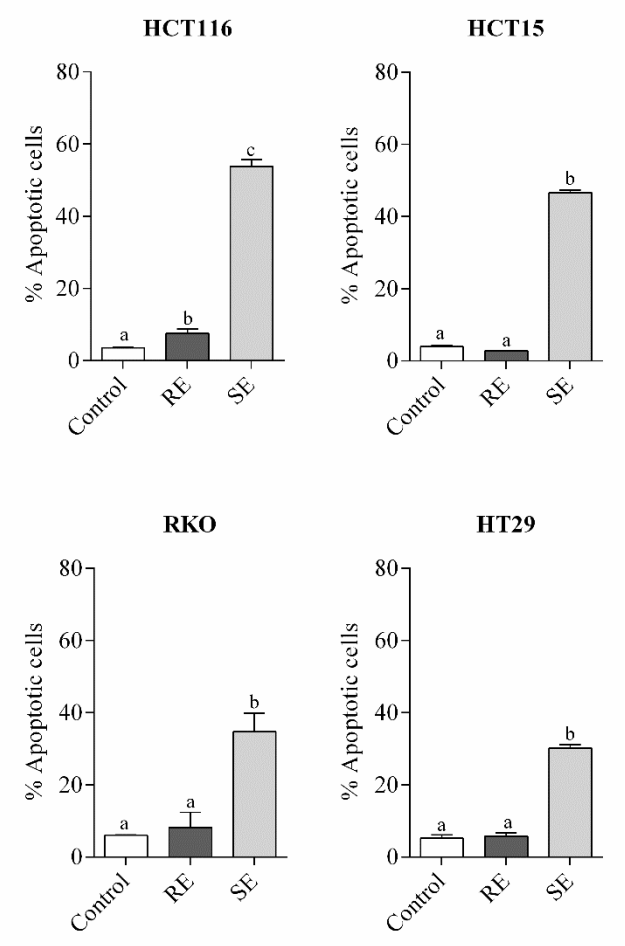

Figure 2. Effect of incubation with 2.0 mg/ml of raw extract (RE) and sprouts extract (SE) of cowpea on cell apoptosis using TUNEL assay. Values are present as mean and standard deviation of three independent experiments. Mean values with unlike letters indicate statistically significant differences (p<0.05).

6.3.4. Enhancement of 5-FU efficacy

In order to understand if a diet rich in cowpea sprouts could have any enhancing effects on the sensitivity to the drug when co-incubated, cell viability was monitored by MTT assay in cells exposed to the extracts and 5-FU. A dose of extract (0.25 mg/ml) with no toxicity was used in combination with the 5-FU drug at a concentration of 2.5 µM for HCT116 and HCT15 or 10 µM for RKO and HT29, according to their sensitivity to the drug. Quercetin was used in the concentration present in the extracts in order to know if the results were due to enrichment in this compound. Overall, the combination of extracts or quercetin with 5-FU showed an additive effect on the decrease of cell viability. Combination of raw extract with 5-FU had significant effects only in HT29. The combination of cowpea sprout extract with 5-FU induced a significant decrease of cell viability on the HCT116, RKO and HT29 cell lines when compared to 5-FU alone. The

effect was more pronounced in RKO and HT29 cells. Quercetin in combination with 5-FU demonstrated significant additive effect only in HT29 cells.

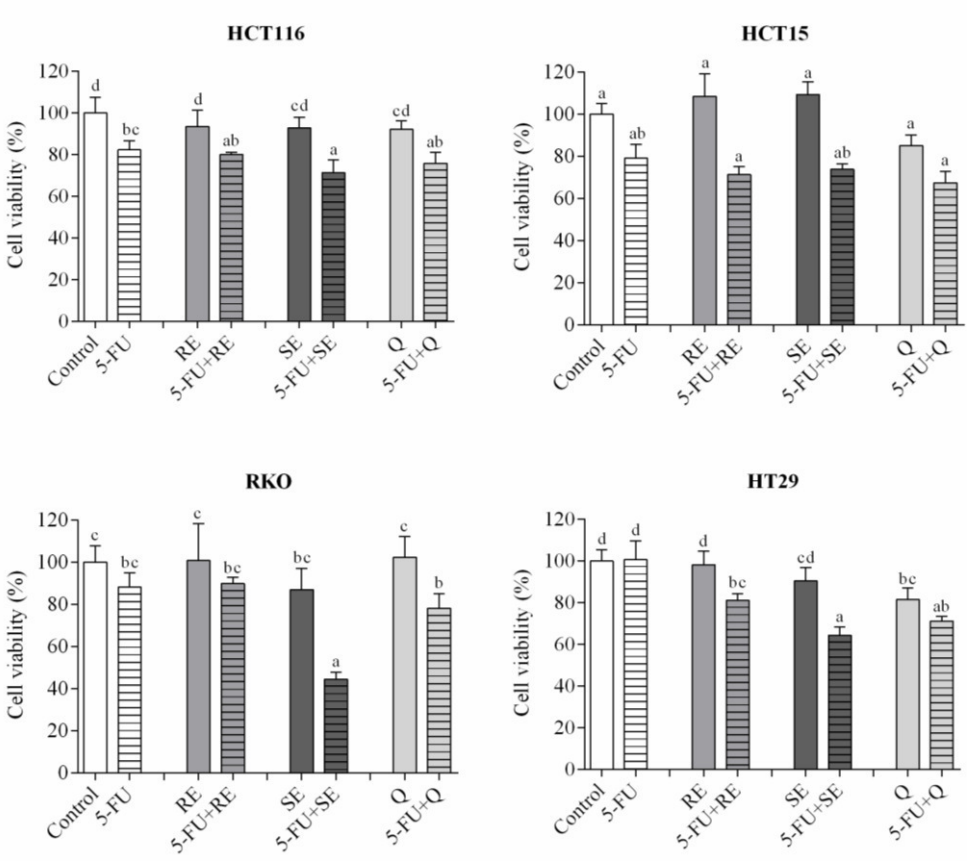

Figure 3. Effects on cell viability of raw extract (RE) and sprouts extract (SE) from cowpea (0.25 mg/ml), Quercetin (Q) (5 µM) and 5-flourouacil (5-FU) (2.5 µM for HCT116 and HCT15 and 10 µM for RKO and HT29) alone, or in combination. Values are present as mean and standard deviation of three independent experiments. Mean values with unlike letters indicate statistically significant differences (p<0.05).

6.4. Discussion

The current study showed that sprouting increased significantly the TFC and antioxidant activity in extracts from cowpea sprouts in comparison with raw seeds, which is in agreement with results with other legume sprouts in back bean (*Phaseolus vulgaris*) (Guajardo-Flores *et al*., 2013; López-Amorós *et al*., 2006), mung bean (*Vigna radiata*) (Guo *et al*., 2012) and chickpea (*Cicer arietinum*) (Tarzi, *et al*., 2012).

The increase in the antioxidant activity correlates with the higher phenolic content observed in extracts of cowpea sprouts when compared to raw cowpea seed extract. Also in studies with peas, beans and lentils sprouts (López-Amorós *et al*., 2006) and soy bean

sprouts (Guzmán-Ortiz *et al*., 2017) a significant increase in the antioxidant activity together with an increase of several phenolic compounds was observed. The increase in quercetin glucosides was very significant in extracts of cowpea sprouts, which is in agreement with reports for sprouts of black (*Phaseolus vulgaris*) (López-Amorós *et al*., 2006) and soy beans (*Glycine max*) (Guzmán-Ortiz *et al*., 2017).

In our study we explored the antiproliferative effect of extracts in different cell lines with different representative molecular signatures of the most common types of CRC. The extracts were applied at different concentrations and exhibited a dose-dependent suppression of cell viability in all tested human CRC cell lines. The decrease in cell viability was more pronounced for extracts of cowpea sprouts in comparison with raw seed extracts, especially in HCT116 and HCT15. In the RKO and HT29, both extracts induce similar suppression of cell viability.

The effects in cell viability is in line with the higher phenolic content/composition and antioxidant activity. Extracts of cowpea sprouts was mainly enriched in quercetin, to which is known to decrease of cell viability in CO115 and HCT15 cells (Xavier *et al*., 2009), HT29 cells (Atashpour *et al*., 2015; Yang *et al*., 2016) and leukemic cells (Srivastava *et al*., 2016).

The different mutation status of the colorectal cancer cell lines used in this study allows the demonstration that different molecular profiles respond with varying levels of sensitivity to the phenolic compound enriched sprout extracts, suggesting that there may be patients who would benefice more than others from the anti-CRC effects of cowpea sprouts extracts.

As previously mentioned, KRAS, PIK3CA, BRAF and TP53 are some of the genes most frequently mutated in CRC (Bracht *et al*., 2010). Mutations in PI3KCA lead to activation of AKT and of numerous effectors that regulate critical cellular functions, namely promoting cell growth and proliferation. The KRAS oncogene is mutated in approximately 35%-45% of colorectal cancers (Tan & Du, 2012), and regulates both MAPK/ERK and PI3K pathways. On the other hand, the BRAF oncogene mutation (Ahmed *et al*., 2013) is present in about 10% of CRC patients and leads to a constitutive activation of MAPK/ERK signaling pathway down stream of KAS, promoting cell proliferation (Barras, 2015). TP53 is a tumor suppressor gene frequently dysregulated in CRC. Patients with mutant p53 gene are often resistant to chemotherapy, conferring poor prognosis (Li *et al*., 2015).

The panel of cell lines used are representative of molecular signatures of CRC. Since the cells used harbor a PI3K activating mutation, effects on the MAPK pathway through KRAS and BRAF targets are possible to infer.

HCT116 and HCT15 cells (expressing PI3K and KRAS mutation) showed higher sensitivity to cowpea sprout extract than RKO and HT29 (with PI3K and BRAF mutation). In cells harboring KRAS mutation, the extract seems to modulate KRAS signaling with effects on both MAPK/ERK and PI3K pathways. RKO and HT29 that harbor BRAF and MAPK/ERK is activated downstream of KRAS which may explain the lower response in the two cell lines. It has been previously reported that treatment of CRC cells with quercetin reduced RAS protein levels and decreased significantly the MAPK/ERK signaling (Xavier *et al*., 2009). These finding are consistent with the higher resistance displayed by BRAF mutation RKO and HT29 (having a downstream activation of MAPK pathway). The p53 protein also plays an important role in the sensitivity to extract since greater toxicity was observed in cells expressing p53, HCT116 in comparison to HCT115 and HT29 that harbor p53 mutations.

Apoptosis is a natural cell death process which cancer cells are able to avoid, continuing to survive and further proliferate. Induction of apoptosis in cancer cells is the preferred way to remove cancer cells from the human body, which is the major goal of chemotherapy treatment (Siah *et al*., 2012).

In order to understand the mechanism behind the suppression of cancer cell proliferation, an investigation to identify apoptotic cell within the population treated with the different extract was carried out. The TUNEL assay showed a greater increased in the induction of apoptosis in cell treated with the extract of cowpea sprouts, especially in HCT116 and HCT15. In the RKO and HT29, these increased was less pronounced however significant. Induction of apoptosis in the preferred way to remove cancer cell from the body. It is known that food compound namely, phenolic compounds are able to induce apoptosis in cancer cells and might contribute to cancer prevention and treatment and is particularly important as a mechanism of action of chemotherapeutic drugs. Quercetin is the major phenolic present in the extract of cowpea sprouts. Previous studies reported a significant induction of apoptosis in HCT15 and CO115 cells (Xavier *et al*., 2009; Xavier *et al*., 2011) and HT29 cells (Atashpour *et al*., 2015) when incubated with quercetin.

5-FU is the pharmacological drug most used in CRC chemotherapy, however, the resistance to this drug remains a concern (Pardini *et al*., 2011; Rejhová *et al*., 2018).

Strategies that enhance 5-FU efficacy by the combination of treatment with other compounds are therefore important. The concentration of 5-FU used to induce a 20 % decrease of cell viability was higher (10µM) in RKO and HT29 cells that harbor BRAF mutation compared with KRAS mutated cell lines HCT11 6 and HCT15 (that were more sensitivity to 5-FU). The effect of extracts on cell viability to 5-FU was more pronounced for sprouts extracts than extracts of raw cowpea except for HCT115 cells (where they were similar). Increased sensitivity to 5-FU by sprout extract was particularly high in the BRAF mutant cell lines (RKO and HT29) that were originally more resistant to the drug. In order to understand if the results were due to the enrichment of extract in quercetin, the co-incubation of 5-Fu with quercetin was also studied. Only HT29 presented a significant sensitivity to 5-FU in combination with quercetin. For these cells, the incubation with quercetin and 5-FU seems to be Based on this observation, the effects of extracts combined with 5-FU was not only due to the presence of quercetin but a synergic effect between the phenolic compounds present in the extract.

6.5. Conclusion

The phenolic cowpea extracts obtained from raw and 5-day sprouts exhibited potential health beneficial properties in cancer prevention and treatment. The beneficial effects included antiproliferative effects and pro-apoptotic properties. Although KRAS mutated cells responded to the extract with more significant decrease in cell viability and increase in apoptosis, it was in BRAF mutated cells (RKO and HT29) that the combination effect of treatment of 5-FU and sprout extracts showed to be more promising, significantly increasing 5-FU sensitivity. This effect did not seem to be due to quercetin alone, but it is most likely results from the complex combination of phenolic compounds in the extract. The present study encourages the utilization of cowpea sprouts in the human diet for its potential health benefits. An improved information on the chemical composition and biological properties in colorectal cancer of cowpea could help to define and valorize cowpea, in terms of nutraceutical potential, and to suggest to consumers.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

- The author Catarina Teixeira-Guedes acknowledges the financial support provided by FCT-Portuguese Foundation for Science and Technology (SFRH/BD/52544/2014), under the Doctoral Program "Agricultural Production Chains – from fork to farm" (PD/00122/2012).

This work was supported by:

- European Investment Funds by FEDER/COMPETE/POCI– Operational Competitiveness and Internacionalization Programme, under Project POCI-01-0145-FEDER-006958 and National Funds by TFCT - Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013.

- Project I&D Interact - Integrative Research in Environment, Agro-Chain and Technology, n.º of operation NORTE-01-0145-FEDER-000017, on the investigation line entitled: Innovation for Sustainable Agro-food Chains - ISAC, co-financed by European Regional Development Fund (FEDER) through NORTE 2020 (Programa Operacional Regional do Norte 2014/2020).

6.6. References

Atashpour, S., Fouladdel, S., Movahhed, T.K., Barzegar, E., Ghahremani, M.H., Ostad, S. N., & Azizi, E. (2015). Quercetin induces cell cycle arrest and apoptosis in CD133(+) cancer stem cells of human colorectal HT29 cancer cell line and enhances anticancer effects of doxorubicin. *Iranian Journal of Basic Medical Sciences*, *18*(7), 635–43. https://doi.org/10.22038/IJBMS.2015.4643

Ayogu, R.N.B., Nnam, N.M., & Mbah, M. (2016). Evaluation of two local cowpea species for nutrient, antinutrient, and phytochemical compositions and organoleptic attributes of their wheat-based cookies. *Food and Nutrition Research*, *60*, 1–8. https://doi.org/10.3402/fnr.v60.29600

Barras, D. (2015). BRAF mutation in colorectal cancer: An update. *Biomarkers in Cancer*, *7*(1), 9–12. https://doi.org/10.4137/BIC.S25248

Bracht, K., Nicholls, A.M., Liu, Y., & Bodmer, W.F. (2010). 5-Fluorouracil response in a large panel of colorectal cancer cell lines is associated with mismatch repair deficiency. *British Journal of Cancer*, *103*(3), 340–346. https://doi.org/10.1038/sj.bjc.6605780

Capelle, L.G., Van Grieken, N.C.T., Lingsma, H.F., Steyerberg, E.W., Klokman, W.J.,

Bruno, M.J., & Kuipers, E. J. (2010). Risk and epidemiological time trends of gastric Cancer in lynch syndrome carriers in the netherlands. *Gastroenterology*, *138*(2), 487–492. https://doi.org/10.1053/j.gastro.2009.10.051

Ceter, M.M., Jemal, A., Smith, R.A., & Ward, E. (2009). Worldwide variations in colorectal cancer. *CA Cancer Journal for Clinicians*, *59*, 366–378. https://doi.org/10.3322/caac.20038.Available

Cruz-Bravo, R.K., Guevara-González, R.G., Ramos-Gómez, M., Oomah, B.D., Wiersma, P., Campos-Vega, R., & Loarca-Piña, G. (2014). The fermented non-digestible fraction of common bean (*Phaseolus vulgaris* L.) triggers cell cycle arrest and apoptosis in human colon adenocarcinoma cells. *Genes and Nutrition*, *9*(1). https://doi.org/10.1007/s12263-013-0359-1

Domínguez-Perles, R., Teixeira, A.I., Rosa, E., & Barros, A.I. (2014). Assessment of (poly)phenols in grape (*Vitis vinifera* L.) stems by using food/pharma industry compatible solvents and Response Surface Methodology. *Food Chemistry*, *164*, 339–346. https://doi.org/10.1016/j.fooDChem.2014.05.020

Doubeni, C.A., Major, J.M., Laiyemo, A.O., Schootman, M., Zauber, A.G., Hollenbeck, A. R., & Allison, J. (2012). Contribution of behavioral risk factors and obesity to socioeconomic differences in colorectal cancer incidence. *Journal of the National Cancer Institute*, *104*(18), 1353–1362. https://doi.org/10.1093/jnci/djs346

Gonçalves, A., Goufo, P., Barros, A., Domínguez-Perles, R., Trindade, H., Rosa, E.A.S., & Rodrigues, M. (2016). Cowpea (*Vigna unguiculata* L. Walp), a renewed multipurpose crop for a more sustainable agri-food system: Nutritional advantages and constraints. *Journal of the Science of Food and Agriculture*, *96*(9), 2941–2951. https://doi.org/10.1002/jsfa.7644

Grady, W.M., & Pritchard, C.C. (2014). Molecular alterations and biomarkers in colorectal cancer. *Toxicologic Pathology*, *42*(1), 124–139. https://doi.org/10.1177/0192623313505155

Guajardo-Flores, D., Serna-Saldívar, S.O., & Gutiérrez-Uribe, J.A. (2013). Evaluation of the antioxidant and antiproliferative activities of extracted saponins and flavonols from germinated black beans (*Phaseolus vulgaris* L.). *Food Chemistry*, *141*(2), 1497–1503. https://doi.org/10.1016/j.fooDChem.2013.04.010

Teixeira-Guedes, C., Oppolzer, D., Barros, A., & Pereira-Wilson, C. (2018). Impact of cooking method on phenolic composition and antioxidant potential of several varieties of *Phaseolus vulgaris* L. and *Glycine max* L. *In Press*.

Guo, X., Li, T., Tang, K., & Liu, R.H. (2012). Effect of germination on phytochemical profiles and antioxidant activity of mung bean sprouts (*Vigna radiata*). *Journal of Agricultural and Food Chemistry*, *60*(44), 11050–11055. https://doi.org/10.1021/jf304443u

Guzmán-Ortiz, F.A., San Martín-Martínez, E., Valverde, M.E., Rodríguez-Aza, Y., Berríos, J.D.J., & Mora-Escobedo, R. (2017). Profile analysis and correlation across phenolic compounds, isoflavones and antioxidant capacity during germination of soys (*Glycine max* L.). *CyTA - Journal of Food*, *15*(4), 516–524. https://doi.org/10.1080/19476337.2017.1302995

Haggar, F., & Boushey, R. (2009). Colorectal cancer epidemiology : Incidence , mortality , survival , and risk factors. *Clinics in Colon and Rectal Surgery*, *6*(212), 191–197. https://doi.org/10.1055/s-0029-1242458.

Hosseinpour-Niazi, S., Mirmiran, P., Hedayati, M., & Azizi, F. (2015). Substitution of red meat with legumes in the therapeutic lifestyle change diet based on dietary advice improves cardiometabolic risk factors in overweight type 2 diabetes patients: A cross-over randomized clinical trial. *European Journal of Clinical Nutrition*, *69*(5), 592–597. https://doi.org/10.1038/ejcn.2014.228

Kuipers, E.J., Grady, W.M., Lieberman, D., Seufferlein, T., Sung, J.J., Boelens, P.G., & Watanabe, T. (2015). Colorectal cancer. *Nat Rev Dis Primers*, *1*(15065), 1–51. https://doi.org/10.1038/nrdp.2015.65.COLORECTAL

Li, X.L., Zhou, J., Chen, Z.R., & Chng, W.J. (2015). P53 Mutations in colorectal cancer-Molecular pathogenesis and pharmacological reactivation. *World Journal of Gastroenterology*, *21*(1), 84–93. https://doi.org/10.3748/wjg.v21.i1.84

Lin, L., Harnly, J.M., Pastor-corrales, M.S., & Luthria, D.L. (2008). The polyphenolic profiles of common bean (*Phaseolus vulgaris* L .). *Food Chemistry*, *107*, 399–410. https://doi.org/10.1016/j.fooDChem.2007.08.038

López-Amorós, M.L., Hernández, T., & Estrella, I. (2006). Effect of germination on legume phenolic compounds and their antioxidant activity. *Journal of Food Composition and Analysis*, *19*(4), 277–283. https://doi.org/10.1016/j.jfca.2004.06.012

Luna-Vital, D.A., Liang, K., González de Mejía, E., & Loarca-Piña, G. (2016). Dietary peptides from the non-digestible fraction of: *Phaseolus vulgaris* L. decrease angiotensin II-dependent proliferation in HCT116 human colorectal cancer cells through the blockade of the renin-angiotensin system. *Food and Function*, *7*(5). https://doi.org/10.1039/c6fo00093b

Marley, A.R., & Nan, H. (2016). Epidemiology of colorectal cancer. *International Journal of Molecular Epidemiology and Genetics*, *7*(3), 105–114. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/27766137%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5069274

Pardini, B., Kumar, R., Naccarati, A., Novotny, J., Prasad, R.B., Forsti, A., & Lorenzo Bermejo, J. (2011). 5-Fluorouracil-based chemotherapy for colorectal cancer and MTHFR/MTRR genotypes. *British Journal of Clinical Pharmacology*, *72*(1), 162–163. https://doi.org/10.1111/j.1365-2125.2010.03892.x

Pereira, E.J., Carvalho, L.M.J., Dellamora-Ortiz, G.M., Cardoso, F.S.N., Carvalho, J L.V., Viana, D.S., & Rocha, M.M. (2014). Effects of cooking methods on the iron and zinc contents in cowpea (*Vigna unguiculata*) to combat nutritional deficiencies in Brazil. *Food and Nutrition Research*, *58*, 1–7. https://doi.org/10.3402/fnr.v58.20694

Rejhová, A., Opattová, A., Čumová, A., Slíva, D., & Vodička, P. (2018). Natural compounds and combination therapy in colorectal cancer treatment. *European Journal of Medicinal Chemistry*, *144*(January 2018), 582–594. https://doi.org/10.1016/j.ejmech.2017.12.039

Siah, S. D., Konczak, I., Agboola, S., Wood, J.A., & Blanchard, C.L. (2012). In vitro investigations of the potential health benefits of Australian-grown faba beans (*Vicia faba* L.): chemopreventative capacity and inhibitory effects on the angiotensin-converting enzyme, α-glucosidase and lipase. *British Journal of Nutrition*, *108*(S1), S123–S134. https://doi.org/10.1017/S0007114512000803

Srivastava, S., Somasagara, R.R., Hegde, M., Nishana, M., Tadi, S.K., Srivastava, M., & Raghavan, S.C. (2016). Quercetin, a natural flavonoid interacts with DNA, arrests cell cycle and causes tumor regression by activating mitochondrial pathway of apoptosis. *Scientific Reports*, *6*(April), 1–13. https://doi.org/10.1038/srep24049

Tan, C., & Du, X. (2012). KRAS mutation testing in metastatic colorectal cancer. *World Journal of Gastroenterology*, *18*(37), 5171–5180. https://doi.org/10.3748/wjg.v18.i37.5171

Tarzi, B.G., Gharachorloo, M., Baharinia, M., & Mortazavi, A. (2012). The effect of germination on phenolic content and antioxidant activity of chickpea. *Iranian Journal of Pharmaceutical Research*, *11*(4), 1137–1143.

Vasen, H.F.A., Tomlinson, I., & Castells, A. (2015). Clinical management of hereditary colorectal cancer syndromes. *Nature Reviews Gastroenterology & Hepatology*, *12*(2), 88–97. https://doi.org/10.1038/nrgastro.2014.229

World Cancer Research Fund. (2017). Diet, nutrition, physical activity and colorectal cancer. Retrieved from https://www.wcrf.org/sites/default/files/Colorectal-Cancer-2017-Report.pdf

World Cancer Research Fund International. (2015). Worldwide data | World Cancer Research Fund International. Retrieved August 28, 2017, from http://www.wcrf.org/int/cancer-facts-Figures/worldwide-data

Xavier, C.P.R., Lima, C.F., Preto, A., Seruca, R., Fernandes-Ferreira, M., & Pereira-Wilson, C. (2009). Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells. *Cancer Letters*, *281*(2), 162–170. https://doi.org/10.1016/j.canlet.2009.02.041

Xavier, C.P.R., Lima, C.F., Rohde, M., & Pereira-Wilson, C. (2011). Quercetin enhances 5-fluorouracil-induced apoptosis in MSI colorectal cancer cells through p53 modulation. *Cancer Chemotherapy and Pharmacology*, *68*(6), 1449–1457. https://doi.org/10.1007/s00280-011-1641-9

Yang, L., Liu, Y., Wang, M., Qian, Y., Dong, X., Gu, H., … Hisamitsu, T. (2016). Quercetin-induced apoptosis of HT29 colon cancer cells via inhibition of the Akt-CSN6-Myc signaling axis. *Molecular Medicine Reports*, *14*(5), 4559–4566. https://doi.org/10.3892/mmr.2016.5818

CHAPTER 7

Final considerations

7.1. General discussion and conclusion

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and the incidence is highly influenced by diet. A range of environmental modifiable lifestyle factors influences the development of CRC, being considered one of the clearest markers of epidemiological and nutritional transition, with incidence rates linked to Western lifestyles. The risk is increased by smoking, alcohol, increased body weight, T2D, high intake of red meat and processed meat. On other hand, consumption of whole grains, fruits, vegetables and legumes is associated with decreased risk. Epidemiological studies have supported the idea that some dietary food components may influence the risk of CRC through modulation of several biological processes, including proliferation, survival and/or cell death.

In this study, we used extracts of the legumes *Phaseolus vulgaris*, *Vigna unguiculata* and *Glycine max* subjected to different cooking methods or after spouting (in the case of cowpea). Characterization of the phytochemical composition and antioxidant activity of legumes extracts were performed. Furthermore, a panel of cell lines derived from several human tumors harboring different mutations representative of different types of colorectal cancer were used to study the anti CRC effects of the different extracts. The effect on CRC cell lines regarding cell cycle modulation and signaling were determined. In order to ascertain whether extracts of 5-day old cowpea sprouts could enhance the efficacy of 5-fluorouracil (5-FU), KRAS (HCT116 and HCT15) and BRAF (RKO and HT29) mutated cells were used.

In chapter 2, the effect of cooking (atmospheric boiling and pressure cooking) on phenolic composition and antioxidant potential of black, pinto, kidney and borlotti bean and soy were investigated. Cooking by the different methods applied increased the total phenolic, flavonoid and *ortho*-diphenol content as well as, antioxidant activity in all common bean varieties but did not significantly increased flavonoid content and antioxidant activity in soy. The phenolic compounds present in all samples were gallic acid and catechin. In common bean, caftaric acid, chlorogenic acid, ferulic acid, caffeic acid and p-coumaric acid were also present in significant amounts, whereas in soy these were not identified. Kaempferol-3-O-glucoside was found in all common beans and, overall, the processing increased its content. Concerning the isoflavones genistein and daidzein, they were only detected in soy and increased in extracts from processed samples, particularly by pressure cooking. In black and kidney bean extracts, myricetin, quercetin, and kaempferol with different glycosylation profiles were also detected. In soy, a modest increase in

antioxidant activity was observed, genistein and daidzein were substantially enriched in the extract of boiled and, particularly, of pressure-cooked compared to raw seeds.

In chapter 3, the anti-colorectal cancer effect of phenolic extracts from cooked cowpea, black bean and soy was evaluated in two isogenic human colorectal carcinoma cell lines HCT116, one expressing p53 (p53wt) and complete knockout for p53 (p53null), in order to investigate p53 dependent effects. A significant inhibition of cell proliferation was observed in both cell lines by the different extracts. HCT116 p53wt cells showed to be more sensitive than HCT116 p53null, in particular to extracts of cooked black bean. The decreased cell viability seems to be correlated with the phenolic content/composition and antioxidant activity of the extracts. Regarding the effects on cell cycle regulation, raw cowpea extracts displayed no significant effect in both cell lines. On the other hand, cowpea extract obtained after boiling or pressure cooking showed to increase the percentage of G0-G1 phase population in both cell lines when compared to control. The incubation with boiled black bean extracts induced an arrest in G0-G1 phase of the cell cycle in both cell lines. Soy showed to be the less efficient inducer of cell cycle arrest, displaying a significant effect only in HCT116 p53wt incubated with the pressure-cooked extract. Effects on molecular regulators of cell proliferation and cell cycle were evaluated by western-blot and qPCR. A decreased activation of PI3K-AKT pathway (lower pAKT/AKT levels) was observed, particularly in HCT116 p53wt for all extracts. The down-regulation of cyclin E and pRB in both cell lines was observed when cells were treated with all cowpea and black bean extracts in both cell lines. Soy extracts had less pronounced effects. Remarkably, in HCT116 p53wt black bean extracts were able of modulating all studied pathways, inducing an increase of the mRNA levels of different cell cycle inhibitors: p53, p27, p21 and p16.

In chapter 4, the effect of cowpea sprouting on polyphenolic content, composition and antioxidant activity over time was investigated. The anti-colorectal cancer potential and the effects on cell cycle modulation was also explored. Total phenolic, flavonoid, *ortho*-diphenol content and antioxidant activity increased significantly in a time-dependent manner after soaking. Quantitative and qualitative differences between the phenolic composition of seeds and sprouts were observed. In dry samples, the concentration of phenolic compound was found to be highest in catechin and decreased in the following order: epicatechin, gallic, chlorogenic and ferulic acid. On sprouts with 5-day old, an increase of catechin, gallic, chlorogenic and ferulic acid was observed. Caftaric and caffeic acids were found only in sprout extract. Sprouting enriched the extracts in flavonoids, such

as quercetin and kaempferol glucosides. The incubation with extracts exhibited a dose-dependent inhibition of cell viability of HCT116, HCT15, CO115 and RKO cancer cell lines, but the effect on HT29 cells was insignificant when incubated with raw extract. Extract from cowpea sprouts shown to be more efficient in decreasing cell viability than extract of raw cowpea. In cowpea sprouts, the lowest IC50 was for CO115 cells and increased in the following order: HCT116, HCT15, RKO and HT29. The analysis of cell cycle distribution of each cell line after incubation with extracts showed that raw cowpea extract did not produce cell cycle arrest in HCT116, HCT15 and HT29. In CO115 and RKO, both cowpea extracts induced cell cycle arrest at G0-G1 phase and in G2-M phase, respectively. The treatment with the extract of cowpea sprouts gave rise to an increased fraction of cells in the G2-M phase in HCT116 and S phase in HCT15. Overall, the sprouting of cowpea seeds significantly increased the total phenolic, flavonoid and *ortho*-diphenol content, as well as, antioxidant activity in a time-dependent manner. The extracts of cowpea sprouts were particularly enriched in quercetin. With regard to the effects of extracts of 5-day old cowpea sprouts, decreased cell viability were in agreement with the results obtained by cell cycle analyze. Cells with different molecular signatures behaved differently with arrest in different phase of the cell cycle (G1, S or G2).

In chapter 5, the enhancement of 5-FU cytotoxic efficiency by the extracts of cowpea seeds or sprouts was characterized. Sprouts extract inhibited the cell proliferation more significantly in HCT116 and HCT15 (cells harboring the PI3K and KRAS mutation) than RKO and HT29 (harboring the PI3K and BRAF mutation). In cells with KRAS mutation, the extract could be regulating the KRAS with effects on both MAPK/ERK and PI3K pathways. In the more resistant cells, which harbor BRAF and PI3K mutations, PI3K/AKT pathways is activated and MAPK/ERK is activated downstream of KRAS, which may explain the lower response in these two cell types. A higher induction of apoptosis was observed for HCT116, HCT15, than RKO and HT29 when incubated with cowpea sprout extract. Raw cowpea extracts induced apoptosis only in HCT116 cells. The combination of extracts with 5-FU showed an additive effect on the decrease of cell viability. Combination of raw extract with 5-FU had significant effects in HCT116, RKO and HT29. Increased sensitivity to 5-FU by sprout extract was particularly high for RKO and HT29, originally more resistant to the drug showing to be more promising. This effect did not seem to be due to quercetin alone, but it is most likely results from the complex combination of phenolic compounds present in the extract.

In conclusion, the results of this work make a significant contribution to the identification of diet interactions and may be used to provide guidelines for dietary recommendations with regard to the inclusion of legumes for cancer prevention as well as for boosting the efficiency of conventional cancer treatments. Sprouting cowpea may provide an innovative added value food product and/or ingredient for functional foods and nutraceuticals.

7.2. Future perspectives

The studies included in this thesis were conducted to explore the anti-colorectal cancer potential of selected legumes and characterize the mechanism of action. Despite the advances obtained in this work, there are many unanswered questions that could be further investigated.

In the present research, we observed that polyphenolic extracts from processed legumes possess anti-cancer potential through PI3K/AKT pathway inhibition and cell cycle modulation (decreased expression of pRB and cyclin E). It would be interesting to explore the compounds present in the extracts responsible for these effects. The effects on the induction of apoptosis and associated signaling pathways should be studied. In addition to the anti-colorectal cancer effects, it would be interesting to characterize the chemoprotective effect of legumes extracts on DNA damage and repair, investigate the antioxidant protection of polyphenolic extracts to stress tolerance and explore the induction of endogenous antioxidant defenses.

Phenolic extracts from cowpea sprouts enhanced the 5-FU efficacy by decreasing the cell viability. Nevertheless, the effect on the induction of DNA damage and induction in apoptosis should be carried out. The effect of this extract in signaling pathways related to cell proliferation, cell cycle and cell apoptosis could be performed in order to clarify the effect observed via MTT assay. An *in vivo* study using mouse models of colorectal cancer could be carried out, in order to find if the combination treatment also enhances the 5-FU anticancer activity *in vivo*.

Recent studies show that dysbiosis in the microbiota is linked to the development of colorectal cancer. The formation of short chain fatty acids (SCFAs) is the result of a complex interplay between diet and the gut microbiota within the gut lumen environment and it has been linked with CRC prevention. It would be important to perform the digestion and fermentation of the legumes used in this thesis and characterize the effects on the microbiota and SCFAs production. Moreover, the extracts produced after fermentation

could be studied in colorectal cancer cell lines in order to understand their anticancer potential.