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COMPOSTOS BIOATIVOS, ATIVIDADE ANTIOXIDANTE E ATIVIDADE ANTIPROLIFERATIVA EM LINHAGENS CELULARES DE CÂNCER DE PRÓSTATA DE EXTRATOS DE CAFÉ OBTIDOS POR EXTRAÇÃO ASSISTIDA POR MICRO-ONDAS (MAE)

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RESUMO
O consumo de café tem sido avaliado como fator de proteção do câncer de próstata (PCa). O café tem efeitos moleculares que podem estar relacionadas com prevenção do PCa. O consumo de café pode estar relacionado à redução do risco de câncer de próstata devido a seus compostos fitoquímicos, como cafeína, ácidos clorogênicos e trigonelina. O processo de torrefação afeta o conteúdo dos fitoquímicos e compostos indesejados podem ser formados. A extração assistida por microondas (MAE) é uma opção para as técnicas de extração convencionais, pois preserva mais compostos bioativos. Portanto, objetivamos avaliar a composição fitoquímica do café e os efeitos preventivos no desenvolvimento do câncer de próstata em 4 graus diferentes de torrefação de café extraídos usando o MAE. Os grãos verdes de Coffea arabica (1) foram processados em torra clara (2), média (3) e escura (4) e essas quatro amostras de café foram extraídas usando o MAE. A capacidade antioxidante dessas 4 amostras foi avaliada pelos métodos DPPH, ABTS, FRAP, Folin-ciocalteu e ORAC. A cafeína, o ácido clorogênico e o ácido cafécico foram medidos por HPLC. As amostras foram testadas em linhagens celulares PCa metastáticas PC-3 e DU-145, quanto aos seus efeitos sobre a viabilidade celular, progressão do ciclo celular e morte celular apoptótica. Os extratos de café verde e torra clara apresentaram a maior atividade antioxidante. O teor de cafeína não foi afetado pela torrefação, o ácido clorogênico foi degradado devido à temperatura e a quantidade de ácido cafécico aumentou na torra clara, com diminuição na torra média e escura. Os extratos de café verde e torra clara promoveram maior inibição da viabilidade celular, causaram parada do ciclo celular em S e G2/M e induziram apoptose, quando comparados aos extratos de café da torra média e escura e os controles. Os extratos de café promoveram maior efeito antiproliferativo na linhagem DU-145 do que nas células PC-3. Os resultados apresentam evidências preliminares de que o consumo de café verde e torra clara pode contribuir para reduzir a progressão do PCa, potencialmente inibindo aspectos relacionados aos subtipos avançados de PCa.

Palavras-chave: café; câncer de próstata; mecanismos moleculares; antioxidante; ácidos clorogênicos; extração assistida por micro-ondas.
ABSTRACT

Coffee consumption has been investigated as a protective factor for prostate cancer (PCa). Coffee has molecular effects that may be related to PCa prevention. Coffee drinking may be related to prostate cancer risk reduction due to its phytochemical compounds, such as caffeine, chlorogenic acids, and trigonelline. The roasting process affects the content of the phytochemicals and undesired compounds can be formed. Microwave-assisted extraction (MAE) is an option to conventional extraction techniques since it preserves more bioactive compounds. Therefore, we aimed to review the molecular mechanisms of coffee on PCa prevention, to evaluate coffee phytochemical composition and the putative preventive effects in prostate cancer development of 4 different coffee-roasting degrees extracted using MAE. *Coffea arabica* green beans (1) were roasted into light (2), medium (3) and dark (4) and these four coffee samples were extracted using MAE. Antioxidant capacity of these 4 samples was evaluated using DPPH, ABTS, FRAP, Folin-ciocalteu and ORAC methods. Caffeine, chlorogenic acid and caffeic acid were measured through HPLC. Samples were tested in PC-3 and DU-145 metastatic PCa cell lines, regarding their effects over cell viability, cell cycle progression and apoptotic cell death. Green and light roasted coffee extracts had the highest antioxidant activity. Caffeine content was not affected by roasting, chlorogenic acid was degraded due to the temperature and the content of caffeic acid increased in light roast with decrease in medium and dark roast. Green and light roasted coffee extracts promoted higher inhibition on cell viability, caused cell cycle arrest in S and G2/M and induced apoptosis, when compared to medium and dark roasted coffee extracts and the controls. Coffee extracts promoted greater antiproliferative effects in DU-145 line than in PC-3 cells. Results provide early evidence that the consumption of green and light roasted coffee may contribute to reduce PCa progression, potentially inhibit aspects related to advanced PCa subtypes.

Keywords: coffee; prostate cancer; molecular mechanisms; antioxidants; chlorogenic acids; microwave assisted extraction.
1. INTRODUÇÃO

O câncer de próstata (PCa) é o tipo mais comum de câncer entre os homens correspondendo a 17.7% de todos os tipos de câncer em homens (WHO, 2018). Muitos fatores contribuem para o desenvolvimento de PCa, incluindo faixa etária e histórico familiar, além de provável contribuição de fatores ambientais, como tabagismo e a dieta (BALLON-LANDA; PARSONS, 2018). Quando o PCa é diagnosticado, o tratamento inclui quimioterapia e abordagem cirúrgica, que apresentam efeitos adversos (LITWIN; TAN, 2017). O uso de extrato de planta têm sido amplamente testado na prevenção de câncer (GREENWELL; RAHMAN, 2015), um deles é o café (PALMIOLI et al., 2017). Foram identificadas correlações inversas entre o consumo de café e a incidência de diversos tipos de câncer, incluindo oral, de faringe, colorretal, hepático, prostático, endometrial e melanoma (WANG et al., 2016).

O café pode auxiliar na prevenção de PCa através de mecanismos moleculares e epigenéticos. Tais efeitos estão relacionados à capacidade antioxidante e propriedades anti-inflamatórias do café. O café é capaz de prevenir danos ao DNA, induzir a ativação de enzimas de fase II, modular fatores de transcrição, melhorar hormônios esteroides circulantes e a resistência à insulina (GROSSO et al., 2017).

A composição bioquímica e bioatividade dos grãos de café têm sido amplamente investigadas devido à sua importância econômica e atributos de qualidade específicos associados à bebida de café e a saúde humana. A cafeína é o composto mais estudado do café, sendo capaz de reduzir o risco de doença de Parkinson e Alzheimer, assim como diabetes tipo 2 e alguns tipos de câncer, além de apresentar efeitos positivos na função hepática (GROSSO et al., 2017). O café também é a maior fonte de ácidos clorogênicos (CGAs), que têm propriedades antioxidante, anti-inflamatória, antidiabética, de diminuição da obesidade, hepatoprotetora, antimicrobiana e anti-hipertensiva (NAVEED et al., 2018). A trigonelina é um dos alcaloides do café que apresenta efeitos antimicrobiano, anticarcinogênico e antihiperplégico. Além disso, já foi descrito com propriedades relacionadas à prevenção da alergia, hiperlipidemia e disfunções renal e hepática (NUGRAHINI et al., 2020)

Apesar do café apresentar tais efeitos fisiológicos, o teor de tais fitoquímicos é influenciado por muitos fatores, incluindo torrefação (SCHENKER; ROTHGEB, 2017), moagem (BLITTERDORFF; KLATT, 2017) e preparação (MESTDAGH; GLABASNIA; GIULIANO, 2017). A torrefação é uma das principais fases de processamento do café, que causa muitas reações químicas, principalmente desidratação, reação de Maillard e degradação.
de Strecker, que formam o aroma e a cor típicos do café, embora reduzam os níveis de compostos bioativos (POISSON et al., 2017).

A cafeína é estável e seu teor é similar no café verde e no torrado, enquanto a trigonelina é parcialmente degradada e convertida em vitamina B3 e compostos voláteis (POISSON et al., 2017). Os ácidos clorogênicos são extensamente perdidos durante a torrefação, através de degradação, formação de lactonas de ácidos clorogênicos e incorporação à melanoidinas (DAWIDOWICZ; TYPEK, 2017). Lipídios, incluindo diterpenos, podem aldeídos através da degradação térmica (POISSON et al., 2017). Além disso, acrilamida é formada durante a torrefação através da condensação de asparagina e açúcares redutores e apresenta efeitos carcinogênicos (DAWIDOWICZ; TYPEK, 2017).

O café é geralmente consumido como uma bebida e há diversas técnicas de preparo, como fervendo (infusão), coando e por pressão (expresso). A extração assistida por micro-ondas (MAE) poderia ser um substituto comercial para o café por infusão. Este processo apresenta vantagens em relação a outros métodos de extração, devido ao menor tempo e temperatura mais baixa, em cujas condições os compostos bioativos são menos degradados. Além disso, compostos voláteis são preservados em um sistema de recipiente fechado e apresenta redução no uso de solventes (LOPES et al., 2020).

Portanto o presente trabalho tem como objetivos: (1) revisar os aspectos moleculares pelos quais o café pode influenciar positivamente a prevenção e tratamento do câncer de próstata; (2) avaliar a alteração da atividade antioxidante e o teor de compostos bioativos do café após torrefação; (3) investigar como diferentes níveis de torra de café extraído por MAE afetam a viabilidade celular, o ciclo celular e indução da apoptose em linhagens metastáticas de câncer de próstata PC-3 e DU-145.
2. MOLECULAR MECHANISMS FOR COFFEE COMPOUNDS ON PROSTATE CANCER PREVENTION

Molecular mechanisms for coffee compounds on prostate cancer prevention

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Molecular mechanisms for coffee compounds on prostate cancer prevention

Prostate cancer (PCa) is the most common type of cancer among men. Coffee may be associated with reduced risk of developing PCa through molecular mechanisms. Coffee has an important antioxidant capacity, that reduced oxidative stress, leading to reduced mutation in cells. Beyond direct antioxidant activity, coffee stimulates phase II enzymatic activity, that are related to detoxification of reactive metabolites. The anti-inflammatory effects of coffee reduce tissue damage related to PCa development. Coffee regulates NFkB pathway and reduces iNOS expression and inflammatory mediators, such as TNF-a, IL-6, IL-8 and CRP. Also, coffee protects DNA against damage, and by activating Nrf2/ARE signaling pathways. Besides NFkB and Nrf2/ARE pathways, coffee modulates other transcriptional factors, such as PI3K/Akt, AP-1, AhR and MAPK. It has been shown that coffee increases testosterone, reduces SHBG, estrogen and PSA. Coffee also enhances insulin resistance and glucose metabolism. All these effects may contribute to protection against PCa development.

Keywords: coffee, prostate cancer, antioxidant, anti-inflammatory, mechanisms
1. Introduction

Prostate is a gland localized between men’s bladder and penis. It surrounds the urethra that secretes prostate fluid that protects sperm. Prostate cancer (PCa) develops when mutated semen-secreting prostate cells start proliferating uncontrollably. If PCa is not treated, it may metastasize, usually to lymph nodes, to hip bone, and then to other organs. Ordinarily, is not diagnosed in the early stages because it is initially asymptomatic (Kgatle et al. 2016). PCa is the most common type of cancer among males, accounting for 17.7% of all cancers in men (WHO 2018).

Cancer in general develops due to successive mutations in genes, which alters cell morphology and physiology. This disorder affects cell cycle and cell division, apoptosis signalling and DNA repair mechanisms (Hassanpour & Dehghani 2017). Besides nonmodifiable risk factor, such as age, family history and ethnicity, PCa development can also be influenced by diet and environmental factors, through epigenetics, which involves changes of gene transcription without any alteration in the nucleotide sequence (Kgatle et al. 2016). Epigenetic modifications include DNA methylation and acetylation, histone and microRNA modifications (Hassanpour & Dehghani 2017).


Coffee has been shown to have molecular effects that would explain those epidemiological findings, related to coffee’s antioxidant and anti-inflammatory properties. Coffee can prevent DNA damage, induce phase II enzymatic activity, modulate transcription
factors, improve circulating steroids and insulin resistance as shown in table 1. Therefore, the aim of this review is to detail molecular mechanisms involved in PCa prevention via coffee consumption.

2. Antioxidant Activity Induced by Coffee

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced endogenously by mitochondrial respiration and exogenously by exposure to oxidizing agents (Liang & Kitts 2015). Oxidative stress may lead to PCa development, and results from cellular production of oxidant molecules surpassing the capacity of antioxidants to overcome these insults (Martini et al. 2016). Then, causes oxidation of crucial biomolecules, causing DNA damage and oxidizing key enzymes involved in DNA methylation (Liang & Kitts 2015).

Coffee contains many antioxidant compounds and, considering the amount of coffee intake in an average diet, it can be a major source of dietary antioxidants (Shaposhnikov et al. 2018). Coffee’s antioxidant compounds include caffeine, phenolic compounds (mostly chlorogenic acids), trigonelline, diterpenes (cafestol and kahweol) and melanoidins (Jabir et al. 2018). A mechanism for coffee’s antioxidant activity is described in Figure 1.

Antioxidant properties observed in coffee are mainly attributed to chlorogenic acids (CGAs), that have one to two aromatic rings linked to hydroxyl groups and donate hydrogen atoms, reducing free radicals. Their oxidation products, phenoxy radical, are promptly stabilized by resonance stabilization. CGAs react with different sources of free radicals at varied pace, their relative efficiency is species-specific (Liang & Kitts 2015).

Some studies have reported a significant increase in total plasma antioxidant capacity following coffee consumption, even after a single serving. These effects of a single dose were
lost in long term, but in recurrent consumption, plasma antioxidant activity was increased in up to 26% (Martini et al. 2016).

Coffee significantly increased antioxidant response element (ARE) activation, which could induce the expression of genes related to the cellular antioxidant system (Choi et al. 2018). ARE proteins are a part of the complex antioxidant system that protects cells from oxidative damage by neutralizing free radicals and oxidizing agents (Priftis et al. 2018). ARE related genes are in the cell defence promoter regions, that include phase II detoxifying enzymes and enzymes involved in antioxidant defence (Pahlke et al. 2019).

It has been observed that coffee consumption increases intracellular the activity of phase I (cytochrome P450) and II enzymes (Shaposhnikov et al. 2018), such as reduced glutathione (GSH), which is a substrate to glutathione peroxidase (GPx) and glutathione S-transferases (GST) synthesis (Han et al. 2017; Jung et al. 2017; Priftis et al. 2018). This increase in GSH has been attributed to polyphenols (Jung et al. 2017). In addition to the increase in GSH, coffee reduced H$_2$O$_2$-induced cell death in vitro via activating PI3K/Akt pathway in MC3T3-E1 cells (Han et al. 2017).

It has been hypothesized that compounds with antioxidant properties generally increase the messenger RNA (mRNA) expression of antioxidant-related enzymes, but these expressions can be downregulated in some antioxidant-treated cells because the compounds may have directly ameliorated the prevailing oxidative stress (Jung et al. 2017).

A low-molecular weight (MW) coffee fraction supplementation in rats was able to reduce noncoding microRNA-124-3p, and increase the expression of mRNA involved in GPx coding, raising the expression of this enzyme, probably due to caffeine (Curti et al. 2019). Noncoding microRNA is an epigenetic factor, can act as tumour suppressors or oncogenes and may be downregulated or upregulated in PCa. They are short regulatory RNA molecules that can’t be translated into amino acids and may disturb the mRNA purpose. This might
influence RNA silencing and gene expression at post-transcriptional and translational levels (Kgatle et al. 2016).

Coffee has also been shown to inhibit oxidative stress through UDP-glucuronosyltransferases (UGT) activation, which catalyse the detoxification of reactive metabolites. Coffee reduced ROS production and Benzo[α]pyrene (BaP) oxidative damage (Kalthoff et al. 2017).

3. Anti-inflammation Effects
Inflammation is a physiological reaction to tissue damage induced by exogenous or endogenous agents. Exogenous factors include pathogens, allergens, foreign bodies and pernicious substances. Endogenous causes originate from cell signalling due to injured or malfunctioning tissues (Liang & Kitts 2015).

Inflammation may be related to PCa development because inflammatory cells are often present in the prostate microenvironment of adult men and are related to PCa precursor lesions, called proliferative inflammatory atrophy. Which is abundant in cells that may be predisposed to genomic mutations and inflammatory stress can provoke epigenetic changes, concomitant with the rupture of the epithelial barrier (Sfanos et al. 2018).

Coffee compounds can decrease chronic inflammation, therefore, protect against DNA degradation, and consequently improve the risk of disease (Frost-Meyer & Logomarsino 2012). The inflammation process is characterized by the raised production of nitric oxide (NO) and pro-inflammatory cytokines, such as C-reactive protein (CRP), interleukins (IL) and tumour necrosis factors (TNF) (Jung et al. 2017).

A proposed mechanism for coffee’s anti-inflammatory effects are described in figure 2. It has been observed in clinical trials, that coffee intake over several weeks had a prevailing anti-inflammatory action evaluated by serum markers, but caffeine presented no explicit
short-term impact on inflammatory reaction (Paiva et al. 2019). In addition to blood inflammatory markers, topical treatment of coffee and caffeine on mice’s paws displayed a considerable inhibition of the carrageenan-induced oedema development (Pergolizzi et al. 2018).

It has been observed that coffee can inhibit TNFα-induced NFkB activity and DNA-binding in PCa cells. Coffee also regulated the expression of inflammatory and cancer related genes probably through NFkB signalling pathway. Coffee down-regulated genes related to invasion (MMP9) and inflammation, such as NFkB2, CD40, EDN1 and ICAM1, and upregulated genes related to antioxidant system, such as NFE2L2, HMOX1, NQO1 and GCLC (Kolberg et al. 2016).

The TNF-a and IL-6 expressions were decreased by light roasted coffee extract, and the levels raised as roasting levels were exacerbated. Inducible nitric oxide synthase (iNOS) expression declined, which produces proinflammatory mediators, such as NO (Jung et al. 2017). Coffee exhibited an inverse relation with CRP, IL-6 and TNFR2, associations were close in caffeinated and decaffeinated coffee (Hang et al. 2019).

Those findings indicate that caffeine is not the only responsible for coffee’s anti-inflammatory effects. Caffeine can reduce TNF-a and IL-6 production in a dose-dependent way and its anti-inflammatory property is due to modulation of nuclear factor kB (NFkB) activation (Choi et al. 2018), which provokes the expression of inflammatory genes, including iNOS, cyclooxygenase-2 (COX2), and cytokines (Jung et al. 2017).

CGAs decrease the generation of inflammatory mediators by inhibition of protein tyrosine phosphatase 1B (PTP1B), minimizing proinflammatory cytokine genes expression, and regulation NFkB activation (Hang et al. 2019). As a result, COX is suppressed, causing a reduction of IL-6 and IL-8 and TNF-a release (Jung et al. 2017; Choi et al. 2018). Great
CGAs concentration repress IL-1B messenger RNA (mRNA), provoking considerably less cell adhesion and inflammation (Frost-Meyer & Logomarsino 2012).

Other coffee compounds can be involved in anti-inflammatory effects. Kahweol lessen COX 2 and monocyte chemoattractant protein-1 (MCP-1) quantities, meaning it could be anti-angiogenic. Kahweol also decreases iNOS in rats’ carrageenan paw oedema. During roasting, trigonelline is fractionated in nicotinic acid, which is a promising anti-inflammatory agent, as it diminishes MCP-1 and enhances adiponectin in adipocytes infused with TNF-a (Frost-Meyer & Logomarsino 2012).

4. Protection Against DNA Damage
A proposed mechanism for coffee protection against DNA damage is described in Figure 3. Coffee’s antioxidant and anti-inflammatory effects results in protection against DNA damage. It has been observed a reduction in spontaneous DNA strand breaks after only 2 hours of coffee consumption, with further decrease when more coffee was consumed (Bakuradze et al., 2016). Similar results were observed in 4 weeks (Bakuradze et al., 2014) and 8 weeks (Pahlke et al. 2019) of coffee intake.

These observations imply a defensive impact of coffee on DNA integrity. It has also been observed strong chemo preventive properties of coffee against DNA damage caused by aflatoxin, probably due to induction of GST (Ferk et al. 2014). Urinary 8-hydroxydeoxyguanosine excretion tended to reduce with coffee intake, which is a biomarker of systemic oxidative DNA damage and repair. This result was associated with lower serum ferritin and indeed, coffee has iron-chelating properties, indicating that coffee might decrease iron storages (Hori et al. 2014).

Coffee constituents, such as CGAs and trigonelline are activators of the Nrf2/ARE signalling pathways (Bakuradze et al., 2016). It has been observed raised levels of Nrf2
dependent enzymes after consumption of coffees rich in CGA. Likewise, an inverse correlation between Nrf2 transcription and DNA strand breaks has been observed after coffee consumption (Bakuradze et al., 2014).

Both Nrf2 and phosphorylated Nrf2 (pNrf2), are involved in the induction of Nrf2/ARE dependent gene transcription. Alterations in Nrf2 translocation mediate the ARE-linked cytoprotective transcriptional response rather than a change in total Nrf2 concentration. There are evidences of pNrf2 translocation to the nucleus and increase in pNrf2 with decrease in Nrf2 due to coffee consumption (Pahlke et al. 2019).

CGA isomers displayed a protective action against X-ray, H2O2 and NH2Cl induced DNA plasmid chromosome breaks. CGAs showed to inhibit methylation of the promoter region of the RAR beta gene through increased formation of S-adenosyl-L-homocysteine (Liang & Kitts 2015). Coffee and its diterpene components kahweol and cafestol, raised the expression of DNA repair protein O6-methylguanine-DNA methyltransferase and some phase II enzymes (Bakuradze et al., 2016).

5. Modulation of Transcriptional Factors
Coffee may prevent PCa development through modulation of transcriptional factors. The mechanism for coffee regulation of transcriptional factors is shown in figure 4. Some of those factors have already been discussed, such as NFKB and Nrf2/ARE pathways. Some other factor coffee may affect are phosphatidylinositol-3-kinase (PI3K/Akt), activator protein 1 (AP-1), aryl hydrocarbon receptor (AhR), and mitogen activated protein kinases (MAPK).

Coffee can downregulate PI3K/Akt signalling pathway (Huang et al. 2016), which is proto-oncogenic and is responsible for metabolism, cell cycle and survival, and angiogenesis. This pathway is frequently engaged in heme-oxygenase 1 (HO-1) expression and transcription
of vary kinds of cells. CGA promotes HO-1 expression and Nrf2 nuclear translocation, which might be related to PI3K/Akt signalling pathway (Han et al. 2017).

There is evidence that CGA secure against cancer caused by external factors. Its defensive actions can be associated to the omission of NFkB, AP-1, and MAPK activation concerning ROS effects (Palmioli et al. 2017).

The MAPK group comprises of three sorts of protein kinases, encompassing extracellular responsive kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. Coffee causes downregulation of MAPK signaling pathways (Huang et al. 2016). There is association between the restriction of cell growth and the lesser activation/phosphorylation of MAPKs, indicating its capacity to decrease cancer cell proliferation (Palmioli et al. 2017).

AhR has an adjusting role in the manifestation of CYP1A1 and CYP1A2, which are embroiled in the metabolization of several substances (Nordestgaard et al. 2020). Coffee can also protect cells against toxicity by induction of UDP glucuronosyltransferases genes through AhR and Nrf2, culminating in raised detoxification and clearance of reactive metabolites (Kalthoff et al. 2017).

6. Controlling Steroids Metabolism
A proposed mechanism for coffee modulation of steroids metabolism is described in figure 5. It was believed that androgens were involved in PCa development. However, a meta-analysis found no relation between levels of testosterone and incidence or increased risk of PCa (Boyle et al. 2016).

Yet, caffeinated coffee is associated to higher concentrations of total testosterone. Probably, testosterone increase observed is linked to SHBG, which is a sex hormones transport protein, functioning as a regulator of their activity. Decreased SHBG may be linked to elevated risk of PCa. Caffeinated coffee is linked to greater SHBG concentration. Caffeine
and SHBG are primarily metabolized by the liver, so caffeine intake might cause SHBG rise by impacting its metabolism (Hang et al. 2019).

Besides that, in vivo assays have evinced caffeine’s ability to affect oestrogen metabolism inhibiting aromatase, the prime enzyme responsible for the transformation of androgen to oestrogen. Serum estrone and oestradiol are associated to a higher risk of PCa and coffee intake is related with reduced levels of both (Hang et al. 2019).

Coffee oil has showed to significantly decrease the prostate-specific antigen (PSA) compared to the control in vivo. PSA concentration are usually remarkably high in PCa. Therefore, a reduction in PSA levels suggests that coffee oil has defensive actions on inflammatory status and against prostate hypertrophy. It has been hypothesized that coffee oil effect was due to 5 alpha reductase enzyme inhibition (Cueto et al. 2016).

Coffee diterpenes (kahweol and cafestol) decreased the nuclear androgen receptor (AR) in AR-positive PCa cells, inhibiting their signals and inducing apoptosis. Coffee diterpenes also caused reduction in CCR2 and CCR5, without raising their ligands (CCL2 and CCL5) (Iwamoto et al. 2019). AR have been associated to increased proliferation and altered migratory potential in PCa cells (Leach & Buchanan 2017).

7. **Enhancing Insulin Resistance**
Fasting serum insulin and insulin resistance are associated to PCa development, especially in men 65 years old or more. Insulin could act as growth factor and probably raise the androgen entry in prostatic cells by reducing the effect of SHBG. Insulin resistance could rise insulin like growth factor (IGF), that has mitogenic and anti-apoptotic properties, which promote cell proliferation (Saboori et al. 2019).

Coffee has been shown to improve glucose metabolism both in short and long term and is associated with lower risk of type 2 diabetes mellitus (T2DM). These effects are
observed both in caffeinated and decaffeinated coffee, however, it might be more significant in decaffeinated coffee (Reis et al. 2018; Reis et al. 2019). Besides its antioxidant and anti-inflammatory activities, coffee exerts specific effects that improve glucose and insulin status. The mechanism of coffee on insulin resistance and glucose metabolism is expressed in figure 6.

It has been observed that caffeinated coffee consumption reduces IGF-binding protein-3 (IGFBP-3), which extends IGF half-life. C-peptide also decreased due to coffee intake, which is a maker for insulin secretions, indicating lower insulin production and resistance (Hang et al. 2019).

It has been observed that incretins raised, and blood glucose reduced after coffee intake. Incretins, such as GLP-1 and GIP, are related to glucose reduction. Additionally, coffee has norharman β-carboline, that inhibits α-glucosidase activity, which lessens carbohydrate absorption, reducing postprandial glucose levels (Reis et al. 2018).

Caffeine acts as an antagonist of the A1 and A2 adenosine receptors, which in the skeletal muscle are related to insulin resistance. Caffeine has a synergic effect with adrenergic hormones, which increases glucose intake by tissues. Most of the population quickly metabolizes caffeine, thus, it has acute effects. There is evidence that habitual caffeine intake leads to tolerance to these effects (Reis et al. 2019).

CGAs are probably responsible for the long-term effects. It has been hypothesized that CGA stimulates insulin release, being a secretagogue. CGA increased the expression of peroxisome proliferator-activated receptor-y (PPAR-y), which is essential in insulin sensitivity. Glucose transporter type 4 (GLUT4) also increased due to CGA, and its expression is stimulated by PPAR-y (Sanchez et al. 2017).
8. Conclusion
Coffee can reduce the risk of developing PCa through many mechanisms. The main mechanisms are probably antioxidant and anti-inflammatory activities, protection against DNA damage, modulation of transcriptional factors, regulation through microRNA, enhancing steroids metabolism and enhancing insulin resistance. However, it is essential more molecular studies to verify the function of those mechanisms. Coffee may impact PCa through other factors, but more studies are necessary to expand the knowledge to verify the balance among consumption and real extension of coffee consumption and its potential PCa risk development.

References:


Table 1. Effects of coffee or coffee compounds on cell or animal models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Coffee Sample/ Compound</th>
<th>Outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP, LNCaP-SF, PC-3, and DU145 cells</td>
<td>Kahweol, cafestol, caffeine, caffeic acid, CGA, and trigonelline.</td>
<td>(-) Proliferation and migration of PCa cells</td>
<td>(Iwamoto et al. 2019)</td>
</tr>
<tr>
<td>Xenograft study of SCID mice</td>
<td></td>
<td>(-) AR, CCR2 and CCR5</td>
<td></td>
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<td></td>
<td></td>
<td>(-) PCa growth</td>
<td></td>
</tr>
<tr>
<td>PC-3 cells</td>
<td>Dark roasted <em>Coffea arabica</em></td>
<td>(-) NF-κB activity and DNA-binding</td>
<td>(Kolberg et al. 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+) Apoptosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+) Modulation of gene expression</td>
<td></td>
</tr>
<tr>
<td>PC-3 xenografts in athymic nude mice</td>
<td>CGA</td>
<td>(-) H₂O₂ damage</td>
<td>(Han et al. 2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+) HO-1 and Nrf2</td>
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<tr>
<td></td>
<td></td>
<td>(+) PI3K/Akt</td>
<td></td>
</tr>
<tr>
<td>MC3T3-E1 cells</td>
<td><em>SCG Coffea arabica</em></td>
<td>(-) PI3K/Akt and MAPK</td>
<td>(Huang et al. 2016)</td>
</tr>
<tr>
<td>B16F10 cells</td>
<td><em>Coffea arabica</em> light, medium, city, and French roasts</td>
<td>(+) GSH</td>
<td>(Jung et al. 2017)</td>
</tr>
<tr>
<td>AML-12 cells</td>
<td><em>Coffea arabica</em></td>
<td>(+) mRNA related to GSH</td>
<td></td>
</tr>
<tr>
<td>RAW 264.7 cells</td>
<td></td>
<td>(-) TNF-a and IL6</td>
<td></td>
</tr>
<tr>
<td>HepG2 and KYSE70 cells</td>
<td>Decaffeinated commercial coffee</td>
<td>(+) UGT</td>
<td>(Kalthoff et al. 2017)</td>
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<tr>
<td></td>
<td></td>
<td>(-) BaP- induced damage</td>
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<td></td>
<td></td>
<td>(-) ROS production</td>
<td></td>
</tr>
<tr>
<td>RINm5F and 3T3-L1 cells</td>
<td>CGA</td>
<td>(+) Insulin secretion</td>
<td>(Sanchez et al. 2017)</td>
</tr>
<tr>
<td>EA.hy926 cells</td>
<td>Green and Light roast <em>Coffea arabica</em></td>
<td>(+) Redox status</td>
<td>(Priftis et al. 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+) GSH</td>
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<tr>
<td>C57BL/6 mice</td>
<td><em>Coffea arabica</em> light, medium, city, and French roasts</td>
<td>(-) liver necrosis</td>
<td>(Choi et al. 2018)</td>
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<tr>
<td></td>
<td></td>
<td>(-) IL-6</td>
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<td></td>
<td></td>
<td>(-) TNF-a</td>
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<tr>
<td></td>
<td></td>
<td>(+) NF-κB pathway</td>
<td></td>
</tr>
<tr>
<td>Sprague Dawley rats</td>
<td><em>Coffea arabica</em> oil</td>
<td>(-) PSA</td>
<td>(Cueto et al. 2016)</td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>Low-molecular-weight from regular and decaffeinated <em>Coffea canephora</em></td>
<td>(-) microRNA-124-3p</td>
<td>(Curti et al. 2019)</td>
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<tr>
<td></td>
<td></td>
<td>(+) mRNA related to GPX</td>
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<td></td>
<td></td>
<td>Decaffeinated coffee had no effect</td>
<td></td>
</tr>
<tr>
<td>Him-OFA rats</td>
<td>Regular and decaffeinated <em>Coffea arabica</em></td>
<td>(-) Hepatic foci frequency</td>
<td>(Ferk et al. 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-) Aflatoxin DNA damage</td>
<td></td>
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<tr>
<td>Wistar rats</td>
<td>Green <em>Coffea canephora</em> and caffeine</td>
<td>(-) carrageenan-induced paw oedema</td>
<td>(Pergolizzi et al. 2018)</td>
</tr>
<tr>
<td>Intervention clinical trial</td>
<td>Green and roasted <em>Coffea arabica</em> blend</td>
<td>(-) Spontaneous DNA strand breaks</td>
<td>(Bakuradze et al., 2016; 2014)</td>
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<tr>
<td>Prospective clinical trial</td>
<td>Caffeinated and decaffeinated coffee</td>
<td>(-) CRP, IL-6, and TNFR-2</td>
<td>(Hang et al. 2019)</td>
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<tr>
<td></td>
<td></td>
<td>(+) Adiponectins</td>
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<td></td>
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<td>(-) estrone and testosterone</td>
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<td>(+) SHBG</td>
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<td></td>
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<td>(-) C-peptide and IGFBP-3</td>
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<tr>
<td>Prospective clinical trial</td>
<td>Coffee intake</td>
<td>(-) Urinary 8-OHdG</td>
<td>(Hori et al. 2014)</td>
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<td></td>
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<td>(-) Ferritin</td>
<td></td>
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<tr>
<td>Prospective clinical trial</td>
<td>Coffee intake</td>
<td>(+) AhR</td>
<td>(Nordestgaard et al. 2020)</td>
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<td></td>
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<td>(+) CYP1A1/A2</td>
<td></td>
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<tr>
<td>Intervention clinical trial</td>
<td><em>Coffea arabica</em></td>
<td>(-) DNA strand breaks</td>
<td>(Pahlke et al. 2019)</td>
</tr>
<tr>
<td>Intervention clinical trial</td>
<td>Caffeinated and decaffeinated coffee</td>
<td>(+) Insulin sensitivity</td>
<td>(Reis et al. 2018)</td>
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<td></td>
<td></td>
<td>(+) GLP-1 and GIP</td>
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</table>

Legend: (-) = reduction/inhibition; (+) = increase/activation/improvement; CGA = Chlorogenic acid; SCG = spent coffee ground.
Figure 1. Mechanism of coffee’s antioxidant activity.
Figure 2. Coffee’s mechanism for anti-inflammatory activity.
Figure 3. Coffee’s Mechanism for protection against DNA damage.
Figure 4. Coffee’s mechanism for modulation of transcriptional factors.
Figure 5. Coffee’s mechanisms for controlling steroids metabolism.
Figure 6. Coffee’s mechanism for enhancing insulin resistance.
3. GREEN AND LIGHT ROASTED COFFEE EXTRACTED BY MICROWAVE ASSISTED EXTRACTION (MAE) INHIBIT PROSTATE CANCER PROGRESSION FEATURES IN DU-145 AND PC-3 METASTATIC-DERIVED CELL LINES

Original Article

GREEN AND LIGHT ROASTED COFFEE EXTRACTED BY MICROWAVE ASSISTED EXTRACTION (MAE) INHIBIT PROSTATE CANCER PROGRESSION FEATURES IN DU-145 AND PC-3 METASTATIC-DERIVED CELL LINES

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Abstract

Coffee consumption has been investigated as a protective factor for prostate cancer. Coffee drinking may be related to prostate cancer risk reduction due to its phytochemical compounds, such as caffeine, chlorogenic acids, and trigonelline. It has been shown that roasting process affects the content of the phytochemicals and undesired compounds can be formed. Microwave-assisted extraction is an option to conventional extraction techniques since it preserves more bioactive compounds. Therefore, this study aimed to evaluate the phytochemical composition and their putative preventive effects in prostate cancer development of 4 different coffee-roasting degrees extracted using microwave-assisted extraction. Coffea arabica green beans (1) were roasted into light (2), medium (3) and dark (4) and these four coffee samples were extracted using microwave-assisted extraction. Antioxidant capacity of these samples was evaluated by five different methods. Caffeine, chlorogenic acid and caffeic acid were measured through HPLC. Samples were tested in PC-3 and DU-145 metastatic prostate cancer cell lines, regarding their effects over cell viability, cell cycle progression and apoptotic cell death. We found that green and light roasted coffee extracts had the highest antioxidant activity. Caffeine content was not affected by roasting, chlorogenic acid was degraded due to the temperature, caffeic acid increased in light roast and decreased in medium and dark roast. Green and light roasted coffee extracts promoted higher inhibition on cell viability, caused cell cycle arrest in S and G2/M and induced apoptosis, when compared to medium and dark roasted coffee extracts and the control samples. Coffee extracts were more effective in DU-145 than in PC-3 cells. Our data provide early evidence that among the 4 tested samples, that the consumption of green and light coffee extracts contribute to inhibit prostate cancer tumor progression features, potentially preventing those aspects related to advanced prostate cancer subtypes.

**Keywords:** Coffee/ Prostate Cancer/ Roasting Degree/ MAE/ Chlorogenic acids/ PC-3/ DU-145.
1. Introduction

Prostate cancer (PCa) is the most common type of cancer among males corresponding to 17.7% of all types of cancer in men (WHO, 2018). Many factors contribute to PCa development, including age, family history, and race, besides probable contribution of environmental factors, such as smoking and diet (Ballon-Landa & Parsons, 2018). Once PCa has been identified, the treatment procedures includes chemotherapy and hormonal therapy, which have adverse effects (Litwin & Tan, 2017). It has been identified inverse correlations between coffee consumption and the incidence of cancer in many body sites, including oral, pharyngeal, colon, liver, prostate, endometrial and melanoma (Wang et al., 2016). The use of plant extracts has been widely tested as preventive agents for cancer (Greenwell & Rahman, 2015), among them coffee consumption (Palmioli et al., 2017).

The biochemical composition and bioactivities of coffee beans have been widely investigated due to their economic importance and the specific quality attributes associated with coffee beverages and human health. Caffeine is the most compound studied, which may reduce the risk of Parkinson and Alzheimer diseases, as well of, Type 2 diabetes, certain cancer types, besides presenting positive effect over liver function (Grosso, Godos, Galvano, & Giovannucci, 2017). Coffee is also a major source of chlorogenic acids (CGAs), which have antioxidant, anti-inflammatory, antidiabetic, anti-obesity, hepato-protective, antimicrobial and anti-hypertensive properties (Naveed et al., 2018). Trigonelline is one of the coffee alkaloids that presents antimicrobial, anti-carcinogenic and anti-hyperglycemic effects. Moreover, it has been reported that it prevents allergy, hyperlipidemia, and liver and kidney dysfunctions (Nugrahini, Ishida, Nakagawa, Nishi, & Sugahara, 2020).

Although coffee displays such physiological effects, these phytochemical’s contents are influenced by many factors, including roasting (Schenker & Rothgeb, 2017), grinding (Blittersdorff & Klatt, 2017) and brewing (Mestdagh, Glabasnia, & Giuliano, 2017). Roasting is one of the main coffee-processing steps, which causes many chemical reactions, mainly dehydration, Maillard reaction and Strecker degradation, which form the typical coffee aroma and color, while reducing the bioactive compound levels (Poisson, Blank, Dunkel, & Hofmann, 2017).

Caffeine is stable and its content is similar in green and roasted coffee, while trigonelline is partially degraded and converted to vitamin B3 and volatile compounds (Poisson et al., 2017). Chlorogenic acids are mostly lost during roasting, through degradation, formation of chlorogenic acid lactones and incorporation to melanoidins (Dawidowicz &
Typek, 2017). Lipids, including diterpenes, can form aldehydes through thermal degradation (Poisson et al., 2017). Moreover, acrylamide is formed during roasting, being generated through condensation of asparagine and reducing sugars, besides presenting carcinogenic properties (Bertuzzi, Martinelli, Mulazzi, & Rastelli, 2020).

Coffee is usually consumed as a beverage and there are many brewing techniques, such as by boiling, filtering or as “expresso”. Microwave Assisted Extraction (MAE) could be a different type of coffee extraction. It has advantages over other methods of extraction, due to shorter time and lower temperature, in which conditions bioactive compounds are less degraded. In addition, volatile compounds are preserved in a closed system and less solvent is needed (Lopes, Passos, Rodrigues, Teixeira, & Coimbra, 2020).

In this context, this study aimed to evaluate the phytochemical composition of 4 different coffee-roasting degrees extracted using MAE and regarding their putative preventive effects in prostate cancer development, by testing their effects over PC-3 and DU-145 PCa metastatic cell lines progression features.

2. Materials and methods

2.1. Sample Obtainment

Green Coffea arabica (Arabica coffee) beans were purchased from producer regions of the Rio de Janeiro state. The green coffee beans (GCB) were processed into 3 different roasting degrees: light roasted coffee (LRC, 12 minutes at 230 °C); medium roasted coffee (MRC, 14 minutes at 240 °C), and dark roasted coffee (DRC, 15 minutes at 245 °C), in the café® Gene roaster (Bauer et al., 2018). These roasting levels are classified by their color, according to the Agrtron Scale. GCB and the three roasted samples were then milled in a grinder (Cuisinart ®, East Windsor, NJ, USA). Then, these 4 milled-coffee samples were standardized according to the grain size, using two sets of sieves (Bertel, São Paulo, Brasil), with 0.850 and 0.600 mm granulometry. Samples were stored in at 4 ºC until extraction.

2.2. Microwave Assisted Extraction (MAE)

In order to prepare the coffee extracts to be tested in prostate cancer cells, the milled samples were prepared using distilled water (aqueous extract - AE) and 50% ethanol (ethanolic extract - EE) as solvents, because the objective was to test in cells, so the extraction solutions cannot be cytotoxic or should be able to be removed from the final solution. A microwave oven (DGT 100 Plus Provecto Analytic, São Paulo, Brazil) was used,
corresponding to a closed system, preventing volatile compounds loss. The MAE conditions were 40 s, at 240 W power at 50 °C in a 8:1 liquid-solid ratio, as previously reported to be effective extracting phenolics (Ranic et al., 2014). After the MAE process, samples were centrifuged at 1300 RPM in order to separate the solid phase from the liquid extract. Then, the coffee powder was subjected to a second extraction cycle to obtain the final extract.

2.3. Antioxidant activity

Antioxidant activity was analyzed by various methods: DPPH, ABTS, FRAP, Folin-ciocalteu and ORAC. Experiments were made using three concentrations of each coffee extract in triplicate (n = 9), except for ORAC.

2.3.1. DPPH

In DPPH method (Rufino, Alves, et al., 2007), antioxidant activity is analyzed based on the capacity of an extract to capture the DPPH radical (2,2-diphenyl-1-picrylhydrazyl). 0.5 mL of each sample reacted with 2.5 mL of DPPH radical in a methanolic solution. A standard curve was prepared, using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Aliquots stood in the dark for 30 min and absorbance was measured at 515 nm in a spectrophotometer (Sequoia-turner, model-340, USA). Antioxidant activity was calculated comparing to the effect of commercial antioxidant Trolox and is expressed in mMol of Trolox Equivalent (TE)/g fresh weight of coffee powder samples.

2.3.2. ABTS

This method is based on ABTS radical (2,2 Azinobis (3-ethyl-benzothiazoline-6-sulfonate)) arrest (Rufino, Brito, et al., 2007). 5 mL of ABTS radical was formed through oxidation by 88 µL of K₂S₂O₈ during 16 h. ABTS was diluted in ethanol and 2.5 mL was added to each extract. Trolox was used to plot a standard curve. Aliquots stood for 6 min and were read in an absorbance of 734 nm in a spectrophotometer (Sequoia-turner, model-340, USA). Trolox equivalent antioxidant capacity (TEAC) were expressed in mMol TE/g of fresh weight of coffee powder samples.

2.3.3. Ferric Reduction Antioxidant Power (FRAP)

FRAP method is based on the ability to reduce Fe (III) to Fe (II) in acid medium (Rufino, Alves, Brito, Morais, & Sampaio, 2006). FRAP reagent was composed by FeCl₃
solution, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) solution in 40 mM HCl and acetate buffer (pH 3.6). 0.3 mL of each extract was added to 2.7 mL of FRAP reagent. Ferrous sulfate was used to make a standard curve. After 30 min of reaction in warm bath, reading was performed in 595 nm in a spectrophotometer (Sequoia-turner, model-340, USA). Results are expressed in µMol of Ferrous Sulfate Equivalent (FeSO₄)/ g fresh weight of coffee powder samples.

2.3.4. Folin-Ciocalteu

Folin-Ciocalteu reagent based spectrophotometric method (Singleton & Rossi, 1965) is usually used to measure total phenolic compounds content. But is based in the compounds’ reducing capacity, therefore, it can be used as an antioxidant assay. Determination was performed adding 2.5 mL of Folin-Ciocalteu reagent 10 % to 0.5 mL aliquots of each coffee extracts. After 5 minutes, 2 mL of sodium carbonate 4 % was added. Gallic acid 0.1 % was used to make a standard curve. The samples stood for 2 h in the dark and absorbance was measured in a spectrophotometer (Sequoia-turner, model-340, USA) at 760 nm. Results were expressed in mg of Gallic Acid Equivalent (GAE)/ g fresh weight of coffee powder samples.

2.3.5. Oxygen Radical Absorbance Capacity (ORAC)

ORAC methodology (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002; Ou, Hampsch-Woodill, & Prior, 2001) is based on the ability of antioxidant compounds to capture the peroxyl radical. In a 96-well microplate, 20 µL of 8 concentrations of each sample were made with PBS buffer (pH 7.4). 120 µL of fluorescein was added and used as substrate. Generation of peroxyl radical was induced by addition of 60 µL AAPH (2,2’-azobis (2-amidinopropane) dihydrochloride). A control without AAPH, a blank with only buffer and a standard Trolox curve were prepared. The microplate was incubated for 3 h at 37°C and read every 5 min by the fluorimeter (SpectraMax i3, Molecular Devices, USA) under excitation at 485 nm and emission at 535 nm. The radical uptake causes a decrease in fluorescence emission. The area under curve (AUC) of the fluorescence decay was calculated and the blank AUC was subtracted from the samples AUC. Antioxidant activity was determined by comparing to the standard Trolox curve and expressed in mMol TE/ g fresh weight of coffee powder samples.
2.4. Phytochemical Composition

Contents were measured using high-performance liquid chromatograph (HPLC) in a Watters-Alliance 2695 chromatograph, photodiode array detector (PDA) 2996 and Empower® software (Waters, Massachusetts, USA).

2.4.1. Determination of caffeine content

For determination of caffeine, a BDS Hypersil C18 column (5 cm x 4.6 mm and 2.6 μm - Thermo Scientific, Massachusetts, USA) was used. The mobile phase was composed of 10 % acetonitrile in 0.5 % acetic acid (v/v) solution. The same solution was also used for sample extraction, using 1 gram in a 25 ml volumetric flask for 10 min in an ultrasound bath. Samples were filtered on rapid filtration filter paper and microfiltered in disposable hydrophilic Teflon filter with a porosity of 0.22 μm. The caffeine external standard was prepared by weighing approximately 30 mg of caffeine in a 30 ml volumetric flask, solubilized and swelled with the mobile phase. Samples were read at 280 nm with a mobile phase flow rate of 0.5 mL/min, with 20 μL of injection volume (Perrone, Donangelo, & Farah, 2008).

2.4.2. Determination of chlorogenic acid content

The determination of chlorogenic acid (5-CQA) was performed using a BDS Hypersil C18 column (5 cm x 4.6 mm and 2.6 μm - Thermo Scientific, Massachusetts, USA). The mobile phase gradient consisted of the initial composition of 5 % methanol (phase A) and 95 % of a 0.5 % formic acid solution (phase B) maintained for 6 minutes. In 8 minutes, the composition of the mobile phase reached 80% of phase A and remained so for up to 10 min. In 11 min, the composition reached the level of 100% of phase A and in 12 to 15 min the composition returned to the initial conditions. Samples were read at 325 nm, with flow rate mobile phase at 1 mL/min and 3 μL of injected volume. The samples were extracted in an ultrasound bath for 20 min with 20 % acetonitrile in ultrapure water (v/v). The samples were then centrifuged, microfiltered in disposable hydrophilic 0.22 μm Teflon filter. The external standard of chlorogenic acid (98 % w/w as 5-CQA) was prepared by weighing 30 mg of the standard for a 25 mL volumetric flask completed with 0.5% formic acid (Trugo & Macrae, 1984).

2.4.3. Determination of caffeic acid
For caffeic acid content, samples were extracted, with the mobile phase for 10 min in ultrasound and filtered (FHUP 0.45 μm) and injected into the HPLC. The system used consists of an isocratic pump, a reversed phase column (Waters Nova Pack C18 - 5 μm (150 mm x 4.6 mm)), UV detector at a wavelength of 330 nm and an integration, acquisition and data processing. Mobile phase was composed of 10% acetonitrile (MeCN) and 0.5% acetic acid (HOAc) (Rivelli et al., 2007).

2.5. Cell Assays

Prostate adenocarcinoma cell lines PC-3 and DU-145 were kindly provided by the National Cancer Institute (INCA). PC-3 is a prostate cancer bone metastasis cell line, while DU-145 is a prostate cancer brain metastasis cell line (Sobel & Sadar, 2005). The prostate cell lines were cultured in RPMI medium supplemented with 10 % fetal bovine serum, 1 % penicillin/ streptomycin and 1 % amphotericin, pH 7.4, under 5% CO2 atmosphere and 37 ºC temperature. Controls used in cell assays were the cell lines in the same medium without any extract.

2.5.1. MTT Cell Viability assay

Cell viability was determined using the MTT (3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyl-tetrazolium bromid) assay (Mosmann, 1983). The cells were plated in 96-well microplates with 2.0 x 10⁴ cells/ well. Cells were treated with 8 distinct concentrations of the samples and then incubated for 24, 48 and 72 h time points. 10 µL of MTT solution (5 g/l) was added to each well. After 4 hours incubation, the culture medium was removed and 50 µL of dimethyl sulfoxide (DMSO) was added in order to solubilize the formed formazan. Samples were read at an ELISA reader (Bio-Rad iMARK) at 570 nm. Cell viability was calculated by comparing to control (100%).

2.5.2. Cell Cycle analysis

Cells were plated in 6-well microplates with 5.0 x 10⁵/well. Prostate cancer cells incubated for 48 h in the presence and in absence of the 4 samples at different concentrations (1000 µg/ml and 5000 µg/ml) were detached using Trypsin solution at room temperature. The cell suspension was analyzed for DNA content by a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). It was used Vindlov’s reagent, reading 30000 cells for each replicate (Pozarowski & Darzynkiewicz, 2004). Cells with lower DNA content than
G₁ in the cell cycle distribution were considered hypodiploid cells (subG₁). Relative proportions of cells with apoptosis (< 2n), diploid G₀/G₁ (2n), S (> 2n, but < 4n), and G₂/M (4n) indicative DNA content were acquired using Cell Quest iPro. The percentage of cell population in each specific phase was estimated with FlowJo v 10.0.6 software and compared to control.

2.5.3. Apoptosis assays

The phosphatidylserine externalization assay using the flow cytometry technique (van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998) indicate the percentage of cells that are probably viable, in apoptosis or non-apoptotic cell death. Prostate adenocarcinoma cells were incubated in a 6-well microplate using 5.0 x 10⁵ cells/well with the extracts for 48 h. The cells were detached using a Trypsin solution, and then the propidium iodide and annexin markers were added. Detection was made by FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA), using Cell Quest iPro software, counting 30000 cells units/replicate. The cell populations analyzed were recognized by their forward scatter (FSC)/ side scatter (SSC) properties. Fluorescein isothiocyanate (FITC) green fluorescence was measured at 530 ± 30nm (detector FL1) and propidium iodide red fluorescence was measured at 585 ± 42 nm (FL2). The percentage of viable cells, and those cells in early or late apoptosis or nonapoptotic death was calculated using FlowJo v 10.0.6 software.

2.6. Statistical Analysis

All assays were made in triplicate and results were expressed by mean ± standard deviation. Data were analyzed using GraphPad Prism (version 5.04, GraphPad Software, San Diego, CA, USA). Results were compared by one-way variance analysis (ANOVA) using Tukey post-test with a confidence level of 95 %. In cell cycle and apoptosis assays it was tested 99 % and 99.9 % of confidence as well.

3. Results and Discussion

3.1. Coffee Extracts Present High Antioxidant Activity

Antioxidant activity is related to health benefits and prevention of chronic diseases. There are several direct and indirect methods for *in vitro* evaluation of antioxidant activity. These methods differ in how to evaluate this activity, and can be based on the reception of H
+ ions or reducing reaction (Cid & de Peña, 2016). Antioxidant activity of the 4 distinct coffee extracts was evaluated using the methods of DPPH, ABTS, FRAP, Folin-ciocalteu and ORAC (Figure 1).

In most tested coffee samples and methods there was no statistical difference between extractors. Usually, alcoholic extracts have higher extraction efficiency when compared to water, but chlorogenic acids, are polar, so both water and 50% ethanol would be able to extract a similar amount of these compounds (Ranic et al., 2014). In addition, water has a higher dielectric constant, which favors MAE because it is heated faster (Lopes et al., 2020).

In DPPH assay, there was no statistical difference between GCB (6.68 ± 0.81 mMol TE/g AE and 10.77 ± 0.77 mMol TE/g EE) and LRC (7.76 ± 0.47 mMol TE/g AE and 10.62 ± 0.27 mMol TE/g EE). Antioxidant activity reduced in MRC (5.71 ± 0.63 mMol TE/g AE and 5.13 ± 0.37 mMol TE/g EE) and DRC CE (5.20 ± 0.63 mMol TE/g AE and 3.38 ± 0.30 mMol TE/g EE).

In ABTS method, there was no statistical difference between AE in GCB, LRC, MRC and DRC (14.79 ± 1.22, 14.17 ± 1.76, 13.52 ± 0.68 and 13.04 ± 0.50 mMol TE/g, respectively). In EE, LRC had higher antioxidant activity (22.72 ± 0.94 mMol TE/g) than GCB, LRC and DRC (11.58 ± 0.82, 10.26 ± 0.06, and 8.58 ± 0.34 mMol TE/g, respectively).

In FRAP assay, antioxidant capacity increased from GCB (304.82 ± 5.54 μMol FSE/ g AE and 206.88 ± 2.57 μMol FSE/ g EE) to LRC (426.92 ± 4.27 μMol FSE/ g AE and 445.29 ± 4.09 μMol FSE/ g EE). Then, it reduced in MRC (318.87 ± 9.70 μMol FSE/ g AE and 202.32 ± 2.87 μMol FSE/ g EE) and DRC (289.79 ± 1.09 μMol FSE/ g AE and 188.11 ± 2.06 μMol FSE/ g EE) without a statistical difference.

In Folin-Ciocalteu method, AE showed no significant difference in GCB (31.74 ± 2.67 mg AGE/ g) and LRC (31.90 ± 2.87 mg AGE/ g). In EE, antioxidant activity increased from GCB (28.00 ± 0.95 mg AGE/ g) to LRC (35.34 ± 1.07 mg AGE/ g). Then, in both extracts, there was a reduction in MRC (13.00 ± 1.85 mg AGE/ g AE and 11.79 ± 0.56 mg AGE/ g EE) and increased in DRC (17.75 ± 0.61 mg AGE/ g AE and 29.59 ± 0.57 mg AGE/ g EE).

In ORAC method, antioxidant activity decreased from GCB (2.41 ± 0.52 mMol TE/g AE and 2.48 ± 0.14 mMol TE/g EE) to LRC (1.47 ± 0.14 mMol TE/g AE and 1.54 ± 0.33 mMol TE/g EE), increased in MRC (2.37 ± 0.30 mMol TE/g AE and 2.08 ± 0.02 mMol TE/g EE) and decreased again in DRC (1.48 ± 0.40 mMol TE/g AE and 1.45 ± 0.45 μmol TE/g EE).
A trend can be observed between methods regarding the antioxidant activity of roasting degrees. Overall, the LRC antioxidant activity was slightly higher than GCB, then the antioxidant activity decreased as the degree of roasting increased in MRC and DRC. This trend is in line with the results described in other studies, which observed that antioxidant activity increases in the beginning of roasting and reduces with further roasting (Liang, Xue, Kennepohl, & Kitts, 2016; Opitz et al., 2017; Sunarharum, Yuwono, & Aziza, 2019).

Dehydration during roasting may contribute to this increase, concentrating antioxidant compounds due to mass loss (Priftis et al., 2018). However, the initial degradation of 5-caffeoylquinic acid (5-CQA) may be involved in this increase as well. 5-CQA is the most abundant phenolic compound in green coffee and is more thermolabile than its isomers (3-CQA and 4-CQA). Initially, 5-CQA degradation can generate products that are potent antioxidants (Kamiyama, Moon, Jang, & Shibamoto, 2015).

An exception to that trend is ORAC, which presented a higher antioxidant activity in GCB and MRC. Although some studies observed a higher antioxidant activity in light roasted coffee than in medium and dark roast in ORAC assay (Bauer et al., 2018; Liang et al., 2016), other studies showed that this difference was not significant, while in other assays was (Mojica et al., 2018; Opitz et al., 2017). The antioxidant activity observed in MRC may be related to incorporation of chlorogenic acids into melanoids, that continue to have antioxidant activity (Perrone, Farah, & Donangelo, 2012).

3.2. Roasting Affects the Content of Caffeine, Chlorogenic Acid and Caffeic Acid

The content of phytochemical compounds is expressed in figure 2. In general, content of phytochemicals in AE was higher than in EE. Caffeine was less affected by roasting than chlorogenic acid and caffeic acid. Caffeine is resistant to temperature and is not significantly degraded during roasting (Vignoli, Viegas, Bassoli, & Benassi, 2014). The increase in caffeine concentration observed can be attributed to mass loss due to dehydration that occurs during roasting process (Priftis et al., 2018).

In AE, caffeine increased from GCB (30.27 ± 0.31 mg/mL) to LRC (32.77 ± 0.25 mg/mL) and decreased in MRC (30.11 ± 0.52 mg/mL) and DRC (26.47 ± 0.45 mg/mL), with no statistical difference between GCB and MRC. In EE caffeine content increased as roasting level raised. It was observed 25.47 ± 0.35 mg/mL in GCB and 28.71 ± 0.41 mg/mL in LRC, with no significant difference between MRC (35.57 ± 0.51 mg/mL) and DRC (35.80 ± 0.36 mg/mL).
As expected, CGA content reduced during roasting. GCB-AE presented higher amount of CGAs (18.52 ± 0.26 mg/mL) than EE (15.95 ± 0.32 mg/mL). This is in line with the results found in antioxidant activity, that was higher in AE in some assays. There was no statistical difference between AE and EE in LRC (8.28 ± 0.23 and 8.14 ± 0.17 mg/mL, respectively), MRC (2.82 ± 0.20 and 2.91 ± 0.12 mg/mL, respectively) and DRC (0.95 ± 0.09 and 0.84 ± 0.11 mg/mL, respectively).

During roasting, CGAs are degraded into volatile compounds, are broke into caffeic acid and quinic acids, and are transformed into chlorogenic acid lactones (Dawidowicz & Typek, 2017). Part of CGA are also incorporated into melanoidins through transglycosylation reaction (Moreira et al., 2017).

The AE presented higher caffeic acid content in GCB (14.70 ± 0.46 mg/mL) and LRC (20.23 ± 0.34 mg/mL) than in EE (11.57 ± 0.44 and 18.77 ± 0.31 mg/mL, respectively). Then, caffeic acid content decreased in MRC (8.69 ± 0.37 mg/mL in AE and 8.05 ± 0.22 mg/mL in EE) and DRC (3.17 ± 0.30 mg/mL in AE and 2.74 ± 0.27 mg/mL in EE), without statistical difference between AE and EE.

Caffeic acid increased from GCB to LRC, due to initial degradation of CGA that releases caffeic acid and decreased in MRC and DRC due to its further thermal degradation (Dawidowicz & Typek, 2017). The content of caffeic acid may explain antioxidant activity observed in the present study, because it has higher radical scavenging power than 5-CQA (Kamiyama et al., 2015).

### 3.3. Influence of Coffee Extracts on Prostate Cancer Cells

As it was found no important difference between extractors in antioxidant activity and phytochemical content, the AE was chosen to be tested in PCa cell lines. Besides that, the AE is non-cytotoxic, more physiological and would be more appropriate for consumption.

#### 3.3.1 Coffee Extracts Influence on Cell Viability Reduction

Extracts were tested in 24 h, 48 h and 72 h in cell viability assay (MTT). For both cell lines within 72 h the extracts showed no difference in cell viability compared to control, indicating that the cells possibly returned to proliferate after the bioactive compounds from the extracts were consumed. Chlorogenic acids are quickly absorbed and metabolized by cells (Clifford, Jaganath, Ludwig, & Crozier, 2017). This indicates that continuous treatment would be required for effective outcomes. The results of the various extract concentrations tested
over viability of both cell lines in 24 and 48 h are shown in Figure 3. The concentration 5000 µg / ml was the most effective and is discussed hereafter.

For PC-3 at 24 h (figure 3A), GCB showed the highest reduction in viability, 17.77% ± 7.08. There was no statistical difference in cell viability among LRC, MRC and DRC (6, 95% ± 1.13, 10.00% ± 2.07 and 4.51% ± 0.56, respectively).

At 48 h (figure 3B), PC-3 viability was further reduced for all samples. GCB and LRC caused higher reduction in cell viability, 53.45% ± 3.72 and 45.16% ± 1.09 respectively. There was no statistical difference for the MRC and DRC, where the viability reduced 33.00% ± 3.56 and 22.52% ± 2.95, respectively.

In DU-145 at 24 h (figure 3C), the reduction in cell viability was more extensive compared to PC-3. GCB and LRC caused a large reduction in viability, 73.21% ± 3.87 and 64.08% ± 3.63 respectively. For the MRC and DRC, there was no statistical difference in viability reduction (43.19% ± 5.52 and 38.45% ± 5.00, respectively).

At 48 h (figure 3D), there was no statistical difference between GCB and LRC, which showed the greatest reduction in viability (56.57% ± 1.54 and 52.37% ± 8, 23, respectively). In addition, these samples showed considerable reduction in cell viability at all concentrations tested. There was no statistical difference between MRC and DRC (38.66% ± 5.62 and 38.21% ± 3.27, respectively).

These results demonstrate that GCB and LRC could possibly be more effective against PCa. These results agree with previous work, which observed a significant reduction in viability of the DU-145 strain in 24 h, mainly in green coffee samples (Bauer et al., 2018).

Many epidemiological studies have investigated the relationship between coffee consumption and PCa risk and have shown varied effects. While some studies show no relationship between coffee consumption and PCa (Arab et al., 2012; Bosire et al., 2013; Geybels, Neuhouser, & Stanford, 2013; Park et al., 2010), many studies showed reduced risk of PCa with increased coffee consumption (Cao et al., 2014; Discacciati et al., 2013; Geybels, Neuhouser, Wright, Stott-Miller, & Stanford, 2013; Li et al., 2013; Liu et al., 2015; Lu et al., 2014).

One possible cause for this discrepancy is that these studies do not consider the type of coffee being consumed by the population. Coffee presents great variability in its composition in relation to factors such as species and variety (Ciaramelli, Palmioli, & Airoldi, 2019; Patay, Bencsik, & Papp, 2016), geographical origin (De Luca et al., 2016; de Toledo et al., 2017), edaphoclimatic conditions (Kitsberger, Scholz, Pererira, da Silva, & Benassi, 2016),
fermentation and processing (dry or wet) (De Bruyn et al., 2017), storage (Abreu, Borém, Oliveira, Almeida, & Alves, 2019), roasting level (Dawidowicz & Typek, 2017; Opitz et al., 2017) and brewing method (Xu et al., 2019) among others. This can influence the amount of bioactive coffee compounds being consumed and influence the long-term effect on PCa risk.

In particular, the level of roasting would be important in these epidemiological data, as lighter roasts are less consumed by the general population. Precisely, green, and light roasted coffee showed higher antioxidant activity and more extensive reduction of cell viability. There are no epidemiological studies specifically investigating light roasted coffee and there could be a greater and more remarkable reduction in PCa risk.

It is interesting to note that coffee caused a faster and more marked reduction in cell viability in the DU-145 cell line than in PC-3. Although PC-3 is more aggressive (Kwon et al., 2018), DU-145 may be equivalent to a more advanced stage of PCa because it represents a brain metastasis compared to a bone metastasis (PC-3). This is in line with some epidemiological studies showing that coffee has a greater risk reduction over more advanced forms of PCa than non-aggressive and localized forms (Lu et al., 2014; Shafique et al., 2012; Wilson et al., 2013, 2011).

3.3.2. Coffee Extracts Cause Cell Cycle Arrest and Induce Apoptosis

In MTT assay, 1000 and 5000 µg/ml concentrations caused the greatest reduction in cell viability and this reduction was more extensive in 48h. Hence, these concentrations were tested at this time for cell cycle and apoptosis assays. Examples of cell cycle histograms are shown in the supplementary material (S1).

Results of coffee extracts effects on PC-3 cell cycle are shown in Table 1. LRC (9.58 % ± 0.25) and MRC (11.45 % ± 1.20) 1000 µg/ml increased the number of cells in subG1 compared to control (p < 0.001), followed by LRC (8.52 % ± 2.54) 5000 µg/ml, DRC 1000 µg/ml (7.38 % ± 1.94) (p < 0.01), GCB 1000 µg/ml (6.29 % ± 0.31) and DRC 5000 µg/ml (6.25 % ± 1.50) (p < 0.05). In G0/G1 phases, GCB 5000 µg/ml (49.71 % ± 1.20) and LRC 1000 µg/ml (61.67 % ± 3.28) caused the greatest reduction in the percentage of cells (p < 0.001), followed by DRC 5000 µg/ml (62.31 % ± 1.57) (p < 0.01) and the concentration of 1000 µg/ml of GCB (64.84 % ± 0.68) and DRC (66.30 % ± 0.48) (p < 0.05), other samples had no statistical difference compared to the control. GCB (12.52 % ± 0.78) and DRC (10.22 % ± 0.99) 5000 µg/ml increased cells in S phase (p < 0.001). Only 5000 µg/ml of GCB (32.93 % ± 1.34) had a significant increase in G2/M phases (p < 0.001).
The effects of the extracts on DU-145 cell cycle are expressed in Table 2. DRC 5000 µg/ml (12.68 % ± 3.25) provoked the highest increase in subG1 (p < 0.001), followed by LRC 1000 µg/ml (11.26 % ± 0.49) (p < 0.01) and MRC 1000 µg/ml (9.96 % ± 2.97) (p < 0.01). In G0/G1 phases, GCB in 1000 µg/ml (43.30 % ± 0.98) and 5000 µg/ml (52.27 % ± 2.74) and MRC in 1000 µg/ml (47.31 % ± 0.48) and DRC in both concentration (49.30 % ± 0.40 and 46.52 % ± 3.17, respectively) caused a significant reduction in cells in G0/G1 (p < 0.001). GCB and DRC in 5000 µg/ml (25.56 % ± 0.56 and 23.20 % ± 1.28, respectively) provoked the most important (p < 0.001) increase in cells in S phase, followed by LRC 1000 µg/ml (7.81 % ± 1.19) and MRC 5000 µg/ml (9.22 % ± 0.31) (p < 0.01). In G2/M phases, GCB 1000 µg/ml (34.83 % ± 1.94) (p < 0.001), MRC 1000 µg/ml (24.22 % ± 0.26) and DRC 5000 µg/ml (12.61 % ± 1.93) (p < 0.05) caused a significant increase in the number of cells.

According to these results, GCB was the most effective in both cell lines compared to the other extracts and to the control. In general, extracts caused more significant alterations in DU-145 than in PC-3, just as it was observed in MTT assay. It can be considered that coffee samples caused a cell cycle arrest in S and G2/M phases.

It has been previously observed that CGAs and its derivatives causes a reduction in cells in G0/G1 phases and cell cycle arrest in S phase in colon cancer cells (Sadeghi Ekbatan, Li, Ghorbani, Azadi, & Kubow, 2018) and prostate cancer cells (Lodise et al., 2019). CGAs are probably the major responsible for cell cycle arrest in S phase in GCB.

Trigonelline is known for regulating cell cycle and promotes G2 phase arrest and may be responsible to results observed in this phase (Mohamadi, Sharififar, Pournamdari, & Ansari, 2018). Other phytochemicals might also be responsible for the cell cycle arrest in G2/M phase observed.

The change in cell cycle due to DRC may be caused by other compounds, since most of CGAs and trigonelline are degraded during roasting (Dawidowicz & Typek, 2017). It is widely known that acrylamide is formed during coffee roasting by the condensation of asparagine and reducing sugars (Bertuzzi et al., 2020; Schouten, Tappi, & Romani, 2020). Acrylamide has been shown to reduce cells in G0/G1 phases and raise the number of cells in S and G2/M phases in astrocytoma cells. Nevertheless, acrylamide negatively influenced repair mechanisms and caused mitochondrial damage, inducing apoptosis in healthy astrocytes cells, that are part of neural tissue and protect neurons from oxidative damage (Chen et al., 2013). Cell cycle alterations caused by DRC and MRC in DU-145 cell line, observed in the present study, may be entailed by acrylamide’s effects.
The effect of the extracts on PC-3 apoptosis is presented in Table 3 and Figure 4 (A). There was no significant difference in the percentage of viable cells in relation to the control (84.77 ± 2.78). Only LRC in 5000 µg/ml concentration significantly increased the number of cells in early apoptosis (8.49 ± 1.41, p < 0.001) and late apoptosis (7.33 ± 4.93, p < 0.05) compared to the control (2.91 ± 0.56 and 5.59 ± 0.76, respectively). In nonapoptotic cell death, unexpectedly, extracts reduced the number of cells in relation to the control (6.23 ± 0.53). LRC (1.05 ± 0.40), MRC (2.05 ± 0.40) and DRC (1.48 ± 0.68) in 5000 µg/ml showed the greatest reduction (p < 0.001), followed by GCB 5000 µg/ml (2.68% ± 1.34) and MRC 1000 µg/ml (3.19% ± 0.49) (p < 0.01).

Results in DU-145 apoptosis are expressed in Table 4 and Figure 4 (B). All samples caused significant reduction (p < 0.001) in viable cells compared to control (84.93 ± 2.05). LRC 5000 µg/ml caused the greatest reduction (58.83 % ± 1.88). No sample showed statistical difference (p > 0.05) in early apoptosis compared to control (7.42 % ± 0.98). In late apoptosis, all samples caused significant (p < 0.001) increase in apoptotic cells, LRC 5000 µg/ml provoked the highest numerical increase (24.33 % ± 1.11) compared to control (5.60 % ± 0.92). Although all samples caused an increase in non-apoptotic cell death, only LRC (10.16 % ± 1.05), MRC (8.88 % ± 1.85) and DRC (9.41 % ± 3.38) samples in 5000 µg/ml (p < 0.001) and MRC 1000 µg/ml (6.44 % ± 0.60) (p < 0.05) caused a statistically significant raise compared to control (2.02 % ± 0.95).

Again, extracts were more effective in DU-145 than in PC-3, in which only LRC showed a greater alteration in apoptosis. In DU-145, all extracts were able to reduce viable cells and increase late-apoptotic cells.

It was expected that higher CGA content in extracts would cause a higher change in apoptosis. However, isomerization and hydrolysis of 5-CQA produces compounds that modulates cell cycle and induces apoptosis (Bauer et al., 2018). This can explain the greater apoptosis induction observed in LRC. In darker roasts CGAs and their derivatives are further degraded (Schenker & Rothgeb, 2017), explaining the reduction of effectiveness observed.

In DU-145 cell line, only roasted samples in the higher concentration caused non-apoptotic cell death. This indicates that nonapoptotic cell death may be caused by compounds formed or released during roasting, such as acrylamide, which is known to be cytotoxic and carcinogenic (Kumar, Das, & Teoh, 2018). Non-apoptotic cell death usually corresponds to necrosis, results in plasma membrane rupture and release of the intracellular content into the microenvironment and occurs due to extreme conditions or toxic compounds (Ye et al., 2018).
This result suggests that the compound formed during roasting may have cytotoxic effects on healthy cells. Thus, it is needed to test toxicity on these extracts.

Coffee provoked no cytotoxic effect in healthy 3T3-L1 cell line (see supplementary material, S2). Furthermore, no cytotoxic effect was observed in healthy macrophages cells (RAW 2647) and hepatocytes (AML-12) when treated with different degrees of roasting, even in high concentrations (Jung, Kim, Park, Jeong, & Ko, 2017). Green coffee cause no significant raise in non-apoptotic cell death, indicating that it is safer to use. Furthermore, green coffee supplementation in bread presented a cytoprotective effect in prostate epithelial cells (Pnt2) (Gawlik-Dziki et al., 2019).

4. Conclusions

Therefore, coffee has high antioxidant capacity, regardless of roasting level, but GCB and LRC have higher antioxidant activity compared to MRC and DRC. CGA was degraded during roasting, caffeic acid content increased in LRC and decreased in further processing. GCB and LRC also caused greater reduction in cell viability in both lines, however, they were more effective in DU-145. LRC caused the greatest alteration in cell cycle and apoptosis. Extracts were also more effective in modulating cell cycle and apoptosis in DU-145 than in PC-3. Therefore, GCB and LRC are likely to be effective against PCa. The higher effectiveness observed in DU-145 than PC-3 indicates that coffee intake may be more powerful against more advanced stages of prostate cancer.

However, further cell experiments are needed to confirm those findings and to evaluate the molecular mechanisms and pharmacokinetics involved in the effects observed. Bioavailability of extracts should be investigated to determine the quantity that would need to be ingested to achieve such effects. Toxicity assays should be done to secure safety. In addition, in vivo and clinical tests would be required to allow the recommendation of the consumption of these types of coffee against prostate cancer.

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Competing Interests: The authors declare no conflict of interest.

References


Figure 1. Antioxidant activity of 4 coffee extracts by different methods: DPPH (A), ABTS (B), FRAP (C), Folin-Ciocalteu (D) and ORAC (E), 50% Hydroethanolic extract (EE) or Aqueous extract (AE) solutions. Different letters indicate statistical difference (p < 0.05). Abbreviations: GCB – green coffee bean; LRC – light roasted coffee; MRC – medium roasted coffee; DRC – dark roasted coffee.
Figure 2. Content (mg/mL) of caffeine (A), Chlorogenic acid (B), and Caffeic acid (C), of 50% Hydroethanolic extract (EE) or Aqueous extract (AE) solutions. Different letters indicate statistical difference (p < 0.05). Abbreviations: GCB – green coffee bean; LRC – light roasted coffee; MRC – medium roasted coffee; DRC – dark roasted coffee.
Figure 3. Cell viability in response to different concentrations of coffee extracts samples was tested by MTT assays: PC-3 24 h (A), PC-3 48 h (B), DU-145 24 h (C) and DU-145 48 h (D).
Table 1. Effect of coffee extracts on cell cycle progression in PC-3 cells after 48 hours.

<table>
<thead>
<tr>
<th>Group</th>
<th>Con.</th>
<th>SubG1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.28±0.89</td>
<td>75.11±1.17</td>
<td>3.64±1.53</td>
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<td>GCB</td>
<td>1000 µg/ml</td>
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<td>64.84±0.68&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5.91±1.69</td>
<td>22.83±1.69</td>
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<td></td>
<td>5000 µg/ml</td>
<td>4.84±2.19</td>
<td>49.71±1.20&lt;sup&gt;***&lt;/sup&gt;</td>
<td>12.52±0.78&lt;sup&gt;***&lt;/sup&gt;</td>
<td>32.93±1.34&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td>LRC</td>
<td>1000 µg/ml</td>
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<td>61.67±3.28&lt;sup&gt;***&lt;/sup&gt;</td>
<td>4.56±1.03</td>
<td>22.17±1.35</td>
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<td></td>
<td>5000 µg/ml</td>
<td>8.52±2.54&lt;sup&gt;***&lt;/sup&gt;</td>
<td>67.24±4.35</td>
<td>3.89±0.19</td>
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<td>MRC</td>
<td>1000 µg/ml</td>
<td>11.45±1.20&lt;sup&gt;***&lt;/sup&gt;</td>
<td>66.95±3.27</td>
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<td>5000 µg/ml</td>
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<tr>
<td>DRC</td>
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<td>66.30±0.48&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5.47±0.86</td>
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<td></td>
<td>5000 µg/ml</td>
<td>6.25±1.50&lt;sup&gt;**&lt;/sup&gt;</td>
<td>62.31±1.57&lt;sup&gt;**&lt;/sup&gt;</td>
<td>10.22±0.99&lt;sup&gt;***&lt;/sup&gt;</td>
<td>20.68±0.67</td>
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</table>

Results are expressed as percentage of total cells. Significant differences between untreated cells (Control) and cells treated with coffee extracts were compared (* p < 0.05; ** p < 0.01; *** p < 0.001). Abbreviations: GCB – green coffee bean; LRC – light roasted coffee; MRC – medium roasted coffee; DRC – dark roasted coffee; Con.-concentration.
### Table 2. Effect of coffee extracts on cell cycle progression in DU-145 cells after 48 hours.

<table>
<thead>
<tr>
<th>Group</th>
<th>Con.</th>
<th>SubG1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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<td>50.49±3.26***</td>
<td>25.56±0.56***</td>
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<tr>
<td>LRC</td>
<td>1000 µg/ml</td>
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<td>63.03±1.72</td>
<td>7.81±1.19**</td>
<td>17.90±1.02</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>6.50±1.86</td>
<td>58.14±2.32</td>
<td>14.28±1.60</td>
<td>21.09±0.39</td>
</tr>
<tr>
<td>MRC</td>
<td>1000 µg/ml</td>
<td>9.96±2.97*</td>
<td>47.31±0.48***</td>
<td>18.51±3.71</td>
<td>24.22±0.26*</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>3.72±1.75</td>
<td>70.19±3.66</td>
<td>9.22±0.31**</td>
<td>16.87±1.70</td>
</tr>
<tr>
<td>DRC</td>
<td>1000 µg/ml</td>
<td>7.20±1.33</td>
<td>49.30±0.40***</td>
<td>21.15±0.14</td>
<td>22.61±1.08</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>12.68±3.25***</td>
<td>46.52±3.17***</td>
<td>23.20±1.28***</td>
<td>12.61±1.93*</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of total cells. Significant differences between untreated cells (Control) and cells treated with coffee extracts were compared (* p < 0.05; ** p < 0.01; *** p < 0.001). Abbreviations: GCB – green coffee bean; LRC – light roasted coffee; MRC – medium roasted coffee; DRC – dark roasted coffee; Con.-concentration.
**Figure 4.** Apoptosis induction in 48 h in PC-3 (A) and DU-145 (B).
**Table 3.** Effect of coffee extracts on programmed cell death in PC-3 cells after 48 hours.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Con.</th>
<th>Viable Cells</th>
<th>Early Apoptosis</th>
<th>Late Apoptosis</th>
<th>Nonapoptotic Cell Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>84.77±2.78</td>
<td>2.91±0.56</td>
<td>5.59±0.76</td>
<td>6.23±0.53</td>
</tr>
<tr>
<td>GCB</td>
<td>1000 µg/ml</td>
<td>90.50±4.16</td>
<td>0.45±0.28*</td>
<td>2.26±0.72</td>
<td>5.62±0.91</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>85.47±2.75</td>
<td>3.48±1.05</td>
<td>8.74±2.33</td>
<td>2.68±1.34**</td>
</tr>
<tr>
<td>LRC</td>
<td>1000 µg/ml</td>
<td>84.55±1.34</td>
<td>2.95±0.83</td>
<td>5.83±1.47</td>
<td>4.89±1.02</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>86.40±4.95</td>
<td>8.49±1.41***</td>
<td>9.82±1.94*</td>
<td>1.05±0.40***</td>
</tr>
<tr>
<td>MRC</td>
<td>1000 µg/ml</td>
<td>86.65±0.78</td>
<td>3.48±0.27</td>
<td>9.07±2.79</td>
<td>3.19±0.49**</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>91.30±2.17</td>
<td>2.96±0.23</td>
<td>3.70±1.47</td>
<td>2.05±0.40***</td>
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<tr>
<td>DRC</td>
<td>1000 µg/ml</td>
<td>87.70±5.23</td>
<td>2.28±0.78</td>
<td>6.63±0.84</td>
<td>5.13±0.35</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>86.63±2.56</td>
<td>5.20±1.15</td>
<td>6.37±0.68</td>
<td>1.48±0.68***</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of total cells. Significant differences between untreated cells (Control) and cells treated with coffee extracts were compared (* p < 0.05; ** p < 0.01; *** p < 0.001). Abbreviations: GCB – green coffee bean; LRC – light roasted coffee; MRC – medium roasted coffee; DRC – dark roasted coffee; Con.-concentration.
Table 4. Effect of coffee extracts on programmed cell death in DU-145 cells after 48 hours.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Con.</th>
<th>Viable Cells</th>
<th>Early Apoptosis</th>
<th>Late Apoptosis</th>
<th>Nonapoptotic Cell Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>84.93±2.05</td>
<td>7.42±0.98</td>
<td>5.60±0.92</td>
<td>2.02±0.95</td>
</tr>
<tr>
<td>GCB</td>
<td>1000 µg/ml</td>
<td>72.50±1.28***</td>
<td>7.88±0.88</td>
<td>15.53±1.24***</td>
<td>4.13±0.91</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>69.00±1.84***</td>
<td>4.42±1.70</td>
<td>16.75±1.06***</td>
<td>4.89±0.96</td>
</tr>
<tr>
<td>LRC</td>
<td>1000 µg/ml</td>
<td>71.47±1.42***</td>
<td>10.03±0.55</td>
<td>15.70±1.22***</td>
<td>2.78±0.40</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>58.83±1.88***</td>
<td>5.71±0.72</td>
<td>24.33±1.11***</td>
<td>10.16±1.05***</td>
</tr>
<tr>
<td>MRC</td>
<td>1000 µg/ml</td>
<td>65.40±1.04***</td>
<td>8.18±0.72</td>
<td>19.97±1.34***</td>
<td>6.44±0.60*</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>67.63±1.72***</td>
<td>6.41±0.49</td>
<td>17.10±0.52***</td>
<td>8.88±1.85***</td>
</tr>
<tr>
<td>DRC</td>
<td>1000 µg/ml</td>
<td>72.50±1.65***</td>
<td>8.43±0.33</td>
<td>15.77±0.93***</td>
<td>3.35±0.53</td>
</tr>
<tr>
<td></td>
<td>5000 µg/ml</td>
<td>61.27±4.34***</td>
<td>7.73±1.43</td>
<td>21.60±2.62***</td>
<td>9.41±3.38***</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of total cells. Significant differences between untreated cells (Control) and cells treated with coffee extracts were compared (* p < 0.05; *** p < 0.001). Abbreviations: GCB – green coffee bean; LRC – light roasted coffee; MRC – medium roasted coffee; DRC – dark roasted coffee; Con.-concentration.
SUPPLEMENTARY MATERIAL

S1. Examples of cell cycle histograms of control (A) and coffee extract treatment (B).

S2. Percentage inhibition in comparison with control of viability of murine fibroblast cell line (3T3 cells) by MTT method, after 24 hours of treatment with coffee extracts (5000mg/m). Abbreviations: CT – control, GCB – green coffee bean, LRC – light roasted coffee, MRC – medium roasted coffee, DRC – dark roasted coffee.
5. CONCLUSÃO

O café tem alta capacidade antioxidante, independentemente do nível de torrefação, mas o café verde e a torra clara do café têm maior atividade antioxidante em comparação com as torras média e escura do café. O ácido clorogênico foi degradado durante a torrefação, o teor de ácido cafêico aumentou na torra clara e reduziu com a continuação do processamento. O café verde e a torra clara do café também causaram maior redução na viabilidade celular em ambas as linhagens, no entanto, foram mais eficazes contra a DU-145. Esses extratos também foram mais eficazes na modulação do ciclo celular e na indução de apoptose na DU-145. Portanto, é provável que o café verde e a torra clara sejam eficazes na prevenção e tratamento do PCa. A maior eficácia observada na DU-145 do que na PC-3 indica que a ingestão de café pode ser mais potente na prevenção de tipos mais avançados de câncer de próstata.

Há evidências de que café pode reduzir o risco de desenvolver PCa através de muitos mecanismos. No entanto, são necessários novos experimentos celulares para confirmar esses achados e avaliar a forma de morte celular e possível toxicidade para garantir a segurança. É essencial mais estudos moleculares para verificar a os mecanismos de ação do café no PCa. Além disso, testes in vivo e clínicos seriam necessários para permitir a recomendação do consumo de café para a prevenção do PCa.
6. REFERÊNCIAS


Molecular mechanisms for coffee compounds on prostate cancer prevention

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<td>Montenegro, Julia; Universidade Federal do Estado do Rio de Janeiro, Food Science, Laboratory of Functional Foods; Freitas-Silva, Otnei; Empresa Brasileira de Pesquisa Agropecuaria Agroindústria de Alimentos; Teodoro, Anderson; Universidade Federal do Estado do Rio de Janeiro, Food Science, Laboratory of Functional Foods</td>
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<tr>
<td>Keywords:</td>
<td>coffee, prostate cancer, antioxidant, anti-inflammatory, mechanism</td>
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</tbody>
</table>

URL: [http://mc.manuscriptcentral.com/lhti](http://mc.manuscriptcentral.com/lhti) Email: nwhartal@wisc.edu
ANEXO 2

Research International Elsevier Editorial System (tm) for Food Manuscript Draft

Manuscript Number:

Title: Bioactive compounds, antioxidant activity and antiproliferative effects in prostate cancer cells of green and light roasted coffee extracted by microwave assisted extraction (MAE)

Article Type: VSI: SLAC 2019

Keywords: Coffee; Prostate Cancer; Roasting Degree; MAE; Chlorogenic acids; PC-3; DU-145.

Corresponding Author: Dr. Anderson Teodoro,
Corresponding Author's Institution:
First Author: Julia Montenegro

Order of Authors: Julia Montenegro; Lauriza dos Santos; Rodrigo de Souza; Larissa Gabrielly Lima; Daniella Mattos; Bruna Viana; Ana Clara Fonseca; Leda Muzzi; Carlos Conte-Júnior; Átel Gizba; Otiel Freitas-Silva; Anderson Teodoro

Abstract: Coffee consumption has been investigated as a protective factor for prostate cancer (PCa). Coffee drinking may be related to prostate cancer risk reduction due to its phytochemical compounds, such as caffeine, chlorogenic acids, and trigonelline. It has been shown that roasting process affects the content of the phytochemicals and undesired compounds can be formed. Microwave-assisted extraction (MAE) is an option to conventional extraction techniques since it preserves more bioactive compounds. Therefore, this study aimed to evaluate the phytochemical composition and their putative preventive effects in prostate cancer development of 4 different coffee-roasting degrees extracted using MAE. Coffea arabica green beans (1) were roasted into light (2), medium (3) and dark (4) and these four coffee samples were extracted using MAE. Antioxidant capacity of these 4 samples was evaluated using DPPH, ABTS, FRAP, Folin-ciocalteu and ORAC methods. Caffeine, chlorogenic acid and caffeic acid were measured through HPLC. Samples were tested in PC-3 and DU-145 metastatic PCA cell lines, regarding their effects over cell viability, cell cycle progression and apoptotic cell death. We found that green and light roasted coffee extracts had the highest antioxidant activity. Caffeine content was not affected by roasting, chlorogenic acid was degraded due to the temperature, caffeic acid increased in light roast and decreased in medium and dark roast. Green and light roasted coffee extracts promoted higher inhibition on cell viability, caused cell cycle arrest in S and G2/M and induced apoptosis, when compared to medium and dark roasted coffee extracts and the control samples. Coffee extracts were more effective in DU-145 than in PC-3 cells. Our data provide early evidence that among the 4 tested samples, that the consumption of green and light coffee extracts contribute to inhibit PCa tumor progression features, potentially preventing those aspects related to advanced PCA subtypes.