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INSTITUTO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA
E
TECNOLOGIA DE ALIMENTOS

TESE

EFEITO PROTETOR DA SALSA (*Petroselinum crispum*) (Mill.) Nym. E CEBOLINHA (*Allium schoenoprasum* L) COMO ANTIOXIDANTES NATURAIS SOBRE A OXIDAÇÃO LIPÍDICA EM SARDINHAS PREPARADAS TERMICAMENTE

FERNANDA SILVA FERREIRA

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Sob a Orientação da Professora
DSc. Dra. Tatiana Saldanha

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Tese submetida como requisito parcial para obtenção do grau de **Doutor em Ciência e Tecnologia de Alimentos,** no Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Área de Concentração em Ciência de Alimentos. Alimentos.

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(Masaharu Taniguchi)

BIOGRAFIA

Fernanda Silva Ferreira, filha de Tarcísio Ferreira de Castro e Francisca da Silva Ferreira, nasceu em Viçosa, Minas Gerais, no dia 16 de abril de 1979. Em 2003 graduou-se Economista Domestica pela Universidade Federal de Viçosa (UFV), MG. Durante a graduação estagiou como voluntária no Laboratório de Microbiologia de Alimentos, Departamento de Microbiologia (UFV). Foi bolsista da FAPEMIG (Fundação de Amparo à Pesquisa do estado de Minas Gerais) e coordenadora do Projeto Reciclar, na área de Educação ambiental, parceria entre a UFV e Prefeitura Municipal de Viçosa, MG. Em 2009 ingressou no Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos (PPGCTA) da Universidade Federal Rural do Rio de Janeiro (UFRRJ), obtendo o grau de Mestre em Ciência e Tecnologia de Alimentos em 2011. Durante o mestrado foi bolsista da CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), desenvolvendo pesquisas na área de Ciência e Tecnologia de Alimentos com controle de qualidade de colorífico de urucum comercializados no Brasil. Atuou como estagiária em docência na disciplina de Análise sensorial. Em 2013 foi aprovada no processo seletivo para doutorado em Ciência e Tecnologia de Alimentos pelo PPGCTA, com bolsa CAPES, desenvolvendo pesquisas na área de Ciência e Tecnologia de Alimentos com oxidação lipídica no processamento térmico de sardinhas adicionadas com ervas aromáticas, como antioxidantes naturais, visando minimizar a degradação de lipídios. Durante o doutorado atuou como estagiária em docência nas disciplinas de Análise de Alimentos e Tecnologia de carnes e derivados, no Departamento de Tecnologia de Alimentos (DTA)/UFRRJ, para os cursos de graduação em Engenharia de Alimentos e Medicina Veterinária, e Lipídeos para os Programas de pós-graduação em Ciência e Tecnologia de Alimentos e Zootecnia, UFRRJ. Possui experiência na área de Ciência dos Alimentos com ênfase em Cromatografia Líquida de Alta Eficiência (CLAE/HPLC, detectores UV, PDA, IR) determinação de fitosteróis, colesterol e óxidos de colesterol por HPLC, determinação de ácidos graxos em produtos de origem animal e vegetal por Cromatografia Gasosa (CG- FID) e análises de atividade antioxidante.

RESUMO

FERREIRA, Fernanda Silva**. Efeito protetor da Salsa (*Petroselinum crispum*) (Mill.) Nym. e cebolinha (*Allium schoenoprasum* L) como antioxidantes naturais sobre a oxidação lipídica em sardinhas preparadas termicamente.** 155 p. Tese (Doutorado em Ciência e Tecnologia de Alimentos). Instituto de Tecnologia, Departamento de Tecnologia de Alimentos, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ. 2019.

Dentre a diversidade de peixes marinhos presentes nas regiões Sudeste e Sul do Brasil, a sardinha brasileira (*Sardinella brasiliensis*) é o recurso pesqueiro mais importante, devido ao seu valor nutricional e baixo custo. Além do alto valor biológico das proteínas que as compõe, este pescado também se destaca por conter significativas concentrações de ácidos graxos poli-insaturados da série n-3, os quais a conferem características nutricionais e funcionais apreciáveis, com diversos efeitos benéficos à saúde. Apesar dessas características desejáveis, a sardinha também contém elevados teores de colesterol, que juntamente com ácidos graxos poli-insaturados são altamente susceptíveis à oxidação lipídica, quando expostos ao processamento térmico a altas temperaturas, induzindo a termo-oxidação do colesterol, formando os produtos de oxidação de colesterol (POCs). Os POCS têm sido associados ao desenvolvimento de enfermidades coronarianas, aterosclerose e outras doenças crônicas e degenerativas. Assim, estudos sobre técnicas alternativas no preparo térmico de pescado, em combinação com fontes alternativas de antioxidantes naturais, são necessários para garantir a qualidade e segurança na ingestão desses alimentos. Essa pesquisa teve como objetivo principal avaliar a composição química, identificar os compostos fenólicos, avaliar a capacidade antioxidante *in vitro* e a capacidade de sequestro de radicais livres *in vivo*, das ervas aromáticas orgânicas, salsa e cebolinha e sua mistura, denominada cheiro-verde. Como também avaliar a eficácia das adições dessas ervas culinárias como antioxidantes naturais, reduzindo a degradação de lipídios e a formação de produtos da oxidação durante o tratamento térmico de filés de sardinhas processadas termicamente por panela do tipo *air-fryer* e *grill* elétrico, justificando assim a realização deste trabalho. Os resultados da caracterização química das ervas estudadas indicam que as mesmas podem ser consideradas como fonte de fibras e minerais. Os principais ácidos graxos foram identificados (g/100g de óleo); os ácidos palmítico (C16:0), ecoisenóico (C20:1 *n*-9), linoleico (C18:2 *n*-6) e ácido alfa-linolênico (C18:3 *n*-3), e como principais fitosteróis os compostos brassicasterol, β-sitosterol, estigmasterol, campesterol, além do 7 cetoestigmasterol. As amostras das ervas culinárias apresentaram consideráveis teores de conteúdo fenólicos (mg GAE/g), flavonoides (mg de quercetina equivalente (QE)/g) e carotenóides totais (µg/g). Nesse estudo, também foram identificados por UPLC-ESI-MS 24 compostos fenólicos nos extratos (80:20) metanol/água nas amostras de salsa, cebolinha e cheiro-verde, dentre os quais, destacaram o ácido protocatecuico, luteolina, kaempferol, quercetina e ácido ferúlico. As ervas aromáticas também apresentaram um bom desempenho em relação às propriedades antioxidantes *in vitro*; DPPH variou de 51,59 a 54,92%; ABTS de 5,12 a 8,29 (µmol TE/g) e FRAP de 6,33 a 8,32 (µmol TE/g). Os testes dos compostos bioativos presentes nos extratos das ervas aromáticas apresentaram ação antioxidante nas leveduras (*Saccharomyces cerevisiae*), reduzindo os efeitos do estresse oxidativo causado pelo peróxido de hidrogênio. Este estudo foi o primeiro a avaliar a formação de

produtos de óxidos de colesterol (POCs) em filés de sardinha processadas termicamente utilizando a fritadeira, sem óleo, *air-fryer*. Os resultados dois processos térmicos, *air-fryer* e *grill*, elétrico, mostraram impacto significativo nos teores de AGPIs n-3, perdas nos teores de colesterol e aumento dos níveis de POCs nas amostras de sardinhas controle. Em contrapartida, foram testados a adição de ervas frescas, salsa, cebolinha e cheiro verde, nos níveis de 2 e 4%, nas sardinhas submetidas ao calor, mostrando-se eficazes como antioxidantes naturais na preservação dos níveis de colesterol e AGPIs, especialmente, os ácidos eicosapentaenoico (EPA) e docosaexaenoico (DHA), e na redução dos níveis totais de POCs. As adições de 4% de salsa e 4% de cheiro verde mostraram-se as mais efetivas contra a oxidação lipídica das sardinhas tratadas termicamente. Sugerindo, assim, um provável efeito sinérgico entre os compostos antioxidantes presentes na salsa e na cebolinha. Desta forma, conclui-se que as ervas culinárias avaliadas neste estudo podem ser consideradas como fontes de antioxidantes naturais alternativas a serem utilizadas na inibição de oxidação lipídica durante o preparo térmico de sardinhas.

Palavras-chave: oxidação lipídica, antioxidantes naturais, colesterol.

ABSTRACT

FERREIRA, Fernanda Silva. **Protective effect of Salsa (*Petroselinum crispum*) (Mill.) Nym. and chives (*Allium Schoenoprasum* L) as natural antioxidants on lipid oxidation in sardines prepared thermally**. 155 p. Thesis (Doctoral Program in Food Science and Technology) – Institute of Technology, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ. 2019.

Among the diversity of marine fish present in the Southeastern and Southern regions of Brazil, the Brazilian sardine (*Sardinella brasiliensis*) is the most important fishing resource due to its nutritional value and low cost. In addition to the high biological value of the proteins, this fish also stands out due to the significant concentrations of n-3 polyunsaturated fatty acids, which confer significant nutritional and functional characteristics with several beneficial effects on health. Despite these desirable characteristics, sardines also contain high levels of cholesterol, which together with polyunsaturated fatty acids are highly susceptible to lipid oxidation, when exposed to thermal processing at high temperatures, inducing thermo-oxidation of cholesterol, forming the products of oxidation of cholesterol (COPs). COPs have been associated with the development of coronary diseases, atherosclerosis, and other chronic and degenerative diseases. Thus, studies on alternative techniques in the thermal preparation of fish, in combination with alternative sources of natural antioxidants are necessary to guarantee the quality and safety in the ingestion of these foods The objective of this research was to evaluate the chemical composition, to identify the phenolic compounds, to evaluate the in vitro antioxidant capacity and the in vivo free radical scavenging capacity, organic aromatic herbs, parsley and chives and their mixture, called *cheiro-verde*. As well as evaluating the efficacy of the additions of these culinary herbs as natural antioxidants, reducing lipid degradation and the formation of oxidation products during the heat treatment of fillets of sardines processed thermally by *air-fryer* pan and electric grill, thus justifying the accomplishment of this work. The results of the chemical characterization of the studied herbs indicate that they can be considered as a source of fibers and minerals. The main fatty acids were identified (g/100 g of oil); (C 18:2 *n*-6) and (C 18:3 *n*-3), and as the main phytosterols the compounds brassicasterol, β-sitosterol, stigmasterol, campesterol, and 7-ketoestigmasterol. Samples of the culinary herbs presented significant phenolic contents (mg GAE/g), flavonoids (mg of quercetin equivalent (Q E)/g) and total carotenoids (μg/g). In this study, 24 phenolic compounds in the extracts (80:20) methanol/water were also identified by UPLC-ESI-MS in the parsley, chives and green-odor samples, among which were protocatecuic acid, luteolin, kaempferol, quercetin and ferulic acid. Aromatic herbs also presented good values of antioxidant properties in vitro; DPPH ranged from 51.59 to 54.92%; ABTS from 5.12 to 8.29 (μmol TE/g) and FRAP from 6.33 to 8.32 (μmol TE/g). The tests of the bioactive compounds of the aromatic herbs the extracts presented antioxidant action in the yeasts (*Saccharomyces cerevisiae*), reducing the effects of the oxidative stress caused by the hydrogen peroxide. This study was the first to evaluate the formation of cholesterol oxides products (COPs) in sardine fillets thermally processed using *air-fryer*, oil-free. The results of the two thermal processes, *air-fryer* and electric grill, showed a significant impact on the levels of n-3 PUFAs, losses in cholesterol levels and increase of COP levels in control sardine samples. On the other hand, were tested the addition of

fresh herbs, parsley, chives and *cheiro-verde* at 2 and 4% levels in sardines submitted to heat, proving to be effective as natural antioxidants in the preservation of cholesterol levels and PUFAs, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, and in reducing the total levels of POCs. The additions of 4% of parsley and 4% of *cheiro-verde* were the most effective against the lipid oxidation of the sardines treated thermally. This suggests a probable synergistic effect between the antioxidant compounds found in parsley and chives. Thus, it is concluded that the culinary herbs evaluated in this study can be considered as alternatives sources of natural antioxidants to be used in the inhibition of lipid oxidation during the thermal preparation of sardines.

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LISTA DE SIGLAS E ABREVIATURAS

AAE	Ascorbic acid equivalent
AGM	Ácido graxo monoinsaturado
AGPIs	Ácido graxo poli-insaturado
AGS	Ácido graxo saturado
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric Pressure Chemical Ionization
COP	Cholesterol Oxidation Product
DHA	Ácido docosaexaenóico / Docosahexaenoic acid
DPPH	2,2-difenill-1-picril-hidraliza
DCNTs	Doenças crônicas não transmissíveis
EAG	Equivalente ácido gálico
EDTA	Ácido etilenodiamino tetra-acético
EPA	Ácido eicosapentaenoico / Eicosapentaenoic acid
EQ	Equivalente quercetina
EROs	Espécies reativas de oxigênio
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization
FAME	Fatty acid methyl ester
GAE	Galic acid equivalente
HPLC	High Performance Liquid Chromatography
IU	International Unit
MS	Mass Spectrometry
ORAC	Oxygen Radical Absorbance Capacity
PDA	Photo Diode Array
POC	Produto da Oxidação do Colesterol
PG	Propil galato
PUFA	Polyunsaturated Fatty Acid
QE	Quercetin equivalent
ROS	Reactive Oxygen Specie
SIM	Selected ion monitoring
TBHQ	Terc-butilhidroquinona
TPC	Total polyphenol content
TFC	Total flavonoid content
TE	Trolox equivalente
UV	Ultravioleta
UPLC	Ultra Performance Liquid Chromatography
ω3 / n3	Ômega 3
ω6 / n6	Ômega 6
ω9 / n9	Ômega 9
5,6α-OH	5,6α-hidroxicolesterol
5,6β-OH	5,6β-idroxicolesterol
7-Keto	7-cetocolesterol
7α-OH	7α-hidroxicolesterol
7β-OH	7β-hidroxicolesterol
20α-OH	20α-hidroxicolesterol
25R-OH	25R-Hidroxicolesterol

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INTRODUÇÃO GERAL

A demanda por pescado tem apresentado um incremento significativo nas últimas décadas, principalmente em função do crescimento populacional e da busca dos consumidores por alimentos mais saudáveis.

Dentre a diversidade de peixes marinhos nas regiões Sudeste e Sul do Brasil, a sardinha brasileira (*Sardinella brasiliensis*) é o recurso pesqueiro mais importante, devido ao seu valor nutricional e baixo custo. Além do alto valor biológico das proteínas presentes na sardinha, também se destaca as significativas concentrações de ácidos graxos poli-insaturados (AGPIs) de cadeia longa, da série n-3, principalmente os ácidos eicosapentaenóico (EPA, C20:5 *n*-3) e docosahexaenóico (DHA, C22:6 *n*-3), desempenham um papel importante para a saúde humana.

Nos últimos anos, inúmeros estudos epidemiológicos referentes aos AGPIs *n*-3 evidenciaram os seus benefícios referentes à promoção da saúde e prevenção de doenças como diabetes, câncer, doença de Alzheimer, demência, depressão, desenvolvimento visual, cognitivo e neurológico.

Apesar dessas características desejáveis, a sardinha também contém concentrações apreciáveis de colesterol, que juntamente com ácidos graxos poli-insaturados são altamente susceptíveis à oxidação lipídica quando expostos ao processamento em altas temperaturas.

Durante o preparo térmico, ocorrem reações químicas e físicas deletérias que alteram o valor nutricional dos alimentos como redução dos teores de compostos termolábeis, como as vitaminas lipossolúveis e os ácidos graxos poli-insaturados, formando compostos citotóxicos. Do ponto de vista tecnológico inúmeros estudos comprovaram as alterações no perfil de ácidos graxos poli-insaturados e a formação de produtos de oxidação do colesterol (POCs) durante o preparo térmico de pescados Os óxidos de colesterol, quando originários da oxidação endógena de colesterol, possuem efeitos benéficos e exercem importantes atividades fisiológicas essenciais ao organismo humano. Eles são intermediários no catabolismo do colesterol, envolvidos síntese de ácidos biliares e na eliminação excesso de colesterol do corpo, atuam como moduladores de permeabilidade celular, receptores de sinalização celular. Como também existem relatos de pesquisa demonstrando que alguns POCs podem apresentar efeitos anticancerígenos, suprimindo a proliferação de células cancerosas.

Entretanto, quando estes compostos são obtidos exogenamente através da dieta, eles podem causar efeitos negativos em outros componentes e estruturas presentes no organismo. A literatura mostra a relação dos POCs com vários efeitos deletérios à saúde. POCs estão associados a potenciais efeitos mutagênicos e citotóxicos. Como por exemplo, no desenvolvimento de câncer de cólon, mama, próstata, pele e pulmão, entre outros. A presença de POCs também afeta a interação de proteínas e peptídeos com a bicamada lipídica e estão associados às doenças neurodegenerativas, como Alzheimer, Parkinson e Huntington. Outro efeito negativo relacionado à ingestão de POCs para a saúde humana é a formação de placas de gordura nos vasos sanguíneos, denominada aterosclerose, sendo considerada como um dos principais eventos envolvidos no comprometimento do sistema cardiovascular.

Devido aos inúmeros efeitos adversos dos POCs, a ocorrência e quantificação destes compostos em alimentos são consideradas de grande importância à saúde

pública, e tem atraído à atenção da comunidade cientifica no intuito de minimizar a formação desses compostos citotóxicos. O processo oxidativo ou peroxidação dos lipídios pode ser evitado com a utilização de substâncias antioxidantes. O desenvolvimento de técnicas para utilização de antioxidantes naturais em detrimento dos sintéticos tem despertado cada vez mais o interesse dos pesquisadores em testar novas fontes de antioxidantes naturais em pescado.

Ervas e especiarias têm sido utilizadas desde a antiguidade por suas qualidades aromatizantes e também por suas propriedades conservadoras e medicinais. Nos últimos anos, pesquisas tem avaliado o potencial do uso desses produtos como antioxidantes naturais, uma vez que contêm diferentes tipos de compostos bioativos capazes de minimizar a oxidação de lipídios em pescado e seus produtos.

A salsa (*Petroselinum crispum*) é uma erva aromática originária das culturas do mediterrâneo, utilizada em diversas partes do mundo desde a antiguidade, tanto na medicina como na alimentação. Outra planta aromática extensivamente utilizada para fins culinários é a cebolinha (*Allium schoenoprasum)*. A combinação das mesmas é conhecida como "cheiro-verde", comumente empregado na culinária brasileira. O potencial do uso da salsa e cebolinha como antioxidantes naturais foram consideradas efetivas como antioxidantes naturais em ensaios *in vitro* e através do emprego em sistemas-modelo em alimentos. Contudo, não existe informação sobre o emprego das mesmas visando à inibição da oxidação lipídica em pescado.

A utilização de processos térmicos na preparação de pescados pode ser considerada como um dos fatores desencadeadores da degradação dos lipídios e consequente formação de POCs. Desta forma, estudos sobre técnicas alternativas no preparo de pescado em combinação com fontes alternativas de antioxidantes naturais são necessários para garantir a qualidade e segurança na ingestão desses alimentos.

Assim, essa pesquisa teve como objetivos avaliar a composição química, identificação de componentes bioativos, capacidade de sequestro de radicais livres *in vitro* e *in vivo*, das ervas aromáticas orgânicas, salsa e cebolinha e cheiro-verde, bem como avaliar a eficácia das adições das ervas culinárias como antioxidantes naturais, reduzindo a degradação de lipídios e a formação de produtos da oxidação do colesterol durante o tratamento térmico de filés de sardinhas em panela do tipo *air-fryer* e *grill* elétrico, justificando a realização deste trabalho.

REVISÃO DE LITERATURA

1 Consumo Mundial de Pescado

A demanda mundial por pescado tem apresentado um incremento significativo nas últimas décadas, principalmente em função do crescimento populacional e da busca dos consumidores por alimentos mais saudáveis (FAO, 2018). Por exemplo, muitos mercados emergentes e exportadores, como Brasil, Índia e Indonésia, ganharam importância graças a melhorias nos sistemas de distribuição e aumento da produção.

Peixe e produtos de peixe são alguns dos itens alimentares mais comercializados no mundo atualmente. Em 2016, cerca de 35 por cento da produção mundial de pescado entrou no comércio internacional de várias formas para consumo humano ou fins não comestíveis (FAO, 2018). Os principais produtos da indústria do pescado incluem peixes inteiros, em pedaços (postas ou filés), resfriados ou congelados, enlatados, produtos secos e curados, óleos de peixe e uma diversidade de outros produtos prontos para o consumo.

A produção total de pescado em 2016 atingiu um recorde histórico de 171 milhões de toneladas (FAO, 2018). Essa produção resultou em um recorde de consumo per capita de 20,3 kg em 2016. A produção pesqueira global de captura foi de 90,9 milhões de toneladas em 2016, uma pequena queda em comparação com os dois anos anteriores. A projeção de produção mundial de pescado para 2030 (peso vivo equivalente) subirá de 170.941 em 2016, para 200.955 milhões toneladas em 2030 (FAO, 2018).

O Brasil se destaca por ser o maior produtor de pescado da América do Sul, apesar de não ter relatado dados oficiais de captura para a FAO desde 2014. Desta forma, os números de produção (225.000 toneladas) para 2015 e 2016 foram dados estimados. Segundo a Organização das Nações Unidas para Alimentação e Agricultura (FAO), o consumo de pescado no Brasil tem como perspectiva alcançar 12,7 kg por habitante em 2025, em torno de 32% a mais do que os 9,6 kg consumidos por ano entre 2013 e 2015. E ainda de acordo com agência das Nações Unidas, o gênero *Sardinella* é largamente distribuído ao redor do mundo, estando presente no Oceano Atlântico e Indo-Pacífico.

2 **Sardinha verdadeira** (*Sardinella brasiliensis*)

De acordo com dados recentes da FAO (2018), dentre as 25 espécies de pescados mais capturados mundialmente nos anos de 2015 e 2016, aquelas do gênero *Sardinella*se destacaram entre as cinco mais capturadas.

A sardinha-verdadeira (*Sardinella brasiliensis*), família *Clupeidae*, é uma das espécies mais importantes para a pesca marinha comercial brasileira (FAO, 2016). A *S. brasiliensis* é um peixe de pequeno porte, corpo lateralmente comprimido e prateado (**Figura 1**). É uma espécie pelágica, ou seja, que habita o mar aberto, ocupando a superfície da coluna d'água e geralmente vivendo em cardumes. Desta forma, possui grande importância industrial, visto que, pode ser capturado em grande quantidade e em menor tempo.

Na costa brasileira, a *S. brasiliensis* é encontrada ao longo da área compreendida entre os estados do Rio de Janeiro (Cabo de São Tomé, 22º S) e Santa Catarina (ao sul do Cabo de Santa Marta Grande, 28º S). No estado do Rio de Janeiro é pescada principalmente na Ilha Grande (Angra dos Reis), arredores de Cabo Frio e na Baía de Sepetiba (FIPERJ, 2018). A espécie é capturada, normalmente, entre as profundidades de 30 e 100 m com estoque variável em função de mudanças oceanográficas (CASTELLO, 2015).

Entre a diversidade de peixes marinhos das regiões Sudeste e Sul do Brasil, a sardinha brasileira (*Sardinella brasiliensis*) se destaca por seu consumo considerável e preço mais baixo que outros peixes (MOREIRA et al., 2015; BALOI et al., 2016).

Figura 1: Sardinha-verdadeira (*Sardinella brasilliensis*).

O Projeto de Monitoramento da Atividade Pesqueira no Estado do Rio de Janeiro– PMAP-RJ monitorou 15 municípios entre Cabo Frio (na região das Baixadas Litorâneas) e Paraty (na região da Costa Verde) no período de julho a dezembro de 2017. As descargas registradas somaram 26.705 t de pescado, sendo a pesca industrial responsável por 72,1% (19.259,1 t), e a pesca artesanal por 27,9% (7.445,8 t). O município de Angra dos Reis, na região da Costa Verde, foi o segundo principal porto pesqueiro, responsável por 23% (6.155,4 t) da produção estadual. Destes, 22,3% (4.303,9 t) da pesca industrial e 24,8% (1.851,4 t) da pesca artesanal (FIPERJ, 2018).

A dieta alimentar do pescado tem grande influência sobre a sua composição química geral e, em especial, sobre a composição de ácidos graxos. De acordo com CASTELLO (2015), os hábitos alimentares de larvas e juvenis de sardinhas (13-65 mm) são compostos por pequenos copépodos, dinoflagelados, larvas de crustáceos decápodes e quetognatos e diatomáceas. Segundo o mesmo autor, supõe-se que haja uma sobreposição alimentar parcial entre a sardinha-verdadeira e a anchoita (*Engraulis anchoita*), já que ambas as espécies coexistem na bacia do sudeste.

As sardinhas caracterizam-se por conter proteínas de fácil digestão e elevado valor biológico, com presença de todos os aminoácidos essenciais, vitaminas (vitaminas A, D, E, e K) e minerais (cálcio, iodo, zinco, ferro e selênio), além da rica composição em lipídios insaturados (FAO, 2016).

O óleo de sardinha é uma importante fonte de ácidos graxos de cadeia longa poli-insaturada do tipo ômega 3 (*n*-3), principalmente os ácidos eicosapentaenóico (EPA, C20:5 n-3) e docosahexaenóico (DHA, C22:6 *n*-3). Diversos estudos destacaram a composição química de sardinhas, incluindo o perfil lipídico dos ácidos graxos (GARCÍA-ARIAS et al., 2003; TARLEY et al., 2004; SALDANHA, BENASSI e BRAGAGNOLO, 2008; BAHURMIZ, ADZITEY e NG, 2017; BANDARRA et al., 2018) bem como a diferença de conteúdos desses compostos de acordo com a época do

ano (CHITRA SOM e RADHAKRISHNAN, 2013; ZOTOS e VOUZANIDOU, 2012; FERNANDES et al., 2014).

Outros ácidos graxos são predominantes em pescado. Dentre estes se destacam, os ácidos mirístico (C14:0), palmítico (C16:0), esteárico (C18:0), oleico (C18:1 *n*-9), linoleico (C18:2 *n*-6) e o araquidônico (C20:4 *n*-6). Entretanto, o perfil lipídico, assim como os teores destes compostos variam consideravelmente de espécie para espécie e mesmo entre indivíduos de uma mesma espécie (OGAWA e MAIA, 1999; NUNES et al., 2008; CHITRA SOM e RADHAKRISHNAN, 2013; BANDARRA et al., 2018; STEINRÜCKEN et al., 2018).

A composição do conteúdo lipídico e de ácidos graxos em peixes varia inter-e intra-espécies, de acordo com os diversos fatores como: sexo, tamanho, ciclo reprodutor, estação do ano, dieta e estado nutricional (ACKMAN, 1967; BANDARRA et al., 1997; SHIRAI, TERAYAMA e TAKEDA, 2002; LUZIA et al., 2003; GARRIDO et al., 2007; ÖZOGUL, ÖZOGUL e ALAGOZ, 2007; TRUSHENSKI, LEWIS e KOHLER, 2008; CHITRA SOM e RADHAKRISHNAN, 2013; FERNANDES et al., 2014; CHRISOLITE et al., 2016; MOHANTY et al., 2016; SPRAGUE, DICK e TOCHER, 2016; BAHURMIZ, ADZITEY e NG, 2017; BANDARRA et al., 2018). De acordo com pesquisadores (HEBERT e ARTS, 2006; BIMBO, 2007; CHAUTON, 2015; SHAHIDI e AMBIGAIPALAN, 2015), dentre as espécies de superfície ou pelágicas, as sardinhas (*Sardinella* sp.) destacam-se como as melhores fontes de EPA e DHA.

3 Importância Nutricional do Pescado para Saúde Humana

O rápido aumento das doenças crônicas não transmissíveis (DCNTs) em todo o mundo constitui um sério problema de saúde pública, tanto nos países ricos quanto nos de média e baixa renda (RAUBER et al., 2018), com consequências sociais, econômicas e de saúde (PULLAR et al., 2018). Estima-se que em um futuro próximo, aproximadamente 70% da mortalidade no mundo estará relacionada às DCNTs (PASSI et al., 2017).

Diversas DCNTs podem estar relacionadas à dieta humana, como doenças cardiovasculares, diabetes mellitus tipo 2, câncer, doenças respiratórias e doenças mentais (WHO, 2018). De acordo com a Organização Mundial de Saúde (OMS), as mortes em decorrências dessas doenças crônicas são amplamente evitáveis (NUGENT et al., 2018). Dentre as medidas para prevenção, a alimentação saudável encontra-se em destaque. Ainda segundo a OMS, o pescado é considerado como um alimento saudável devido a sua composição química, além de conter nutrientes funcionais importantes para a saúde humana (FAO, 2016).

Os peixes são considerados a principal fonte de AGPIs (*n*-3) na dieta humana, especificamente (EPA) e (DHA). Desta forma, nos últimos anos, inúmeros estudos epidemiológicos evidenciaram os efeitos benefícios dos AGPIs *n*-3 para a saúde por apresentarem atividades anti-inflamatórias, anti-cancerígenas e efetivos positivos no tratamento de algumas doenças crônicas neurológicas como demência, Parkinson e Alzeimer (GIL e GIL, 2015; PAWEŁCZYK et al., 2016; PARVATHY et al., 2016; ALEXANDER et al., 2017; COLUSSI et al., 2017; ELTWERI et al., 2017; GOPINATH et al., 2017; LEE et al., 2017; SIOEN et al., 2017; ZÁRATE et al., 2017; LAI et al., 2018; SHAHIDI e AMBIGAIPALAN, 2018).

Os principais ácidos graxos da família *n*- 3, relacionados aos efeitos benéficos à saúde são, principalmente, os ácidos EPA e DHA, que desempenham um papel

importante na promoção da saúde e na prevenção de doenças (LAYE et al., 2018; SIOEN et al., 2017). Os AGPIs, EPA e DHA são considerados essenciais, pois não são produzidos pelo organismo humano, sendo, portanto, obtidos através da dieta. Os AGPIs *n*-3 (**Figura 2)** desempenham importantes funções na modulação do sistema imune, atuando como cofatores enzimáticos, e também como componentes celulares, garantindo a manutenção da fluidez e das funções da membrana e dos fosfolipídios plasmáticos (BARBALHO et al., 2016).

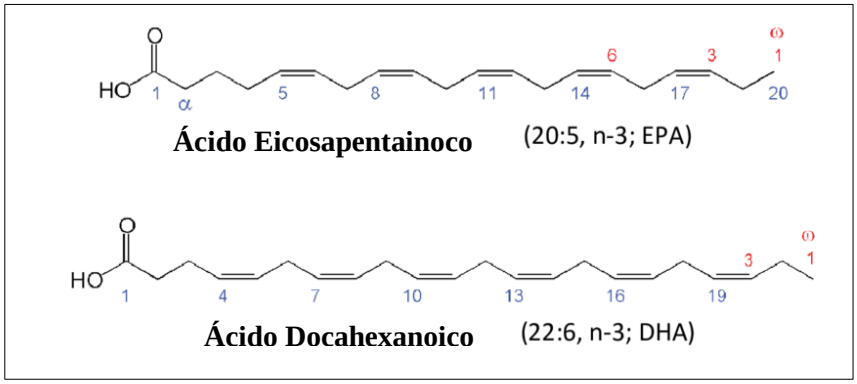

Figura 2. Exemplos das estruturas químicas dos AGPI (*n*-3) EPA e DHA.
Fonte:https://www.researchgate.net/figure/Chemical-structures-of-EPA-and-DHA_fig1_289536833

O ácido DHA tem um papel importante no desenvolvimento do cérebro e da retina durante o desenvolvimento fetal e nos primeiros 2 anos de vida, com influencias positivas no neurodesenvolvimento, principalmente a acuidade visual e as funções cognitivas (WADHWANI, PATIL e JOSHI, 2018). De acordo com a LAYE et al. (2018), as vias de sinalização lipídica cerebral que estão alteradas nos distúrbios neurológicos, podem ser alvos viáveis para o desenvolvimento de novas terapias, em particular, os efeitos de AGPIs *n*-3 e seus metabólitos que regulam o fenótipo e a função da micróglia, exercendo suas atividades anti-inflamatórias no cérebro.

Os AGPIs *n*-3 possuem propriedades anticancerígenas (MOLOUDIZARGARI et al., 2018). Estudos recentes sugerem que os mesmos podem ser eficazes como coadjuvantes com agentes quimioterápicos e outros compostos anticâncer naturais no tratamento do câncer de cólon (LEE et al., 2017) e outros tipos de câncer (DA SILVA PAIXÃO et al., 2017; ELTWERI et al., 2018). Além disso, o DHA é responsável por melhorar muitas respostas celulares, incluindo os efeitos anti-inflamatórios (MOCELLIN et al., 2016; NEWELL et al., 2017).

Nas últimas décadas a importância dos AGPIs n-3 para o sistema cardiovascular esteve sob os holofotes dos cientistas (BOWEN, HARRIS e KRIS-ETHERTON, 2016; ALEXANDER et al., 2017; COLUSSI et al. et al., 2017; LAI et al., 2018), devido ao fato de compostos serem considerados eficazes na prevenção de inflamações, ateroscleroses, doenças crônicas e eventos cardiovasculares (principalmente acidente vascular cerebral e infarto agudo do miocárdio), especialmente em pessoas com alto risco cardiovascular.

Embora tenham sido apresentados inúmeros relatos na literatura sobre os efeitos benefícios da ingestão de AGPIs *n*-3 para a saúde humana, existem evidências recentes que apresentam controvérsias sobre a eficácia dos mesmos em prevenir doenças

cardiovasculares (BOWEN, HARRIS e KRIS-ETHERTON, 2016; ALEXANDER et al., 2017; COLUSSI et al., 2017; MAKI et al., 2017; SISCOVICK et al., 2017; SHAHIDI e AMBIGAIPALAN, 2018).

Um estudo de meta-análise foi realizado por COLUSSI et al. (2017) sobre o impacto dos AGPIsn-3 na função vascular e na pressão arterial. Essa pesquisa destacou que esses ácidos graxos estão presentes em quantidades variáveis nas membranas celulares de espécies marinhas e seu conteúdo afeta uma variedade de funções celulares. Além disso, reportaram evidências obtidas de estudos em animais e humanos sugerem que os AGPIs n-3 afetam muitas etapas do processo aterosclerótico, melhorando a função endotelial nos vasos sanguíneos e promovendo vasodilatação pelo relaxamento das células musculares lisas. Ainda, de acordo com os autores, esses compostos também exercem ações antioxidantes, anti-inflamatórias e antitrombóticas. Desta forma, podem retardar o desenvolvimento de placas ateroscleroticas e reduzir o endurecimento das paredes vasculares.

Esse efeito pode estar relacionado ao conteúdo da membrana de "pré-tratamento" dos AGPIs *n*-3, e isso explica algumas inconsistências entre os ensaios de intervenção (COLUSSI et al., 2017). Os autores dessa pesquisa concluíram que embora resultados encorajadores tenham sido inicialmente obtidos com o uso de suplementos de AGPIs n-3 em ensaios de prevenção secundária, as meta-análises não confirmaram a capacidade desses ácidos graxos em reduzir os riscos de desenvolvimento de doenças coronarianas e cerebrovasculares. Assim, pode-se dizer que os AGPIs *n*-3 estão associados à melhora significativa da função vascular e à redução da pressão arterial. No entanto, a evidência atualmente apoiando um papel desses ácidos graxos na prevenção cardiovascular é inconsistente e precisa de mais investigação.

Outras pesquisas também estão de acordo com essas discussões apresentadas acima (ALEXANDER et al., 2017; MAKI et al., 2017; SISCOVICK et al., 2017). Explicações potenciais para os resultados discrepantes incluem estudos com baixas amostragens e taxas de eventos, participantes com alto consumo de peixe/frutos do mar, dosagem de EPA e DHA abaixo do ideal, duração da suplementação, idade no início do estudo, tempo de seguimento e padrão concorrente, cuidados para o tratamento de doenças cardiovasculares (DCV) (BOWEN, HARRIS e KRIS-ETHERTON, 2016).

Desta forma, embora existam controvérsias na relação de prevenção de doenças cardiovasculares pelo consumo de eicosanoides (EPA e DHA), a ingestão dos mesmos ainda é extremamente recomendada por médicos e nutricionistas, devido aos efeitos benéficos à saúde humana. Pois, a deficiência desses eicosanoides pode causar sintomas neurológicos, redução da acuidade visual, lesões de pele, retardo no crescimento e diminuição da capacidade de aprendizado (KITESSA et al., 2014). Portanto, recomenda-se a ingestão de peixes gordos 2 a 3 vezes por semana (SIMOPOULOS, 2016).

4 Oxidação do Colesterol e Formação dos POCs

A sardinha, além de fonte dos ácidos graxos n-3 essenciais, também apresenta concentrações elevadas de colesterol (SALDANHA, BENASSI e BRAGAGNOLO, 2008; CARDENIA et al., 2013; SCHERR et al., 2015).

Colesterol é o principal composto da família dos esteróis e está presente em células de origem animal, representando o lipídio mais proeminente em células eucarióticas (MCLEAN, HANS e MUNRO, 2012). Além disso, a molécula de colesterol atua no controle da fluidez e permeabilidade das membranas celulares e

também na síntese de ácidos biliares, vitamina D e hormônios esteroides, sendo um componente crucial na composição de lipoproteínas que estão envolvidos no transporte e metabolismo de lipídios no corpo (ZHAO e DAHLMAN-WRIGHT, 2010; MORZYCKI, 2014).

A molécula de colesterol ($C_{27}H_{46}O$) (**Figura 3)**, por ser um composto insaturado é altamente instável e suscetível à oxidação lipídica, acarretando a formação de radicais livres. O colesterol pode estar presente em sua forma livre, combinada com ácidos graxos de cadeia longa, ou como ésteres de colesterol (MORZYCKI, 2014).

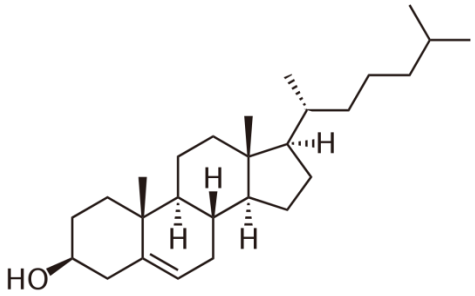

Figura 3. Estrutura química do colesterol (BRZESKA, SZYMCZYK e SZTERK, 2016).

O colesterol pode ser oxidado enzimaticamente em sistemas ou através de mecanismos químicos (RODRIGUEZ, CLARK, LEE e CURCIO, 2014). A auto-oxidação é o principal mecanismo de oxidação em alimentos, estando associada à reação do oxigênio com ácidos graxos insaturados através de um mecanismo de reações em cadeia de radicais, que são espécies químicas instáveis e de alta reatividade que contém um ou mais elétrons não pareados. Desta forma, a auto-oxidação ocorre em três etapas distintas: iniciação, propagação e terminação (HALLIWELL e GUITTERIDGE, 2000; LAGUERRE, LECOMTE e VILLENEUVE, 2007; KUMAR et al., 2015).

A etapa de iniciação ocorre formação dos radicais dos ácidos graxos, que são formados a partir da abstração de hidrogênio de um ácido graxo, ocasionando a formação de um radical de ácido graxo com um elétron não pareado no carbono. Para que isso ocorra é necessária uma elevada energia de ativação nessa reação em presença de $^3O_2$ (forma menos reativa do oxigênio), que são normalmente inicadas a partir da ação de agentes iniciadores como luz, calor ou íons metálicos (LAGUERRE, LECOMTE e VILLENEUVE, 2007; KUMAR et al., 2015). Normalmente, a abstração de hidrogênio ocorre preferencialmente nos átomos de carbono onde a energia de dissociação da ligação é baixa, como por exemplo, quando um átomo de carbono é adjacente a uma ligação dupla, a ligação covalente C-H torna-se mais fraca, requerendo menor energia de dissociação. Em contrapartida, os ácidos graxos saturados são bastante estáveis e não se oxidam a uma velocidade significativa (GORDON, 2004).

Durante esse processo de iniciação, a retirada do hidrogênio de ácidos graxos insaturados depende do número de ligações duplas na molécula, sendo que, a abstração de hidrogênio de um grupo metileno de um sistema 1,4-pentadieno ocorre mais facilmente do que em um simples grupamento alélico. Por conseguinte, o radical 1,4-dieno gerado é estabilizado de modo mais eficiente por ressonância, isto é, pelo deslocamento de elétrons pelos cinco átomos de carbono. As diferenças nas velocidades de reações de auto-oxidação de ácidos graxos insaturados, explicam o fato da

velocidade de oxidação nos alimentos ser muito maior quando ácidos graxos poli-insaturados estão presentes (LAGUERRE, LECOMTE e VILLENEUVE, 2007).

Na etapa seguinte, conhecida como propagação, o radical proveniente da fase de iniciação, reage rapidamente com o oxigênio atmosférico gerando novas espécies de radicais livres, como os peróxidos, que quando provenientes de ácidos graxos poli-insaturados apresentam formas de ressonância estabilizadoras. As reações de formação dos radicais peróxidos ocorrem rapidamente devido a baixa energia de ativação requerida, consequentemente, formando uma elevada concentração destes radicais no meio. Além disso, a elevada energia dos radicais peroxil permite que eles promovam a abstração de hidrogênio de outro ácido graxo gerando produtos primários da oxidação, como por exemplo, os peróxidos e hidroperóxidos (LAGUERRE, LECOMTE e VILLENEUVE, 2007; MEDINA-MEZA, BARNABA e BARBOSA-CÁNOVAS, 2014; KUMAR et al., 2015).

Durante a etapa de terminação ocorre a interrupção das reações devido a redução do número de ácidos graxos no sistema, assim, os radicais passam a reagir entre si formando compostos estáveis (NICKI et al., 2005; MASUDA et al., 2010). A **figura 4** apresenta o esquema geral do mecanismo de oxidação lipídica.

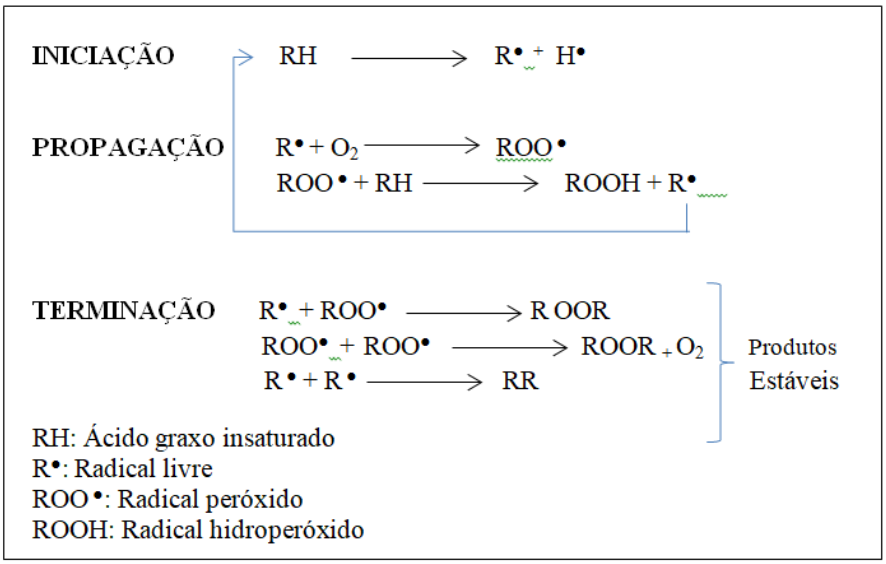

Figura 4. Esquema geral do mecanismo de oxidação lipídica (Adaptado de: RAMALHO e JORGE, 2006).

Na etapa de terminação, podem ser formados produtos secundários por meio de transformações de hidroperóxidos por reações de decomposição, pela cisão da ligação dupla adjacente ao grupo hidroperoxil com a formação de aldeídos, hidrocarbonetos, álcoois, cetonas voláteis e outros compostos não voláteis (LAGUERRE, LECOMTE e VILLENEUVE, 2007).

Além dos ácidos graxos insaturados, o colesterol também pode ser degradado por meio de reações oxidativas via auto-oxidação, acarretando a formação de produtos oxidados do colesterol ou óxidos de colesterol. Na sua forma livre, o colesterol é uma cadeia longa de álcool policíclico com um anel tetracíclico comum aos esteróis que possui um grupo hidroxila em C3 e uma insaturação entre o C5e C6, e uma cadeia lateral alifática (HUR et al., 2007). A estrutura química do colesterol é suscetível a

processos oxidativos que resultam na formação de compostos mono-ou polioxigenados, chamados POCs (SMITH, 1987).

Os compostos predominantes são os originados a partir da oxidação do carbono C-7, sendo formados simultaneamente 7α- e 7β-hidroperóxidos, mas o 7β -hidroperóxido é predominante, pois é termodinamicamente mais estável. Em seguida, são reduzidos aos seus álcoois correspondentes (7α- e 7 β -hidroxicolesterol) e a 7-cetocolesterol. Este último composto pode ser degradado termicamente em colesta-3,5-dien-7-ona (SMITH, 1987). A oxidação dos carbonos terciários C20 e C25 da cadeia lateral do colesterol também pode ocorrer, originando 20α-hidroxicolesterol e 25-hidroxicolesterol, respectivamente (SMITH, 1987) (**Figura 5)**.

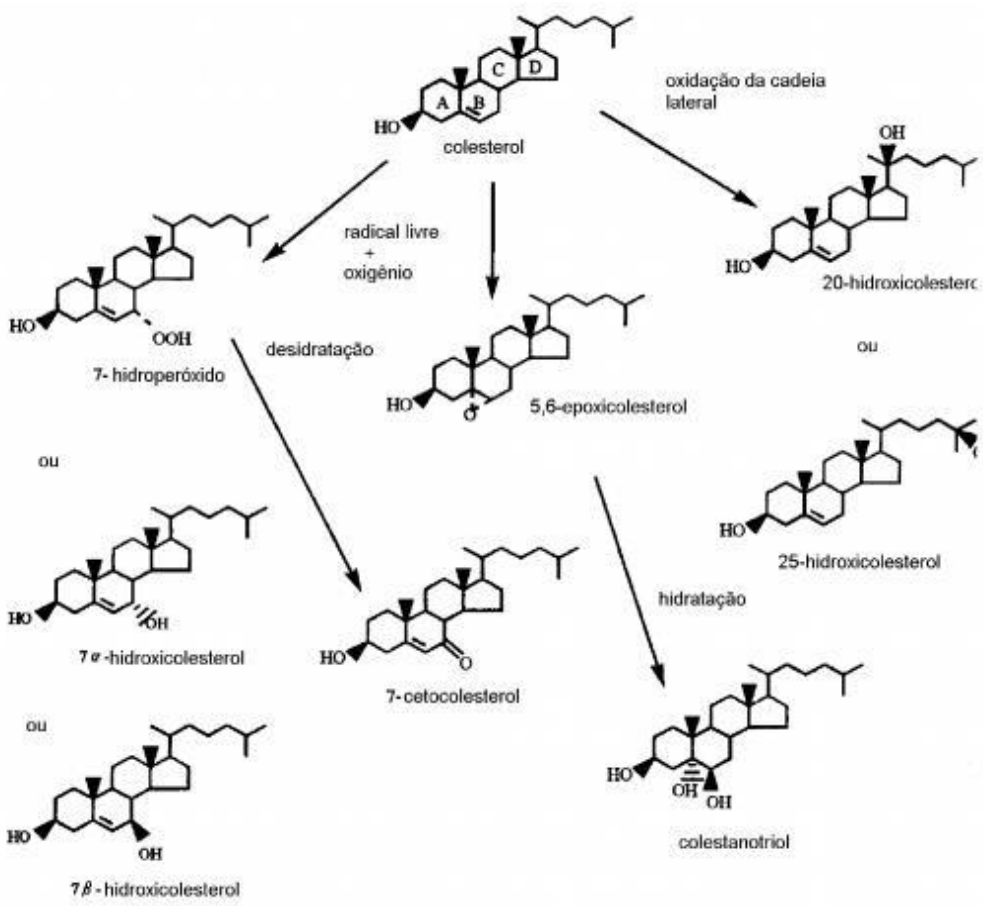

Figura 5. Mecanismo de auto-oxidação do colesterol (SMITH, 1987)

Os produtos 5α, 6α e 5 β, 6β-epoxicolesteróis foram identificados como produtos da oxidação do colesterol pelo ar atmosférico, embora seu mecanismo de reação seja mais complexo. Eles não são formados apenas pela reação do colesterol com o oxigênio triplete ($^3O_2$) e singlete ($^1O_2$), mas por diferentes espécies de oxigênio ativas (GUMULKA et al., 1982, GUARDIOLA et al., 1996). Os 5,6-epóxidos apresentam graus de estabilidade diferentes conforme o meio e o pH (MAERKER, 1987; KIM e NAWAR, 1993).

No caso dos alimentos, a oxidação do colesterol ocorre de forma não enzimática em um processo auto-oxidativo através de reações em cadeia complexas, que são

baseadas na formação de radicais livres, sendo os principais locais suscetíveis à oxidação, a dupla ligação entre os carbonos 5 e 6, o carbono (alílico) 7, e os carbonos terciários C20 e C25 (SMITH, 1987) (**Figura 6**).

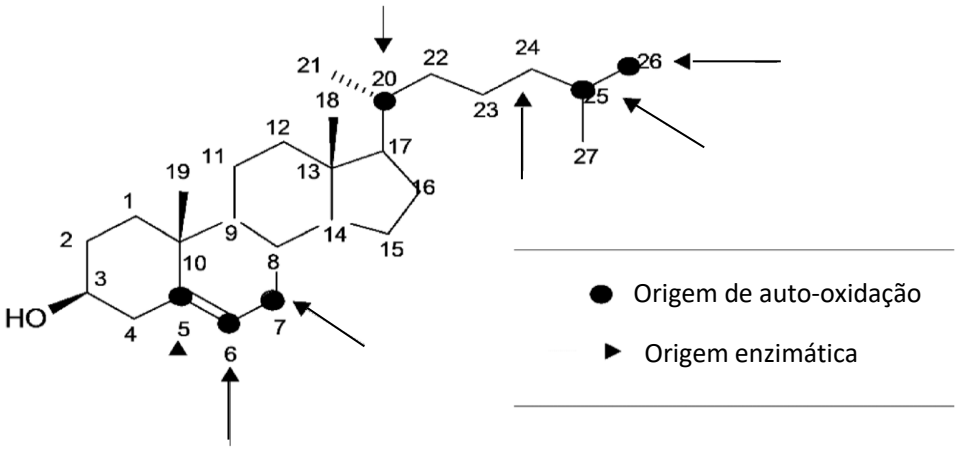

Figura 6. Posições da molécula de colesterol que são suscetíveis ao ataque oxidativo
Fonte: SMITH (1987).

A oxidação do colesterol ocorre de maneira semelhante à oxidação de outros lipídios insaturados, que são suscetíveis a ataques por espécies reativas de oxigênio (EROs). EROs são compostos de radical e moléculas não radicais, tais como grupos de hidroxila (OH) e peroxil (ROOH), peróxido de hidrogênio (H_2O_2), oxigênio singlete ($^1O_2$), triplete ($^3O_2$) e outros. Essas espécies podem ser formadas enzimaticamente, quimicamente, fotoquimicamente, por irradiação e decomposição de hidroperóxidos, e também podem se decompor ou interagir uns com os outros para formar novos radicais. Este processo é autocatalítico, gerando assim produtos de oxidação primária, e reações subsequentes levarão à formação de uma ampla variedade de produtos de oxidação secundária (TAI, CHEN e CHEN, 1999; MEDINA-MEZA e BARNABA, 2013; DANTAS et al., 2015).

Os POCs mais comuns presentes nos alimentos são 7-cetocolesterol, 20α-hidroxicolesterol, 25-hidroxicolesterol, α, β-epoxicolesterol e 7α, 7β-hidroxicolesterol (HUR et al., 2007; ORCZEWSKA-DUDEK et al., 2012). O perfil dos produtos formados e suas concentrações são influenciadas por fatores como atividade de água, tempo de oxidação, pH, exposição à luz e irradiação, temperaturas elevadas, estado físico do colesterol e composição alimentar (presença de ácidos graxos insaturados) (DANTAS et al., 2015; MEDINA-MEZA e BARNABA, 2013; BARNABA et al., 2016). A presença de ácidos graxos insaturados na matriz alimentar pode acelerar a oxidação do colesterol, pois gera um ambiente pró-oxidante, devido à presença das duplas ligações, gerando assim zonas de ressonância que resultam em radicais como hidroperóxidos (BARNABA et al., 2016).

Os óxidos de colesterol (7α-hidroxicolesterol, 7β-hidroxicolesterol, 5,6α, β-epoxycolesteróis, 7-cetocolesterol e colestanetriol) são predominantes em pescado, embora a oxidação da cadeia lateral do colesterol também podem formar isômeros dos carbonos 20, 24, 25 e 26 (KORAHANI et al.,1982; MAERKER, 1987).

Os óxidos de colesterol quando são originários da oxidação do colesterol de forma endógena, ou seja, a partir do próprio metabolismo lipídico no organismo, possuem efeitos benéficos, os quais exercem importantes atividades fisiológicas

essenciais ao corpo humano, como por exemplo, eles são intermediários no catabolismo do colesterol, principalmente na síntese de ácidos biliares e estão envolvidos na eliminação do excesso de colesterol do corpo (BJORKHEM, 2013). Além disso, esses compostos formados endogenamente atuam como moduladores de permeabilidade celular (KULIG et al., 2015), reguladores da homeostase do colesterol, como biomarcadores (KULIG et al., 2016), e receptores de sinalização celular (LEONARDUZZI et al., 2014; VURUSANER et al., 2014). Estudos relatam ainda, que alguns POCs endógenos podem apresentar efeitos anticancerígenos, suprimindo a proliferação de células cancerosas como no caso de câncer na próstata, mama, cólon e pulmão (LIN et al., 2013). Recente pesquisa reportou os benefícios desses POCs que podem atuar também nas paredes de células-tronco mesenquimais, exibindo propriedades de inibir a diferenciação de adipócitos (LEVY et al., 2017).

Em contrapartida, quando estes compostos são obtidos exogenamente através da dieta, eles podem causar efeitos deletérios em outros componentes e estruturas presentes no organismo. Durante a preparação de alimentos, em altas temperaturas, a oxidação do colesterol é iniciada principalmente no carbono 7, formando hidroperóxidos 7α e 7β simultaneamente, e então, esses compostos são reduzidos a seus álcoois correspondentes, 7α- e 7β-hidroxicolesterol, ou 7-cetocolesterol (CHIEN, WANG e CHEN, 1998; SMITH, 1987). O colesterol também pode degradar e levar à formação de outros produtos, como hidrocarbonetos de cadeia curta, aldeídos, cetonas e álcoois (SMITH, 1987). Na presença de ar, o colesterol pode formar epóxidos como 5α, 6α-epoxicolesterol e 5β, 6β-epoxicolesterol (CARDENIA et al., 2013). Além disso, esses epoxicolesteróis também podem ser formados por reações com diferentes espécies de oxigênio ativo, como radicais hidroxila ($^{\bullet}OH$), alcoxila ($RO^{\bullet}$), peroxila ($ROO^{\bullet}$), ozônio ($O^{\bullet}_3$) e peróxido de hidrogênio (H_2O_2) (GUMULKA, PYREK e SMITH, 1982; GUARDIOLA et al., 1996).

Durante o processamento térmico, alterações como perda de atividade antioxidante enzimática, ruptura das membranas celulares, que trazem ácidos graxos poli-insaturados em contato com pró-oxidantes, e decomposição térmica de hidroperóxidos em espécies pró-oxidantes são responsáveis pelo aumento da taxa de oxidação do colesterol (HUR et al., 2007). Desta forma, a formação dos POCs de origem exógena é considerada prejudicial à saúde, visto que possuem efeitos adversos à saúde humana, acarretando danos às células, como a ocorrênica de citotoxicidade, aterogênese e carcinogênese. Esses efeitos negativos relacionados aos POCs exógenos têm sido relatados em muitos estudos que apresentam a correlação entre a presença de POCs e alterações nas propriedades das membranas celulares com o desenvolvimento de doenças crônicas e neurodegenerativas (HUR et al., 2014; ALFONSO-GARCÍA et al., 2014; HUR, SEO e AHN, 2015; KULIG et al., 2015; KULIG et al., 2016; OLIVIER et al., 2017; ROSA-FERNANDES et al., 2017)

A presença de POCs também afeta a interação de proteínas e peptídeos com a bicamada lipídica, tal como os peptídeos β-amiloides associados à doença de Alzheimer, que apresentam maior interação com a bicamada lipídica quando na presença de óxidos de colesterol (PHAN et al., 2013; ZARROUK et al., 2014). Outras doenças neurodegenerativos como Parkinson e Huntington também podem ser associadas à ingestão de POCs. Estudos mostraram alterações nos níveis plasmáticos de 24-hidroxicolesterol e 27-hidroxicolesterol em pacientes com doença de Huntington (LEONI e CACCIA, 2015).

Além dos efeitos deletérios citados, os POCs estão relacionados a outros potenciais efeitos mutagênicos e citotóxicos. Evidências de pesquisas relataram que

vários órgãos, como cérebro, olhos, coração, cólon, pâncreas, fígado e próstata podem ser adversamente afetados por esses compostos citotóxicos (ALFONSO-GARCÍA et al., 2014). Como também, estão associados ao desenvolvimento de câncer de cólon, mama, próstata, pele e pulmão, entre outros (THANAN et al., 2014; JAVITT, 2015; MARWARHA et al., 2017).

Outro efeito negativo relacionado à ingestão de POCs para a saúde humana é a formação de placas de gordura nos vasos sanguíneos, denominada aterosclerose, sendo considerada como um dos principais eventos envolvidos no comprometimento do sistema cardiovascular. É caracterizado pelo acúmulo de colesterol nos macrófagos nas paredes das artérias, levando à formação subsequente de placas ateroscleróticas, que gradualmente contribuem para o desenvolvimento de várias doenças. Os principais POCs encontrados em lesões ateroscleróticas são 27-hidroxicolesterol e 7-cetocolesterol, seguidos de 7α- e 7β-hidroxicolesterol (KHATIB e VAYA, 2014).

Evidências de estudos clínicos também comprovaram a presença de POCs associadas ao desenvolvimento de outros efeitos negativos no corpo humano, como, diabetes tipo 2 (SOTTERO et al., 2015), perda neurossensorial da audição (MALGRANGE et al., 2015), processos crônicos de inflamação da vesícula biliar, problemas renais (KULIG et al., 2016), doenças oculares (RODRIGUEZ et al., 2014; GAMBERT et al., 2017) e osteoporose (SATO et al., 2017).

Embora um grande número de pesquisas tenha sido conduzidas demonstrando os diversos efeitos deletérios dos POCs (citotóxico, aterogênico e carcinogênico), ainda não foi determinada a ingestão diária mínima, necessitando assim, a realização de estudos *in vivo* relacionados ao papel metabólico, sobre a bioabsorção destes compostos pelo organismo humano.

5 Oxidação do Colesterol em Pescados Processados Termicamente

Apesar do consumo de peixes crus ter aumentado nos últimos anos (KAWAI et al., 2012), o tratamento térmico é ainda um dos principais métodos de preparo de pescado. Devido aos elevados níveis de óxidos de colesterol encontrados em pesquisas com peixes processados termicamente (DANTAS et al., 2015), os efeitos do processamento térmico na composição lipídica em pescado, resultando na formação de compostos oxidados, como óxidos de colesterol, tem sido objeto de estudos científicos nos últimos anos (SALDANHA, BENASSI e BRAGAGNOLO, 2008; SALDANHA e BRAGAGNOLO, 2010; VAISALI, 2016; TARVAINEN et al; 2016).

As frações lipídicas do peixe, geralmente compostas principalmente de AGPIs e com altos níveis de colesterol são particularmente suscetíveis aos processos de oxidação quando exposto a fatores favoráveis durante o processamento e armazenamento (SALDANHA et al., 2008; DANTAS et al., 2015; FREITAS et al., 2015).

Os peixes geralmente são submetidos a diferentes formas de tratamento térmico para seu consumo. Os diferentes métodos de aquecimento são um fator chave no processo de oxidação do colesterol. Maiores quantidades de POCs são formados quando o alimento é submetido a uma maior intensidade de calor (MORGAN e ARMSTRONG, 1992). Altas temperaturas produzem grandes quantidades de radicais livres devido à aceleração da propagação reações e a decomposição de hidroperóxidos lipídicos (OTAEGUI-ARRAZOLA et al., 2010). Estudos concluíram que os óxidos de colesterol são produzidos após tratamento térmico de 120 °C e que a composição dos produtos formados está diretamente relacionada à temperatura e o tempo de

aquecimento. Além disso, segundo os autores, a produção de óxidos de colesterol atinge um máximo quando aquecidos a 150 °C (OSADA et al., 1993a). Também observaram que o colesterol mostrou-se instável, especialmente quando aquecido com gorduras insaturadas, quando os autores observaram redução na concentração total e aumento na produção de óxidos de colesterol. Esse fato revela que a presença de lipídios insaturados favorece a oxidação do colesterol (OSADA et al., 1993 b).

O efeito de diferentes métodos de cocção (fervura, vapor e cozimento) e secagem, sobre os teores e formação de óxidos de colesterol em lulas (*Todarodes pacificus*) foram avaliados por HOMNG et al., 1996, onde a presença de 22-hidroxicolesterol e colestanetriol foram detectados, mas não quantificados.

Em um estudo sobre os efeitos da fritura por imersão em óleo, cozimento e sob o aquecimento de microondas, HEE-KIM et al. (2000) avaliaram a relação do processamento com a formação de POCs em sauri (*Cololabis seira*). Os autores relataram a produção de altas concentrações de 7α e 7β-hidroxicolesterol, bem como 5,6α e 5,6β epóxidos em todas as amostras; as concentrações mais elevadas foram encontradas nas amostras fritas por imersão em óleo. 7-cetocolesterol e o 25-hidroxicolesterol foram observados em amostras submetidas ao cozimento e em microondas. No entanto, o colestanetriol, o mais tóxico dos POCs, foi observado apenas nas frituras por imersão.

ECHARTE et al. (2001) avaliaram a formação de óxidos de colesterol comparando 3 maneiras diferentes no preparo de salmão; frito com azeite, frito com óleo de soja (com 30 mL de azeite e 30 mL de óleo de soja, respectivamente, em frigideira convencional a 180 °C/4 min, 2 min. para cada lado) e assado (com 45 mL de azeite em forno a 200 °C por 30 min.). Os autores da pesquisa identificaram os seguintes óxidos: colestanetriol, 7-cetocolesterol, 5,6α-epoxicolesterol, 25-hidroxicolesterol, 7α-hidroxicolesterol e 7β-hidroxicolesterol, com aumento significativo nas concentrações de óxidos após o aquecimento de lipídios nas amostras cruas para 2,98 μg/g emas amostras fritas em azeite, a 3,35 μg/g nas amostras fritas em óleo de soja e 7,38 μg/g de lipídios nas amostras de salmão assado. O principal óxido observado em todas as amostras foi o 7-cetocolesterol.

AL-SAGHIR et al. (2004) analisaram amostras de salmão submetidas a para preparação térmica por fritura (180 °C/ 6 min, 3 min. de cada lado) com diferentes tipos de óleos (azeite, óleo de milho e óleo vegetal hidrogenado), fritos sem óleo (6 min, 3 min cada lado) e no vapor (66 a 67 °C/12 min). Os autores observaram um aumento significativo nas concentrações no total de óxidos de colesterol das amostras aquecidas. Os produtos de oxidação do colesterol (POCs) aumentaram após os processos de aquecimento, a partir de 0,86 μg/g nas amostras cruas, para 5,98 μg/g nas amostras fritas a óleo, 3,98 μg/g com azeite, 4,38 μg/g em óleo de milho, 3,34 μg/g em óleo vegetal parcialmente hidrogenado e a 9,88 μg/g nas amostras cozidas no vapor. Entre os métodos de processamento térmico, o vapor resultou em maiores níveis de POCs, neste estuo, provavelmente devido ao tempo de aquecimento mais longo (12 min) em comparação com as amostras fritas.

Os efeitos dos óleos vegetais (milho, canola, soja e azeite) foram avaliados sobre a oxidação do colesterol em amostras de salmão assado, submetido a diferentes temperaturas (150, 175 e 200 °C) e tempos de processamento (10, 20 e 30 min). De acordo com os resultados da pesquisa, o principal óxido (22-cetocolesterol) não foi detectado após aquecimento a 125 °C durante 30 min. No entanto, no salmão aquecido a 200 °C durante 10 min, os autores observaram o teor de 0,98 μg/mL. Dentre os diferentes óleos estudados, o óleo de soja obteve a maior capacidade para evitar a

oxidação do colesterol, devido aos níveis mais elevados de tocoferóis, que atuaram como componentes antioxidantes (ZHANG, 2005).

ASTIASARÁN et al. (2007) compararam os efeitos de diferentes métodos de cocção (microondas, fritura em azeite, grelhados e assados) na formação de POCs em amostras de salmão e camarão tratados termicamente. Segundo os autores, o microondas usado 650 W, e as temperaturas nos outros processos foram: 180 °C com azeite; 95 a 100 °C nas amostras assadas e 180 °C (1,5 min de cada lado) nas amostras grelhadas. Os resultados obtidos apresentaram a presença dos seguintes óxidos: 7α-hidroxicolesterol, 19-hidroxicolesterol, 7β-hidroxicolesterol, 5,6β-epoxicolesterol,5,6α-epoxicolesterol, colestanetriol, 25-hidroxicolesterol, e 7-cetocolesterol. Após o tratamento térmico, um aumento significativo do total de POCs foi encontrado no salmão, sendo que o uso do microondas apresentou um aumento maior de óxidos em relação à fritura e assado, aumentando o teor de POCs 42 vezes em relação às amostras cruas (de 0,016 para 0,674 mg/100 g de amostra).

Um estudo comparou dois tipos de peixes (pescada e sardinha) grelhados sobre o efeito do tratamento térmico na formação de POCs. Os autores observaram redução de colesterol (P <0,02) após grelhar (175 °C/2 min em pescada) e um aumento significativo de óxidos de colesterol nas duas espécies de peixe, representando um total de 20,62 para 43,28 μg/g de POCs em sardinhas e de 8,79 a 19,35 μg/g em pescada, em base seca. Entre os POCs identificados estavam o 7-cetocolesterol, 19-hidroxicolesterol, 22(S)-hidroxicolesterol, 25(R)-hidroxicolesterol, 25-hidroxicolesterol e 24 (S)-hidroxicolesterol (SALDANHA e BRAGAGNOLO, 2010).

Diferentes tipos de processamento térmico foram testados por FREITAS et al. (2015) para avaliar os níveis de 7-cetocolesterol em pescada do Atlântico (*Merlucciushubbsi*) e filés de *smooth weakfish* "Rabeta brasileira" (*Cynoscion leiarchus*), submetidos aos seguintes métodos: assado em forno elétrico, (200 °C/30 min) ou microondas (15 min); cozimento (180 °C/25min), grelhado (220 °C/8 min), cozido a vapor (150 °C/30 min), fervido (96 °C/18 min), em forno eléctricos de convecção forçada (180 °C/4 min) e fritos (180 °C/3 min). Todas as amostras apresentaram 7-cetocolesterol. O vapor produziu o nível mais baixo de 7-cetocolesterol nas amostras com 6,90 μg/g em pescada e 6,47 μg/g de lipídios, em filés de *Cynoscion leiarchus*. Os tratamentos utilizando altas temperaturas, como assados no forno comum e nos fornos eléctricos de convecção foram associados com um aumento dos níveis 7-cetocolesterol (11,54 e 13,94 μg / g, respectivamente).

A oxidação durante o processamento de alimentos de origem animal pode causar uma perda de qualidade dos alimentos e a formação de compostos oxidados, considerados tóxicos ao organismo. O processo oxidativo ou peroxidação dos lipídios pode ser evitado com a utilização de substâncias com potencial antioxidante. Por essa razão, vários estudos têm sido realizados utilizando antioxidantes nos alimentos como uma estratégia para prevenir a oxidação durante processamento e armazenamento, consequentemente, com o intuito de inibir ou minimizar a formação de óxidos de colesterol e assim reduzir a ingestão desses óxidos pela alimentação (CABONI, FREGA e LERCKER, 2005; LEE et al., 2008; BARRIUSO et al., 2015, 2016; SAMPAIO et al., 2012; FIGUEIRÊDO et al., 2015; BIERZUŃSKA et al., 2017).

6 Uso de Antioxidantes Naturais na Inibição da Formação de POCs em Pescado

Antioxidantes são compostos que inibem ou retardam as reações de oxidação por mecanismos diferentes. Eles variam de acordo com a estrutura química e podem ser

classificados em sintéticos ou naturais (DE e CHATTERJEE, 2015; EMBUSCADO, 2015). Os antioxidantes sintéticos são amplamente utilizados na indústria alimentar, e os mais comumente usados são butilhidroxianisol (BHA), butil hidroxitolueno (BHT), propil galato (PG) e terc-butil hidroquinona (TBHQ) (DOLATABADI e KASHANIAN, 2010). No entanto, devido aos potenciais efeitos tóxicos e carcinogênicos, e consequentemente aos possíveis danos à saúde causados pela ingestão de produtos sintéticos (DE e CHATTERJEE, 2015; ESKANDANI, HAMISHEHKAR e DOLATABADI, 2014), nos últimos anos, há uma crescente tendência na substituição destes compostos pelos antioxidantes naturais (BARRIUSO et al., 2015; DE e CHATTERJEE, 2015; KUMAR et al., 2015; MI et al., 2016).

Com base nos resultados de efeitos adversos à saúde humana na utilização de antioxidantes sintéticos reportados em diversas pesquisas científicas (NAGAI, OKUBO, USHIYAMA, SATOH e KANO, 1996; YU, TAN e KONG, 1997; OKUBO, YOKOYAMA, KANO e KANO, 2003; KASHANIAN e DOLATABADI 2009; BOTTERWECK, VERHAGEN, GOLDBOHM, KLEINJANS e VAN DEN BRANDT, 2000; NAGAI et al., 1996; ESKANDANI et al., 2014; WANG et al., 2014), deve-se ser reavaliado o uso generalizado na indústria de alimentos e os possíveis riscos para o consumo humano.

Consequentemente, o uso de produtos naturais como antioxidantes tem atraído à atenção de consumidores e da comunidade científica. Pois, em geral, são considerados mais seguro do que os antioxidantes sintéticos (DE OLIVEIRA et al., 2018). Desta forma, os compostos bioativos além de fornecerem nutrientes, são capazes de adicionar sabores característicos aos alimentos como no caso do uso de ervas aromáticas e especiarias. Além disso, estudos têm mostrado os efeitos protetores como antioxidantes naturais na oxidação lipídica, aumentando a vida de prateleira de alimentos (KUMAR et al., 2015; RAEISI, SHARIFI-RAD, QUEK, SHABANPOUR e SHARIFI-RAD, 2016). Os antioxidantes naturais incorporados em alimentos são assimilados pelo organismo e apresentam potencial valor nutricional e propriedades terapêuticas proporcionando benefícios adicionais à saúde (EMBUSCADO, 2015; CALEJA et al., 2016).

Os mecanismos de ação dos antioxidantes utilizados em alimentos contendo lipídios insaturados e colesterol atuam retardando a oxidação dos lipídios de duas maneiras: protegendo os lipídios dos iniciadores da reação ou inibindo a oxidação na fase de propagação (LAGUERRE, LECOMTE e VILLENEUVE, 2007). E ainda, de acordo com o mecanismo de ação, os antioxidantes podem ser classificados em antioxidantes primários e antioxidantes secundários (KUMAR et al., 2015).

Os antioxidantes considerados como primários são capazes de doar elétrons ou hidrogênio para o radical formado nas fases de oxidação lipídica, como a iniciação e propagação, convertendo-as em produtos termodinamicamente estáveis e/ou formando o complexo lipídico-antioxidante, que pode reagir com outro radical (KUMAR et al., 2015). Os antioxidantes secundários reduzem a taxa de oxidação através de diferentes mecanismos de ação, como a absorção da radiação UV, inibição de enzimas pró-oxidantes e decomposição de hidroperóxidos em espécies não-radicais, o que leva à inibição de reações em cadeia, a fim de evitar a abstração contínua de hidrogênio a partir dos substratos (RICE-EVANS, MILLER e PAGANGA, 1997; SHAHIDI e AMBIGAIPALAN., 2015). Além disso, devido à ação catalítica de íons metálicos e oxigênio, os antioxidantes secundários reduzem a taxa de oxidação lipídica removendo ou sequestrando esses catalisadores e tornando-os inativos (EMBUSCADO, 2015).

Como o processo de oxidação do colesterol é semelhante ao processo que envolve outros lipídios insaturados (SMITH, 1987; MEDINA-MEZA e BARNABA,

2013), pode-se considerar que os antioxidantes apresentam o mesmo mecanismo de ação para todas as moléculas lipídicas.

Os antioxidantes mais eficazes são aqueles que atuam desorganizando reações dos radicais em cadeia. Estes compostos são geralmente constituídos por anéis aromáticos que promovem a remoção ou inativação dos radicais reativos doando átomos de hidrogênio para essas moléculas, interrompendo a reação em cadeia. O átomo de hidrogênio ativo do composto antioxidante é abstraído pelos radicais reativos mais fácil do que os hidrogênios alílicos das moléculas insaturadas. Assim, as espécies inativas são formadas, tornando-se incapaz de iniciar ou propagar as reações oxidativas (NAWAR, 1991).

No entanto, a ação dos antioxidantes também depende da estrutura molecular da matriz alimentar a ser adicionado, como também é influenciada por outros fatores relacionados aos lipídios envolvidos, como a natureza lipídica, equilíbrio hidrofílico-lipofílico do antioxidante, e outras interações. Antioxidantes que são eficazes em um sistema podem não ser aplicáveis em outro. Assim, estudos comparativos são necessários entre diferentes compostos para determinar o melhor antioxidante para cada sistema específico (VAISALI, BELUR e REGUPATHI, 2016).

Os antioxidantes encontrados nos extratos naturais são representados por categoria heterogênea de moléculas e sua eficácia antioxidante está diretamente relacionada às características químicas e físicas desses compostos, como tamanho, número de cargas e grau de hidroxilação e metilação, que diferem em quantidade e natureza, dependendo da fonte específica (TUBEROSO et al., 2013; GARCÍA-CASAL, PEÑA-ROSAS e MALAVÉ, 2016; JIANG e XIONG, 2016).

Os compostos fenólicos estão entre os principais compostos bioativos presentes em produtos de origem vegetal e são responsáveis pela as propriedades antioxidantes dessas plantas e produtos vegetais. Os principais constituintes fenólicos são ácidos fenólicos (ácidos gálico, cafeico e rosmarínico), diterpenos fenólicos (carnosol e ácido carnósico), flavonóides (quercetina, catequina, apigenina, kaempferol, naringenina e hesperetina) e óleos voláteis (eugenol, carvacrol, timol e mentol) (BREWER, 2011).

Os fenólicos variam estruturalmente de uma única molécula fenólica para complexos de polímeros de alto peso molecular (SHAHIDI e AMBIGAIPALAN, 2015), que possuem anel aromático com um ou mais hidroxilos e também podem ter outros grupos substituintes em sua estrutura, tais como ésteres metílicos e glicosídeos (HAN, SHEN e LOU, 2007).

Extratos naturais de plantas frequentemente contêm altas concentrações de compostos fenólicos, que por sua vez, possuem forte atividade de doação de H ou têm alta capacidade de absorção de radicais (VIJI et al., 2015). Devido a esses mecanismos, os compostos fenólicos podem retardar e prevenir a oxidação do colesterol, pois controlam as concentrações de pró-oxidantes e inativam os radicais livres, como os formados nas posições C-7 e C-25, fornecendo 7α-hidroperoxicolesterol, 7β-hidroperoxicolesterol, e 25-hidroperoxicolesterol (MEDINA-MEZA e BARNABA, 2013). O potencial antioxidante dos compostos fenólicos depende do número e da posição dos grupos hidroxila (SHAHIDI e AMBIGAIPALAN, 2015). Por exemplo, o número e a localização dos grupos hidroxila livres na estrutura dos flavonóides definem o potencial de eliminação dos radicais reativos. Desta forma, estruturas poliméricas com alto número de grupos hidroxilas apresentam maior potencial antioxidante (KUMAR et al., 2015).

Outro grupo importante de compostos ativos são os carotenoides, sendo efetivos sequestradores de oxigênio e radicais singlete (BÖHM, EDGE e TRUSCOTT, 2012),

consequentemente, os carotenoides permitem a eliminação de outras espécies radicais (CAROCHO e FERREIRA, 2013). As reações de carotenóides com radicais são mais complexas e dependem principalmente de a natureza do radical, em um mecanismo envolvendo a transferência de elétrons e abstração de hidrogênio (BÖHM et al., 2012). O oxigênio singlete é um dos EROs mais reativos na oxidação do colesterol, principalmente, a oxidação por fotooxidação do colesterol iniciado pelo oxigênio singlete, levando à formação hidroperoxicolesteróis, como 5α-hidroperoxicolesterol, que podem sofrer um rearranjo alílico para dar origem tanto a 7α- hidroperoxicolesterol e 7β-hidroperoxicolesterol (BOSELLI, CARDENIA e RODRIGUEZ-ESTRADA, 2012). Assim, os carotenoides são considerados compostos importantes, que podem controlar a oxidação do colesterol.

Além destes compostos naturais mencionados anteriormente, alguns minerais (Se, Zn) e vitaminas (vitamina A, vitamina C e vitamina E) também atuam como co-fatores para enzimas antioxidantes e também são considerados antioxidantes naturais. Além disso, os peptídeos naturais são capazes de neutralizar os radicais livres e o íons metálicos prooxidativos quelantes (JIANG e XIONG, 2016).

A utilização de antioxidantes naturais permite manter ou melhorar a qualidade dos alimentos e suas características sensoriais durante o processamento e armazenamento dos mesmos. O processo oxidativo ou peroxidação dos lipídios pode ser evitado com a utilização de substâncias antioxidantes. O desenvolvimento de técnicas para utilização de antioxidantes naturais em detrimento dos sintéticos é uma demanda dos consumidores, da legislação, e tem despertado cada vez mais o interesse dos pesquisadores em testar novas fontes de antioxidantes naturais em pescado. Diversas pesquisas foram realizadas para avaliar os efeitos de diferentes fontes de antioxidantes naturais (ARBELOA et al., 2010; FAN et al., 2010; SANCHO et al., 2011; SEKHON-LOODU et al., 2013; FARVIN et al., 2014; FIGUEIRÊDO et al., 2015; VAISALI, BELUR, REGUPATHI, 2016).

A influência de tocoferóis foi testada para inibir a oxidação do colesterol em peixes, com resultados positivos em relação à formação de óxidos de colesterol em alimentos processados e durante o armazenamento. Concluindo que a adição de vitamina E ou alguma forma natural de tocoferóis atingiu as expectativas (LI et al., 1996). Outros autores testaram os efeitos da adição de tocoferóis e BHA na oxidação de colesterol durante o processamento e armazenamento subsequente de anchovas cozidas e secas, e o BHA apresentou melhores resultados que os tocoferóis. Os níveis de 7α-hidroxicolesterol, 7-cetocholesterol, e colesterol-5,6-α- e β-epóxidos na amostra com BHA foram menores, inibindo quase 50% da formação desses óxidos (SHOZEN et al., 1997).

SANCHO et al. (2011) avaliaram o potencial das sementes de urucum visando minimizar a oxidação do colesterol em amostras de almôndegas de pescada branca cozidas em água durante 30 min. A especiaria foi avaliada contra a oxidação do colesterol isolada e em combinação com coentro. Entretanto, os autores observaram que não houve alteração nos teores de óxido colesterol nas almôndegas após o cozimento, indicando que o tratamento térmico não foi significativo ($P > 0,05$), desta forma, sendo que não houve formação de POCs após cozimento das almondegas, não tem como concluir o efeito das especiarias sobre a oxidação do colesterol em pescado nesse estudo.

A interação entre o óleo de fritura (soja) e a formaçãode óxidos de colesterol em amostras de camarões grandes de água doce (*Macrobrachium acanthurus*) foi avaliada por SIMON et al. (2012). Esses autores observaram a presença de 7-cetocolesterol e

colestanetriol nas amostras cruas. No entanto, após fritar em óleo de soja (50 mL, 5 min a 160 °C), não houve diferenças significativas nos níveis dos produtos de oxidação avaliados. Carotenóides naturalmente presentes no camarão, e os tocoferóis do óleo de soja podem ter atuado sinergicamente, protegendo as amostras da oxidação do colesterol.

HERNANDEZ-BECERRA et al. (2014) monitoram a formação de POCs e as mudanças no conteúdo de astaxantina, um carotenoide responsável pela cor característica dos camarões durante os processos de cozimento. Este carotenóide tem um alto potencial antioxidante, no entanto, os autores verificaram uma alta formação de POCs durante o processamento. Assim, os autores concluíram que as condições do processo levaram à degradação da astaxantina, reduzindo sua atividade como antioxidante.

Em um estudo realizado por TARVAINEN et al. (2016), o efeito de três diferentes extratos: folhas de alecrim, folhas de orégano, e uma mistura de extratos de sete ervas aromáticas (açafrão, orégano, lúpulo, cravo, salvia, ajowan e alcaçuz) foi investigado contra a oxidação do colesterol em filetes de salmão do Atlântico durante a preparação térmica (180 °C durante 20 min). Os resultados apresentaram 14 μg/g no total de POCs nas amostras controle após o tratamento térmico. Em contrapartida, em todas as amostras contendo os extratos naturais, foi observado valores bem inferiores (<1 μg/g) após o processamento, confirmando assim o potencial desses antioxidantes naturais no retardamento da termo-oxidação do colesterol.

Tendo em vista a importância do pescado como fonte de nutrientes funcionais para a saúde humana, e as inúmeras pesquisas a respeito dos efeitos benéficos dos ácidos graxos n-3, aliados à formção de óxidos de colesterol durante o processamento térmico dos peixes; esses fatos sugerem que medidas preventivas sejam tomadas para evitar a formação desses compostos citotóxicos. Desta forma, a utilização das ervas aromáticas, salsa, cebolinha e cheiro verde apresentam grande potencial como antioxidantes naturais com o propósito de minimizar a formação de compostos oxidados em pescado, com o propósito de garantir a segurança no consumo destes alimentos. Assim, a utilização dessas ervas culinárias em sardinhas processadas termicamente, por panela do tipo *air-fryer e grill* elétrico, mostrou-se como uma interessante fonte alternativa de se estudar os efeitos protetores das mesmas contra a oxidação do colesterol e ácidos graxos insaturados presentes nas sardinhas.

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CAPÍTULO I

REVIEW: POTENTIAL PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITIES OF PARSLEY (*Petroselinum crispum*) (Mill.) Nym. AND CHIVES (*Allium schoenoprasum* L)

ABSTRACT

The increased demand for food or products without synthetic additives has attracted the attention of researchers towards the employment of new natural antioxidants sources. The antioxidant effects of various plant and their extracts as well as individual phenolic compounds have been evaluated and tested in numerous studies. Aromatic herbs such as parsley (*Petroselinum crispum*) and chives (*Allium schoenoprasum* L.) have been used worldwide for a long time both in cooking as natural seasonings and flavorings, in traditional medicine for the prevention of many diseases, as well as in many tests in the medical and cosmetic areas. They are a very important source of phytochemicals, which contain bioactive compounds with great chemistry and biological activities. The *in vitro* and *in vivo* studies have demonstrated the potential of these aromatic herbs as antioxidants, digestive stimulants, antibacterial, anti-inflammatory, antiviral, and anti-cancer activities. The aim of this review is to present the literature data related to the bioactive compounds and their main effects present in parsley and chives as functional foods in different applications.

Keywords: Aromatic Plants. Bioactive Compounds. Phenolic Compounds

RESUMO

O aumento da demanda por alimentos ou produtos sem aditivos sintéticos tem atraído a atenção dos pesquisadores para o emprego de novas fontes antioxidantes naturais. Os efeitos antioxidantes de várias plantas e seus extratos, bem como compostos fenólicos utilizados individualmente foram avaliados e testados em diversos estudos. As ervas aromáticas como a salsa (*Petroselinum crispum*) e a cebolinha (*Allium schoenoprasum* L) têm sido mundialmente utilizadas tanto na culinária como temperos naturais e aromatizantes, quanto na medicina tradicional para prevenção de muitas doenças, bem como em diferentes testes na área médica e de cosméticos. Estas ervas são consideradas fontes importantes de fitoquímicos, que contêm compostos bioativos com capacidades antioxidantes. Ambos os estudos, *in vitro* e *in vivo* demonstram o potencial dessas ervas aromáticas como antioxidantes, estimulantes digestivos, entre outras diferentes atividades, como antibacterianas, anti-inflamatórias, antivirais e anticâncer. Esta revisão teve como objetivo apresentar os dados da literatura relacionados aos compostos bioativos e seus principais efeitos benéficos presentes na salsa e cebolinha como alimentos funcionais, ou seja, que oferecem benefícios à saúde, além de suas funções nutricionais básicas. Eles podem, por exemplo, reduzir o risco de doenças crônicas degenerativas, como câncer e diabetes, entre outras.

Palavras-chave: Plantas Aromáticas. Compostos Bioativos. Compostos Fenólicos.

1 INTRODUCTION

In recent years, the use of natural antioxidants has attracted the attention of consumers and researchers worldwide, due to the harmful effects of the synthetic antioxidants. In addition, there is also been a growing interest of the food industry in incorporating natural compounds for direct addition or to be used in synergy with other compounds has been increasing or this purpose to have beneficial properties for health (CAROCHO et al., 2014; MAQSOOD et al., 2014; VALLVERDÚ-QUERALT et al., 2014; DE OLIVEIRA et al.,2018).

Traditionally, culinary herbs are immensely used in cooking to give flavor and aroma to food; they can be used fresh, dried, chopped or ground. Furthermore, they can be employed as preservatives, since they are constituted by components with antioxidant and antimicrobial properties (WONG e KITTS, 2006; VIUDA-MARTOS et al., 2010). Certain herbs are also listed as "medicinal plants" by providing compounds, which present beneficial effects to human health such as anti-inflammatory actions, anti-viral and anti-cancer (KAISER et al., 2009; VIUDA-MARTOS et al., 2011; CHARLES, 2013; PÁPAY e ANTAL, 2014; PARVU et al., 2014; ABOU KHALIL et al., 2016; SĘCZYK et al., 2016; BERETTAet al., 2017; RABOU e EID, 2017; VENKATESH, 2018).

Aromatic herbs contain many chemicals and phytochemical constituents such as minerals, vitamins, carotenoids, fatty acids, phytosterols, and phenolic acids, among others. The phenolic compounds are omnipresent in the plant kingdom (ROBARDS, 2003); additionally, they contribute to the intake of natural antioxidants, consequently promote the protection of important cellular components such as DNA, proteins, and lipid membranes against the action of reactive oxygen species (MATÉS et al., 1999; SEIFRIED et al., 2007; LOBO et al., 2010; LUSHCHAK et al., 2014, GASCHLER et al., 2017; GHOSH et al., 2017; ISLAM et al., 2017; LAURINDO et al., 2018).

Among the aromatic herbs, including parsley (*Petroselinum crispum*) and chives (*Allium schoenoprasum* L) both plants have been studied in several areas. Parsley is native from Mediterranean cultures, and has been used in many parts of the world since ancient times in both food and medicine (DIAZ-MAROTO, 2003; FARZAEI et al., 2013). Parsley contains several bioactive compounds such as luteolin, folic acid, vitamins C and E, thiamine, riboflavin and minerals (P, K, Na, Ca, Mg, Fe, Mn, Zn and Cu) (SOYSAL, 2004; SANTOS et al., 2014). It is also considered a medicinal plant with numerous proven pharmacological properties such as: antioxidant, hepatoprotective, neuroprotective, antidiabetic, analgesic, spasmolytic, immunosuppressive, anti-coagulant, anti-ulcer, laxative, estrogenic, diuretic, hypotensive, antibacterial and antifungal agents (FARZAEI et al., 2013). Thus, the results of researches indicate a potential role in the extracts of phenolic compounds derived from parsley as natural antioxidants beneficial to health (SOYSAL, 2004; ZHANG et al., 2006; YANTIZ et al., 2008; LANTTO et al., 2009; SANTOS et al., 2014), as well as for the development of new drugs (LANTTO et al., 2009).

Chives are extensively used for culinary purposes throughout the world. It is a perennial herb that can be cultivated and harvested many times throughout the year. In addition, unlike the spicy taste of garlic and onion, chives have a softer flavor (PARVU et al., 2014). The main constituents of chives are carbohydrates, proteins and amino acids and additionally it also contains several vitamins such as α-Tocopherol (Vitamin E), β-carotene (Pro-vitamin-A), ascorbic acid (C), thiamine (B1), riboflavin (B2),

nicotinamide (B3), pantothenic acid (B5), pyridoxine (B6) and minerals (P, K, Na, Ca, Mg, Fe, Mn, Zn and Cu) (SANTOS et al., 2014). *Allium schoenoprasum* L can be used to reduce blood pressure, relieve sunburn, sore throats, as well as used as microbial and antifungal agents (RATTANACHAIKUNSOPON e PHUMKHACHORN, 2008).

The mixture of parsley (*salsa*) and chives (*cebolinha*) is popularly known in Brazil as *cheiro-verde* (literally "green aroma"). They are widely consumed, usually freshly chopped, as key seasoning for major Brazilian dishes, including fish, meat, chicken, rice, beans, stews, soups, vegetables, salads, condiments, sauces, and stocks. *Cheiro-verde* (**Figure 1**) is commonly sold in food markets as a bundle of both types of fresh herbs.

Figure 1- Mixture of chives and parsley = *cheiro- verde. * Seasoning usually used in Brazilian cuisine*

Numerous studies were carried out on parsley and chives on chemical characterization and beneficial effects on health. Thus, the purpose of this review is to provide comprehensive information on the phytochemistry and biological properties as natural antioxidants on both herbs in order to explore and deepen the knowledge regarding their bioactive potential. The current state of the art is evaluated the trends of these culinary herbs for future food industry applications.

2 PARSLEY (*Petroselinum crispum* (Mill.) Nym.)

Parsley (*Petroselinum crispum*, (Mill.) Nym. Apiaceae family) is an aromatic herb (**Figure 2**), which is a biennial plant in temperate climates, or an annual herb in subtropical and tropical areas. This herb is a native plant of southern Europe, originated from the Mediterranean culture, it is one of the most common culinary herb consumed globally (DIAZ-MAROTO, 2003; FARZAEI et al., 2013).

In central Europe, Eastern Europe, and southern Europe, as well as in western Asia, freshly chopped green parsley is used as an ingredient in stocks, soups, and sauces, on boiled or mashed potatoes, rice dishes (risotto or pilaf), on fish, fried chicken, lamb, goose, and steaks, as well in meat or vegetable stews including shrimp creole, beef bourguignon, goulash, or chicken, as well this fresh herbs are used on toppings in green salads, and sandwiches with cold cuts or pâtés dishes.

Figure 2: Parsley (*Petroselinum crispum*)

In Brazil it was introduced by the Portuguese settlers, the fresh and dried parsley is also widely used as flavoring in many different food products due to its powerful aromatic odor. This aromatic herb has also been utilized around the world since antiquity in food as well as in folk medicine, cosmetics, pharmaceutical and medicinal tests purposes (DIAZ-MAROTO et al., 2003; SOYSAL, 2004; VIUDA-MARTOS et al., 2010; PAPAY et al., 2012; FARZAEI et al., 2013).

2.1 Chemicals and Phytochemical Constituents in Parsley

Diverse studies have shown the chemical characterization of *Petroselinum crispum* and numerous phytochemicals compounds were identified in this culinary herb. The fresh leaves of *Petroselinum crispum* are highly nutritious and can be considered a natural vitamin and mineral supplement (TRIFUNSCHI e ARDELEAN, 2012). Although it is not commonly eaten in large quantities, parsley is also a good natural source of carotene (pro vitamin A), vitamins B1, B2, and C, as well as iron and other minerals (PAPAY et al., 2012). Furthermore, this herb contains other bioactive compounds such as luteolin, folic acid, vitamins E, thiamine, riboflavin, and minerals (P, K, Na, Ca, Mg, Fe, Mn, Zn and Cu) (SOYSAL, 2004; PAPAY et al., 2012; SANTOS et al., 2014). In **Table 1** are shown the nutritional and recommendation (%) content of parsley, according with USDA Nutrient Database (2018).

Table 1. Data adapted from nutritional content of parsley (*Petroselinum crispum*), USDA Nutrient Database (2018).

Parsley- *Petroselinum crispum*, fresh	
Nutritional value per 100 g (3.5 oz)	
Energy -151 kJ (36 kcal)	
Carbohydrates	
Total Carbohydrates	6.33 g
Sugars	0.85 g
Dietary fiber	3.3 g
Fat	
Total Fat	0.79 g
Total fatty acids	0.132 g
Total monounsatured fatty acids	0.295 g
Total polyunsatured fatty acids	0.124 g
Phytosterols	5 mg
Protein	
Total Protein	2.97 g
Vitamins	**value per 100 g**
Vitamin A	8424 µg
Beta-Carotene	5054 µg
Lutein zeaxanthin	5561 µg
Thiamine (B1)	0.086 mg
Riboflavin (B2)	0.098 mg
Niacin (B3)	1.313 mg
Pantothenic acid (B5)	0.400 mg
Vitamin B6	0.090 mg
Folate (B9)	152 µg
Vitamin C	133 mg
Vitamin E	0.75 mg
Vitamin K	1640 µg
Minerals	**value per 100 g**
Calcium,Ca	138 mg
Iron, Fe	6.20 mg
Magnesium, Mg	50 mg
Manganese, Mn	0.160 mg
Phosphorus, P	58 mg
Potassium, K	554 mg
Sodium, Na	56 mg
Zinc, Zn	1.07 mg

Units: g = micrograms; mg = milligrams; IU = International units.
Data adapted from: USDA Nutrient Database (2018).

Studies have reported different contents of vitamin C present in parsley; CĂTUNESCU et al. (2012) ie 179.69 mg ascorbic acid /100g FW, higher than AZEEZ (2008), who reported for parsley a content of 110–200 mg vitamin C/100g edible material. On the other hand, LISIEWSKA et al. (2003) found greater content of vitamin C in fresh parsley leaves: 310 mg/100g for Hamburg type parsley, and 257 mg for the leafy type. KUŹMA et al. (2014) observed similar levels of vitamin C (248.31 mg/100 g dry matter) in parsley leaves.

LEAHU et al. (2016) also reported higher vitamin C content in parsley extract (347.6 mg /100). VIOLETA NOUR et al. (2017) in a research of bioactive compounds, antioxidant activity and nutritional quality of different culinary aromatic herbs, observed 206.32 (mg/100 g fw) of vitamin C in parsley, and the following minerals: Na, K, Ca, Mg, with their respectively contents: 18.32; 654.31; 142.47; 54.63 (mg/100 g fresh weight).

Another important component found in salsa is iron. *Petroselinum crispum* is often used in the popular medicine as an anti-anemic product, and is claimed to be rich in iron. Previous reports have stated iron contents in leaves varying between 126.2 and 1100 mg/kg (ANCUCEANU et al., 2018). Recently, a study has measured iron contents in the vegetative organs (roots, stem sand leaves) of parsley cultivated in Romania on three different soils, at three development stages. The iron contents in all vegetative organs of the plant varied between 21.6 in stems and 645.2 mg/kg in leaves. The results showed that iron contents tended to increase with the development stage and the soil type may have very limited influence on the iron contents (ANCUCEANU et al., 2018).

In relation to the lipid profile, the results of a study carried out by PARRY et al. (2006) showed that parsley seed oil had the highest oleic acid content, 81 g/100 g total fatty acid (FA), and the lowest saturated fat among the tested oils. The ratio of oleic to linoleic acid was about 7.4:1. Similar results were obtained by GUNSTONE (1991); they reported that parsley seed oil contained 81.9% oleic acid, with an oleic to linoleic acid ratio of 6.6:1. Palmitic (C16:0) and stearic (C18:0) acids were the most abundant fatty acids found in this plant (LOPEZ et al., 1999). The concentrations of oleic acid in the parsley seed oils were significantly higher than the concentrations commonly found in olive oil, which normally range between 68 and 73% (PARRY et al., 2006). The authors concluded that parsley seed oil may be an excellent source of monounsaturated fatty acids (MUFA).

Carotenoids and tocopherols are well recognized for their potential health benefits. In parsley leaves it is also noted the presence of carotenoids, including β-carotene, lutein, violaxanthin, and zeoxanthin (PARRY et al.,2006; LUTHRIA, 2008; YILDIZ et al., 2008; FARZAEI et al., 2013; KAISER et al., 2013). PARRY et al. (2006) found 989.1 µg/kg of β-carotene, 216.4 µg/kg of lutein, 20.55 mg/kg of zeaxanthin, and 1.43 (mg/kg) of cryptoxanthin, totaling 40.49 µmol/kg of total carotenoids. According to these authors, parsley may serve as dietary source of carotenoids, especially zeaxanthin. KAMEL et al. (2013) studied the effect of microwave drying process on some bioactive compounds of parsley, and they observed 40.00 mg/kg carotenoids in parsley leaves blended in water. KUŹMA et al. (2014) observed (31.28 mg/100 g dry matter) of carotenoids.

Other lipid compound present in plants is phytosterol. Although they are of great importance in human health, due the ability of plant sterols and stanols (phytosterols/phytostanols) to reduce serum low-density lipoprotein (LDL)-cholesterol level; however, there are few studies characterizing these compounds in parsley

(BOLDIZSÁR et al., 2013). PIIRONEN et al. (2003) studied the content of plant sterols in vegetables and observed the presence of brasicasterol, campesterol, stigmasterol, and sitosterol (2, 12, 115, and 136 mg/kg^{-1}dw) in parsley. BOLDIZSÁR et al. (2013) found two phytosterols, including stigmasterol and β-sitosterol, in parley fruits (PFr) and parsley leaves (PLe) samples. The concentrations of stigmasterol and β-sitosterol in PFr were 3.36 and 2.77 μmol/g, and in PLe samples were 2.77 and 2.76 μmol/g, respectively. ITO, MEIKO et al (2017) observed the presence of stigmasterol, campesterol and β-sitosterol (0.77, 0.64 and 0.45 mg/g tissue) in fresh parsley.

2.2 Phenolic Compounds

Phenolic compounds are omnipresent in the plant kingdom but their distribution depends on the part of plant/tissue (ROBARDS, 2003). They are secondary metabolites of plants, presenting an aromatic ring bearing one or more hydroxyl substituents and derive from phenylalanine and tyrosine (MUCHUWETI et al., 2007; NACZK e SHAHIDI, 2004). Therefore, the antioxidant capacities of phenolic compounds depend on the position and degree of hydroxylation of the molecule (ROBARDS, 2003). These compounds are one of the major groups contributing for aromatic plants properties, including the prevention of cancer, cardiovascular, and neurodegenerative diseases (COSTA et al., 2015).

The phenolic compounds are the main bioactive phytochemicals identified in parsley in several studies (apigenin, apennine, malonyl-apennine, luteolin, crisoeriol, cosmosiin, quercetin, kaempferol, *p*-coumaric acid, myricetin and isorhamnetin) (PÁPAY et al., 2012; FARZAEI et al., 2013). Flavonoids are phenolic compounds isolated from a wide variety of plants, and are valuable for their multiple properties; they act in vegetables as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening. Therefore, flavonoids are the most common in our diet, considered as primary antioxidants, chelators and superoxide anion scavengers (DE BEER et al., 2017; LAWSON et al., 2017).

Different total phenolic contents were reported in parsley. VIOLETA NOUR et al., 2017 observed 360.89 (mg GAE/100 g), higher levels than OPARA e CHOHAN (2014), ie 89.27 mg GAE/100 g, but lower than those presented by SHAN et al. (2005) ie 636 mg GAE/100 g fw or ČÍŽ et al. (2010) 599.7 mg GAE/100 g in parsley. LUTHRIA et al (2006) reported 12.1 mg (GAE/g) of phenolic compounds extracted of parsley.

TRIFUNSCHI e ARDELEAN, 2012 evaluated studied the influence of different solvent extracts of parsley leaves and observed that the ethanol extract showed (54.20) of polyphenolics and (42.10) mg/g contents of flavonoid, higher than observed in chloroform (15.20 and 4.50), and methanol (35.60 and 25.12) extracts, respectively.

According to the authors, the bioactive antioxidant components can be isolated by further separation of ethanol extract. And also, the high scavenging property of parsley may be due to hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary component as a radical scavenger. Based on their results, they indicate that this plant material may become an important source of compounds with health protective potential.

In herbs the main flavonoid constituents are flavonol aglycones such as quercetin, myricetin, kaempferol, and their glycosides. This is in agreement in a research carried out with parsley presented by VIOLETA NOUR et al. (2017). Their results presented high total flavonoid content (260.55 mg QE/100 g) in methanolic

extract, value in good agreement with the high content of myricetin (151.03 mg/100 g) and quercetin (71.33 mg /100 g) recorded in parsley.

VIOLETA NOUR et al. (2017) reported the quantification of other several phenolic compounds found in parsley, shown on **Table 2**.

Table 2. Results of the quantification of phenolic compounds observed in parsley.

Phenolic compounds	Parsley Contents (mg/100 g fresh weigh)
Gallic acid	0.75
Catechinhidrate	3.19
Vanillic acid	2.79
Chlorogenic acid	1.81
Caffeic acid	2.53
Siringic acid	0.26
Epicatechin	2.67
Coumaric acid	1.21
Ferulic acid	6.12
Sinapic acid	7.68
Salicylic acid	10.73
Rutin	4.32
Ellagic acid	6.31
Myricetin	151.03
Trans- cinnamic acid	27.34
Quercetin	71.33

Source: Data adapted from (VIOLETA NOUR et al., 2017).

Other researchers also presented different reports related to the identification and quantification of phenolic compounds in parsley. JUSTESEN et al. (1998) observed high amount of apigenin (185 mg/100 g fresh weigh) in this plant, but lower levels (1.1 mg/100 g fresh weigh) in both kaempferol and luteolin, respectively. Compounds such as quercetin, luteolin, kaemferol and apigenin were also identified from parsley extracts by STAN et al. (2012) and PÁPAY et al. (2012).

MATTILA et al. (2000) found high values for apigenin (1484.2) in parsley, as well observed the presence of luteolin (21.7), and isorhamnetin (36.4) µg/100 g dry weight (dw), respectively. In agreement with their study, apigenin was found at high concentration in parsley 1521 (winter) and 1636 µg/g (summer) (HUBER et al., 2009). The authors also compared the quantitative variation of phenolic compounds in four brands of dehydrated parsley; but they did not find significant difference in the apigenin contents. In agreement, CAO et al. (2010) observed very low level of apigenin in parsley, only 4.4mg/kg, and lower than the other contents (14.2, 18.5, 5, and 11.2 mg/kg) of luteolin, kaempferol, quercetin, and isorhamnetin, respectively.

The concentrations of phenolic and other secondary metabolites in vegetables are influenced by many factors, including soil, variations in cultivar, growing location, irrigation, agricultural practices and processing, and climatic conditions (CAO et al., 2010). In addition, soil cultivation of crops may also result in year-to-year variability in the composition of phytochemicals and in total yield (BOURGAUD et al., 2001). In general, aromatic plants are complex matrices regarding their content on phenolic compounds. Their composition can be affected by the chemical structure of the studied analytes, the selected methods, the composition/nature of the aromatic plant and storage conditions (COSTA et al., 2015).

JUSTESEN e KNUTHSEN (2001) evaluated the composition of flavonoids in fifteen fresh herbs. Among the studied fresh herbs, higher levels of flavonoids were found in parsley (apigenin 510-630 mg/100g). Other flavonoids identified in their study were: isorhamnetin, kaempferol, luteolin, and quercetin. The authors also evaluated the calculation of flavonoid intake suggesting an increase in the daily ingestion of parsley due to the high levels of flavonoids observed.

A research of parsley extracts in a combined HPLC-CUPRAC (cupric ion reducing antioxidant capacity) has identified the essential antioxidant compounds after 4 hour hydrolysis of 70% methanolic extract, such as the following sequence of phenolic compounds: *p*-coumaric acid, myricentin, and apigen (YILDIZ et al., 2008). Other study using HPLC on identification of parsley compounds showed the presence of keampferol and apigenin (GADI et al., 2012). This was in accordance with other studies on phytochemical screening of parsley that revealed the presence of those flavonoids (FEJES et al., 2000) such as the flavone (apigenin) and flavonol (kaempferol) (GEBHARDT et al., 2005).

Most flavonoids occur naturally as glycosides, thus, soluble phenolic compounds are generally extracted using water, methanol, ethanol or acetone. In other study regarding the influence of experimental conditions of phenolic compounds in parsley, was found Apennine malonyl-Apennine as the main phenolic compounds (LUTHRIA, 2008).

From aqueous extract of parsley, were isolated and identified the following flavonoids: apigenin,apigenin-7-O-glucoside or cosmosiin, apigenin-7-O-apiosyl-(1→2)-O-glucoside or apiin, and coumarin 2’’,3’’-dihydroxyfuranocoumarinor oxypeucedanin hydrate (CHAVES et al., 2011).

ALEZANDRO et al. (2011) evaluated the flavonoids content for functional foods development of three different commercial brands of spices for industrial ingredients (**Table**3). The authors observed the presence of apigenin, and luteolin. On the other hand, VERMA e TREHAN (2013) found 4.20 mg/g of quercetin in methanolic parsley extract by HPLC analysis, and TIVERON et al. (2012) identified concentrations (%) of phenolic compounds such as 2,5- dihydroxybenzoic acid, 2- dihydroxybenzoic acid, caffeic acid, 2,4- dihydroxybenzoic acid (0.06, 0.82, 0.14, 0.41), respectively, in ethanolic extracts of parsley, analyzed by GC-MS.

Table 3. Flavnoids contents of species from A, B and C brands of parsley (mg/100g f.w.)

Brands of Parsley	Apigenin	Luteolin	Total
Parsley A	560	34	594
Parsley B	708	64	772
Parsley C	525	24	549

Data adaptaded from ALEZANDRO et al., 2011

STAN et al. (2012) testing different extraction techniques identified flavonoids, such as flavones (apigenin and luteolin), and flavonols (quercetin and kaempferol) in parsley.

KAISER et al. (2013) evaluated the effect of bleaching on stability of polyphenols present in pasty parsley, observing the presence of the following phenolic compounds: *p*-coumaric acid, isorhamnetin, apigenin, and malonyl. Phenolic acids such caffeic acid and flavonoids were also observed by others authors in parsley (SHAN et al., 2005; TIVERON et al., 2012).

The identification and quantification of flavonoid aglycones, sugars, sugar alcohols, carboxylic acids, were assessed by GC-MS. Apiose, glucose and the aglycone flavonoid had their origin spaced from the enzymatic hydrolysis, while gradual formation of 2-O-apiosil-apiose is attributed to the disaccharide of specific glycosidase process (BOLDIZSÁR et al., 2013).

The research results presented indicate a potential role in the extracts of phenolic compounds derived from parsley as natural antioxidants. Moreover, there is increasing interest in the use of culinary herbs as natural preservative agents due to their abundance of bioactive phytochemicals (YASHIN et al., 2017, SHARIF et al., 2017, CHAKRABORTY et al., 2017, GANDHI et al., 2018). Thus, aromatic plants can contribute to the promotion of human health due to their antioxidant properties.

2.3 Antioxidants Properties of Parsley

In recent years, the assessment of the antioxidant potential of food has increased (XU et al., 2017). Antioxidants can be defined as any substance which, even at low concentrations, as compared to an oxidized substrate, are considered effective by retarding or inhibiting the oxidation of the substrate (EMBUSCADO, 2015). These compounds in food may have important roles as health-protecting factors, as well as are widely used as additives in fats and oils and in food processing to prevent or delay deterioration of foods (LAGUERRE, LECOMTE e VILLENEUVE, 2015).

Several techniques have been used to determine the *in vitro* antioxidant capacity in order to allow rapid screening of promising substances and/or mixtures. Indirect methods involving electron transfer reactions, such as ABTS • +, DPPH • and FRAP, are easier to apply (TIVERON et al., 2012). On the other hand, direct methods, such as β-carotene is characterized by their ability to inhibit or suspend lipid oxidation in model systems, based on the measurement of changes in concentration of oxidized compounds, oxygen depletion, or in the formation of oxidation products (BECKER, NISSEN e SKIBSTED, 2004). Other assay that is used in antioxidant capacities of extracts from natural products is related to the ability to absorb oxygen radicals (ORAC). Effective extraction and proper evaluation of antioxidants from foods and medicinal plants are crucial to promote the application in functional foods, pharmaceuticals and food additives (XU et al., 2017; GRANATO et al., 2018).

Spices and some herbs have received increased attention as sources of many effective antioxidants; thus, researchers have evaluated the potential of the antioxidant capacity of parsley. ZHENG e WANG (2001) evaluated the antioxidant capacity in parsley and their results showed 1.12 mg (GAE/ g of fresh weigh) of total phenolic, and 11.03 (µmol of TE/g of fresh weigh) ORAC.

GUERRA e LAJOLO (2005) observed that the ether extract (EE) of parsley and coriander increased the substrate stability of lipid to the level of 8%. Parsley's performance overcame the others antioxidants used in this study, including the synthetics. The compounds found in parsley were considered better antioxidants than coriander.

PARRY et al. (2006) in a study with cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin, and milk thistle seed oils observed that parsley seed oils exhibited the strongest DPPH● scavenging activity, quenched 87-91% of the radicals and presented the highest oxygen radical absorbance capacity (ORAC) value of 1098 (µmol Trolox equiv/g oil). The total phenolic content (TPC) in parsley was 2.27 GAE

(mg/g oil).VIOLETA NOUR et al. (2017) found high antioxidant capacity 987.51 (mg Trolox/100 g) in parsley.

Antioxidant activities of freeze-dried and irradiated parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) leaves and stems were determined on methanol and water extracts. Parsley presented higher concentration of phenolic compounds than cilantro (WONG e KITTS, 2006).

YILDIZ et al. (2008) reported the total antioxidant capacity in parsley using CUPRAC (cupric ion reducing antioxidant capacity) method, between 60 and 77% (by different hydrolysates of extracts, and solid samples), respectively.

ČÍŽ et al. (2010) investigated the antioxidant properties of selected vegetables, using the total peroxyl radical-trapping parameter (TRAP), oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (HORAC) methods, presenting the following contents of: ORAC (113.5µmol TE/g FW), TRAP (68.1 µmol TE/g FW), and HORAC (55.0 µmol GAE/g FW); values well correlated with total phenolic contents (605.6 mg GAE/100 g FW) observed in parsley. Thus, their results indicated a good correlation between the methods for measuring antioxidant capacity.

ALEZANDRO et al. (2011) also presented the correlation between 1.40 of total phenolic contents (catechin equivalent/100g f.W.), and 3.7 (mmoles Trolox equivalents/100g) DPPH (scavenging ability).

TIVERON et al. (2012) performed a study to evaluate the phenolic content and antioxidant capacity of vegetables commonly consumed in Brazil. The results in parsley showed 30.7 (µmol Trolox/g DW) ABTS, 60.7(%) β-carotene, 130.8 FRAP (µmol Fe^{2+}/g DW), 1.05 Rancimat (protection factor), and 10.8 DPPH (µmol Trolox/g DW).

RAMKISSOON et al. (2013) evaluated the presence of bioactive compounds and the antioxidant capacity in parsley. They found (1.583 mg GAE/mL) of total polyphenol content (TPC), (0.942 mg AAE/mL) of ferric reducing antioxidant power (FRAP), and (0.343 mg AAE/mL) of 2,2-diphenyl-2-picrylhydrazyl (DPPH).

PRIECINA e KARLINA (2013) conducted a study to compare the antiradical activity, total polyphenol content (TPC) and total flavonoid content (TFC) in dried in conventive and microwave-vacuum dryers of parsley. Total flavonoid content found in dried microwave- vacuum parsley was 180 (mg catechin equivalent (CE) 100/g in dry weight), the value of DPPH antiradical activity was 11.98%. Moreover, higher total polyphenol content in parsley was reached using steaming 1.5 min and dried in microwave-vacuum dryer 1800 (mg GAE/100g). Thus, it was observed that steaming had positive effect on total polyphenol and a negative effect on total flavonoid content in parsley. The study concluded that steaming process and microwave-vacuum drying has positive effect on antiradical activity and may be considered as useful tool in improving nutritional and phytochemical properties of spices and vegetables.

CHANDRA et al., 2014 evaluated a comparative assessment of total phenolic and flavonoid content, antioxidant properties in parsley. The authors observed the following results: 18 (mg GAE/g dw) of total phenolic, 14.35 (mg quercetin acid equivalent (QE)/g dw) of total flavonoids, and 8.69% DPPH scavenging activity, respectively.

SHEHATA et al.(2014) conducted a study to determine the total polyphenols and flavonoids, estimation of antioxidant activity *in vitro* by two methods (DPPH radical-carotene) in parsley extracted by cold and boiled water for 10, 30 and 60 minutes. The authors found differences in the treatments with cold water and hot water for all analysis. Their results demonstrated that the antioxidant capacity was directly related to the total amount of phenolics and flavonoids. Thus, they concluded that

parsley leaves can be used as an easy and safe accessible source of natural antioxidants, as food supplements, or in the pharmaceutical and medical industries.

PÁPAY e ANTAL (2014) evaluated different extracts of parsley with the objective to examine the changes and the main mode of actions of antioxidant capacity and apigenin content. The antioxidant capacity of the extracts was investigated for 7 days, and the results showed that the total capacity (DPPH) decreased; on the other hand, the apigenin content has increased during extraction at higher temperature (40 °C and 60 °C). This study confirms the antioxidant potential of parsley extract which was influenced by particle size and temperature.

KUŹMA et al. (2014) investigated the influence of different solvents and times of extraction on natural antioxidants content of parsley. Their results suggested that the best solvent for polyphenols was acetone 70%, and for catechins was distilled water. All extracts examined displayed the antioxidative activity, but water was the best solvent in the method of assaying the activity against $ABTS^{\bullet+}$ and Fe^{2+} ions chelating capability, whereas methanol turned out to be the least effective in this respect. Opposite results were observed in the case of determining the activity against DPPH•. Thus, the prolongation of the extraction time enhanced or decreased anti radical activity in some cases.

TANG et al. (2015) evaluated the performed of various extractions (hexane, dichloromethane, ethyl acetate, methanol and water) in parsley. The dichloromethane extract of parsley exhibited the highest phenolic content 42.31(mg GAE/g), and ferric reducing ability 0.360 (mmol/g). The extract showed DPPH radical scavenging activity with an IC50 value of 3310.0±80.5 μg/mL. The study concluded that parsley has health-promoting properties with the potential to prevent oxidative stress-related diseases and can be developed into functional food.

LEAHU et al. (2016) determined the content of polyphenolics and antioxidant activity in the extracts of three medicinal and aromatic plants. The results of total phenolic and DPPH activity presented in parsley were 211.9 (mg GAE/100g fresh weight), and 8.62 (fresh), 23.84 (dried parsley) IC50 value (scavenging of 50% DPPH radical), respectively.

As observed, several studies have reported the existence of significant antioxidant properties of parsley. However, in food systems there is little information available about its effect across stability of food added with parsley.

JIA et al. (2012) evaluated the application of parsley in a food system, to observed the effects of this culinary herb in a food system (materials were freshly processed at a fish meal factory in Mie Prefecture, contained moisture 8.6%, crude protein 57.8%, crude fat 11.9%, ash 18.5% and crude fiber 2.5% on fresh weight basis). Their result of the extract of parsley was 37.8% (DPPH radical-scavenging activity) on the antioxidant capacity. According to the authors, parsley showed effective on the oxidative stability of parsley during the food system storage, suggesting that parsley can be used to suppress lipid oxidation. Thus, this finding represents an important potential as a food additive in the food industry.

PEREIRA e TAVANO (2014) investigated the effect of herbs and spices (parsley, onion, parsley, spring onion, laurel and coriander) as potential natural antioxidants in cooked beans. Parsley showed great results as an antioxidant: 69.86 micromol of DPPH inhibited/g, 5.40 (mg GAE/g) total phenolic, and 4.25 (mg CE/g) total flavonoids. In the *in vitro* test for digestibility of the protein of the baked beans, parsley achieved the greatest result (81.12%). The study concluded that parsley presented the best result as natural antioxidant compared to the others.

SĘCZYK et al. (2015) tested the addition of parsley aimed food fortification in pasta. Part of wheat flour was replaced with powdered parsley leaves from 1% to 4% (w/w). The fortified pasta presented a positive effect on its phenolic contents and antioxidant properties. The highest phenolic levels and antioxidant capacity of pasta were obtained by addition with 4% of parsley leaves.

In another study, SĘCZYK et al. (2016) examined the nutraceutical fortification with parsley (phenolics content, antioxidant activity, biological activity) and nutritional potential (starch and protein digestibility) of wheat pasta supplemented with 1–4% of powdered parsley leaves. Compared to the control, the potentially bioaccessible fraction of pasta fortified with 4% parsley leaves was characterized by 67% increased phenolics content, a 146% higher antiradical ability and 220% additional reducing power.

Thus, the use of this natural antioxidant in the food industry should be considered as an alternative to substitute traditional synthetic (JIA et al., 2012, DE OLIVEIRA et al., 2018).

2.4 Potential of Parsley for Health

Due to the deleterious effects of chemical drugs may have, the use of medicinal plants has a long history in treatment of different diseases and for health maintenance, and moreover, their pharmaceutical application is increasing (ROUHI-BOROUJENI et al., 2017). In recent years, great interest in secondary metabolite production, leads to possibility of altering the production of bioactive compounds via tissue culture technology (KUMAR e KUSHWA, 2017). Thus, the medicinal plants have biological activities, low toxicity and economic viability (RAO et al., 2013). Naturally occurring molecules with antioxidant activity, which can possible, have a therapeutic application against oxidative damage induced diseases. In a complex system of bioactive ingredients, the therapeutic effects are the result of the synergistic effect of various compounds (VIUDA-MARTOS et al., 2010, PÁPAY e ANTAL, 2014). These compounds have many beneficial biological effects, proved in diverse studies.

Parsley has been widely employed around the world in folk medicine as a cure against arterial hypertension, diabetes (ZIYYAT et al., 1997, BNOUHAM et al., 2002; EDDOUKS et al., 2002, FARZAEI et al., 2013), renal diseases (JOUAD et al., 2001) and anti-coagulant effect (GADI et al., 2012, FARZAEI et al., 2013).

According to FARZAEI et al. (2013), this aromatic herb are used as gastro tonic, anti-urolithiasis, anti-inflammatory and for the treatment of amenorrhea, dysmenorrhea, gastrointestinal disorder, cardiac disease, otitis, sniffle, antioxidant, neuroprotective, analgesic, spasmolytic, immunosuppressive, anti-ulcer, a laxative, estrogen, antibacterial, and antifungal. Effects as hepatoprotective, diuretic, antiseptic of urinary, and also various dermal diseases were related by PÁPAY et al. (2012). As well as, it has been reported that this plant has antihyperglycemic (YANARDAG et al., 2003), and antihyperlipidimic (YAZICIOGLU e TUZLACI, 1996). Parsley seed was also used traditionally as a carminative to decrease flatulence and colic pain (LARIJANI et al., 2016).

Potential biological effects of apigenin are described *in vitro* and *in vivo* in parsley, which is high in apiin (apigenin-7-O-apiosylglucoside) (NIELSEN et al., 1999). Apigenin is described as non-mutagenic and non-toxic human cells (WANG et al., 2017). The substance is suggested to be chemopreventive, a modulator of certain signal transduction pathways (VAN DROSS et al., 2003), an *in vivo* inhibitor of UVB-induced skin tumor in mice (WEI et al., 1990), and an inhibitor of several protein kinases and

enzymes which may play a major role in tumor promotion (cyclooxygenase-2). In addition, the substance induces apoptosis in certain human carcinoma cells, e.g. breast, prostate, thyroid, leukemia, melanoma, and colon, but not in normal cells (WAY et al., 2004; SHUKLA e GUPTA, 2004; SHUKLA e GUPTA, 2006). Apigenin is also estrogenomimetic compound and interacts with P 450 and phase II enzymes involved in the metabolism of estrogens (GRADOLATTO et al., 2004). Furthermore, apigenin possesses anti-inflammatory, anti-proliferative and free radical-scavenging properties in many *in vitro* systems (YANG et al., 2001; SHUKLA e GUPTA, 2004; 2010).

A study was conducted to assess the effect of parsley intake (absorption and excretion after the ingestion of apiin-rich food), the apigenin concentrations in plasma and urine allowing conclusions on apigenin bioavailability. The results have revealed that small portion of apigenin provided by food reaches the human circulation and, therefore, may reveal biological effects (MEYER et al., 2006). Previous study also showed apigenin was detected in urine samples after parsley consumption (NIELSEN et al., 1999).

Parsley has health-promoting properties with the potential to prevent oxidative stress-related diseases and can be developed into functional food. Mouse fibroblasts (3T3-L1) pre-treated with 400 µg/mL of parsley leaves extract showed 50.9% protection against H_2O_2-induced DNA damage, suggesting its potential in cancer prevention. The extract (300 µg/mL) inhibited H_2O_2-induced MCF-7 cell migration by 41%. As cell migration is necessary for metastasis of cancer cells, inhibition of migration is an indication of protection against metastasis (TANG et al., 2015).

Recently, researchers have investigated the effects of parsley root extract (PCE) on DNA synthesis performance, metabolic activity and cytotoxicity in malignant and benign breast cells. PCE was found to contain a substantial ratio of lignans. According to the BrdU proliferation assay analysis, PCE demonstrated significant DNA synthesis inhibition of up to 80% at concentrations of 10, 50, 100 and 500 µg/ml in both cell lines. Based on the MTT assay analysis, only at a concentration of 500 µg/ml, PCE demonstrated a statistically significant inhibition of cellular metabolic activity of 63% in MCF7 and 75% in MCF12A of their respective normal capacity (SCHROEDER et al., 2017).

The elevation of the parameters reported in the study of fortified pasta parsley was accompanied by an augmentation of its antiproliferative effect on carcinoma cells, which confirms their biological relevance (SĘCZYK et al., 2016). YOUSOFI et al. (2012) investigated the suppressive effects of parsley on mouse splenocytes and macrophages cells. Their results indicated that this herb may be able to suppress the cellular and humoral immune response. It can also suppress both: no production and the functions of macrophages as the main innate immune cells. These results may suggest that parsley is a proper suppressant for different applications.

Although parsley has been used to treat allergy, autoimmune and chronic inflammatory disorders, the mechanism underlying its beneficial effects in these immune-mediated diseases have been rarely investigated (KARIMI et al., 2012). These authors studied the immunomodulatory effects of this plant. They found that its inhibitory effect on PHA-stimulated splenocytes might be due to the production of cytokines such as IFN-g and IL-2, which is vital for T-cell proliferation, or it may influence the signaling pathways. Their results indicated that parsley modulate the activity of macrophages without exerting cytotoxic effect, in addition, may identify it as a useful natural candidate to treat some autoimmune and allergic diseases.

In a screening study, conducted on several medicinal plants, crude aqueous extract of parsley has shown a significant *in vitro* inhibition of rat platelet aggregation (MEKHFI et al., 2004). Other study also, has demonstrated that this extract also inhibited *ex vivo* platelet aggregation and prolonged *in vivo* bleeding time in rat. They have suggested that this effect could be attributed to phenolic compounds such as flavonoids which are likely concentrated in the crude aqueous extract (GADI et al., 2009).

The inhibitory activity toward clotting formation and platelet aggregation was assessed from parsley aqueous extract (Pc), which were isolated and identified the flavonoids apigenin (1), apigenin-7-O-glucoside or cosmosiin (2), apigenin-7-O-apiosyl-(1→2)-O-glucoside or apiin (3) and coumarin 2'',3''-dihydroxyfuranocoumarin or oxypeucedanin hydrate (4). It was observed that Pc didn't show inhibition on clotting activity when compared with the control. However, a strong antiplatelet aggregation activity was reported for Pc (IC_{50} = 1.81 mg/mL), apigenin (IC_{50} = 0.036 mg/mL), and cosmosiin (IC_{50} = 0.18 mg/mL). In this way, the results showed that Pc, apigenin and cosmosiin interfered on haemostasis inhibiting platelet aggregation (CHAVES et al., 2011).

GADI et al. (2012) investigated the effect of genins (aglycone flavonoids without sugar group) isolated from parsley leaves *in vitro* on human platelet aggregation and adhesion to a collagen-coated surface under physiologic flow conditions. The strongest effect was observed in collagen induced aggregation (IC50 = 0.08 mg/ml). In addition, adhesion of human platelets to collagen was greatly decreased (over 75%) by genins (0.3 mg/ml). While the mechanism by which genins act is unclear, the authors suggest that these compounds may interfere with a multiple target step in the haemostasis process. Therefore, their results show that genins isolated from parsley has a potent antiplatelet activity, and also, it may be an important source of beneficial antiplatelet compounds that decrease thrombosis and cardiovascular diseases. The aggregation tests showed that these compounds have anti-aggregating activity. Suggestion that flavonoids isolated from parsley inhibit platelet aggregation probably by a blockage of the cyclooxygenase pathway.

Other studies on the medicinal species with parsley showed that its aerial parts aqueous extract was able to inhibit platelet activity induced by ADP (MEKHFI et al., 2004), which, a crude extract at 10 mg/mL inhibited 78% of platelet aggregation. Flavonoids and other phenolic substances are able to interfere in the platelet system. Therefore, apigenin was shown to block the inducer collagen and ADP in platelet-rich plasma (JANSSEN et al., 1998; MLADĚNKA et al., 2004). A diet rich in phenolic compounds may favorably contribute for reducing risks of cardiovascular diseases through several mechanisms. Several studies on the cardiovascular protective effect of flavonoids have been reported, suggesting that both apigenin and luteolin may act as competitors with the receptor of thromboxane A2 (TXA2), an inducer of platelet aggregation (GUERRERO et al., 2007).

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and ongoing research efforts are focused on understanding the underlying pathophysiology of hepatic steatosis with the anticipation that these efforts will identify novel therapeutic targets. A study investigated the parsley extract in hepatic steatosis in rats fed with fructose enriched diet. Hepatic function and structure was evaluated in these rats. Modified fructose diet produced dyslipidemia, hepatic steatosis and infiltration of inflammatory cells in the liver and higher plasma hepaticmarkers. Parsley

extract reversed metabolic changes such as abnormal attenuated chronic changes in modified fructose diet induced NAFLD (NAIR et al., 2015).

Due the numerous of therapeutic properties of *Petroselinum crispum,* this plant was also evaluated on the effect on sperm parameters, testis tissue and serum nitric oxid levels in mice, since sperm dysfunction is one of the main causes of male infertility. The results of parsley administration (100, 150 and 200 mg/kg) significantly increased mean percentage of sperm motility, testis and prostate weight and serum nitric oxide compared to the control group ($P < 0.05$). Based on the results of this study, it seems that the hydroalcoholic extract of parsley can affect some reproductive indices such as weight of testis and prostate, sperm motility and decrease nitric oxide level in blood serum. However, further studies are required to shed light on the mechanisms of these compounds in the reproductive system (JALILI et al., 2015).

HAIDARI et al. (2011) purposed a study to investigate the effects of oral administration of parsley, and its major flavonol constituents (kaempferol and quercetin). The results showed that parsley and its flavonol did not cause any significant reduction in the serum uric acid levels in normal rats, but significantly reduced the serum uric acid levels of hyperuricemic rats in a time-dependent manner. All treatments significantly inhibited liver xanthine oxidoreductase activity. Parsley, kaempferol and quercetin treatment led also to a significant increase in total antioxidant capacity and decrease in malondialdehyde concentration in hyperuricemic rats. Therefore, since increased serum uric acid is known to be a major risk related to the development of several oxidative stress diseases, these features observed in parsley and its flavonols make them as a possible alternative for allopurinol, or at least in combination therapy to minimize the side effects of allopurinol to treat hyperuricemia and oxidative stress diseases.

As shown in this review, parsley is a plant with various biological activities. Therefore, research was conducted to investigate on both morphologically and biochemically whether parsley, which is used as by the popular medicine to decrease blood glucose, has any antidiabetic effect on pancreatic B cells of rats. Hence, the blood glucose levels in the diabetic group given the plant extract were reduced in comparison to the control diabetic group. In addition, a decrease was observed in the weight of the control diabetic group and the diabetic group given the plant extract. The study concluded that the plant therapy can provide blood glucose homeostasis, but cannot regenerate B cells of the endocrine pancreas (YANARDAG et al., 2003).

Parsley is a medicinal plant widely used in urolithiasis, as this plant is known to maintain kidney's health; in addition, histopathological examinations confirmed the potential prophylactic and curative roles of parsley aqueous extract regarding inhibition of formation of renal stones associated with decreased calcium kidney content (MORAM, 2016; AL-YOUSOFY et al., 2017). Furthermore, kidney failure can lead to death in diabetes (CHEN et al., 2007; PRAKASH et al., 2007). In addition, research has reported that maternal hyperglycemia is associated with neonates that are at greater risk for future development of negative health outcomes such as insulin resistance, obesity, metabolic syndrome and type 2 diabetes mellitus (CALKINS e DEVASKAR, 2011). Diabetes can cause nephropathy, destroys distal tubular epithelium, cytoplasmic changes, thickness in small arteries and glomerulosclerosis which causes thickened glomerular basement membranes with increased extracellular matrix deposition (MAEDA e SHIIGAI, 2007; KURT et al., 2012).

In this way, supportive therapy in treatment of diabetes during pregnancy takes place by anti-diabetic plants such as parsley. A recent study has been undertaken to

investigate the possible anti-diabetic and antioxidant role of aqueous parsley extract on streptozotocin (STZ) induced gestational diabetes mellitus in rats. Fetuses of the diabetic mothers showed some developmental changes such as very thin skin, very thin muscle layer under the skin, absence of eyelid and ear pinna, exencephaly and kyphosis. On the other hand, fetuses of the diabetic mothers which were treated with parsley leaves extract showed somewhat normal morphological development. According to the biochemical histopathological observations, the parsley leaf extract succeeded to minimize the drastic changes, which were observed in the diabetic rats and their fetuses. The study concluded that the administration of parsley leaf extract has the ability to minimize the damage of hyperglycemia (RABOU e EID, 2017). Other research has evaluated the use of parsley in the treatment of diabetes (RAMKISSOON et al., 2013, ABOU KHALIL et al., 2016). Parsley was also used as a probe in an experiment to prevent the behavioral, morphological and biochemical changes in the newborn brain following the administration of cadmium (Cd) to the pregnant mice. The low dose of parsley 10 g/kg/day exhibited significant effects in neutralizing and reducing the deleterious changes due to Cd exposure during pregnancy on the behavioral activities, neurotransmitters, oxidative stress, and brain neurons morphology of the mice newborns (ALLAM et al., 2016).

Another study testing chronic cadmium (Cd) administration, employed parsley to prevent the behavioral, biochemical and morphological changes in the brain tissue of the albino mice. The researchers concluded that the low dose (5 g/kg/day) of parsley exhibited beneficial effects in reducing the deleterious changes associated with Cd treatment on the behavior, neurotransmitters level, oxidative stress and brain neurons of the Cd-treated mice (MAODAA et al., 2016).

Oxidative stress has been shown to play a principal role in the pathogenesis of stress-induced gastric injury, since parsley contains many antioxidants such as flavanoids, carotenoids and ascorbic acid, a study was carried out on the histopathological and biochemical results of nutrition with a parsley-rich diet in terms of eliminating stress-induced oxidative gastric injury in forty male *Wistar* albino rats. The results showed that the oral administration of parsley was effective in reducing stress-induced gastric injury by supporting the cellular antioxidant defense system (AKINCI et al., 2017).

Since, the use of medicinal plants in the therapy is emerging, and the exact dose and mode of action of the medicinal plants are still an unfolding area; therefore the study on the main mode of action, the antioxidant capacity is important, to improve the formulation (PÁPAY e ANTAL, 2014). In this sense, further researches in this area are still need.

In addition to all the benefits of bioactive compounds, parsley was also used to produce bioplastics. The natural elements present in these plants also have a wide range of mechanical properties (BAYER et al., 2014). Comparing its mechanical properties with the synthetic oil base, this study showed that these bioplastics have mechanical properties equivalent to non-biodegradable plastics. This opens possibilities for replacing some of the non-biodegradable polymers by bioplastics obtained from agro-industrial waste.

The current trend is to find a biological solution to minimize the perceived hazardous impacts from synthetic herbicides in agriculture production and investigation on all chemicals that could lead to the development of natural herbicides (RAVAZI et al., 2010). Apiaceous species are known for their high diversity of secondary metabolites, including many groups that represent a rich source of structural diversity.

Researches proposed a work to evaluate the phytochemical composition and phytotoxicity of parsley. Thus, their results showed that the methanol extract induced complete inhibition of lettuce germination and shoots growth and 97% inhibition of root growth (SBAI et al., 2016).

3 CHIVES (*Allium schoenoprasum* L)

Allium schoenoprasum L. (family Amaryllidaceae), commonly known as chives or as *green-onions*, it has been used for centuries for their exceptional flavour as vegetables and spices besides being valued as ornamentals, and widely used worldwide in ethno medicine for prevention of various diseases (SINGH et al., 2018). The name '*schoenoprasum*' is derived from the Greek word, skhoínos (means kind of grass) and *'prason'* (means leek). It is believed that chives is originated form Siberai from there it spread to Asia, Europe and North America (ŠTAJNER et al., 2011). It is one of the largest monocot genus which has beautiful perennial flowering herbaceous plants, that can be grown and harvested many times throughout the year, it's tolerant to changes in temperature has adaptability to different environments (RATTANACHAIKUNSOPON e PHUMKHACHORN 2008; ZDRAVKOVIĆ-KORAĆ et al., 2010; VLASE et al., 2013; PARVU et al., 2014).The green cylindrical (long tube-shaped) (**Figure 3**) is the smallest edible species of the Allium genus (ŠTAJNER et al., 2011).

Figure 3-Chives (*Allium schoenoprasum)*

Unlike the spicy flavor of garlic and onion, chives has a more soft and mild flavor, more easily acceptable to the taste. Fresh or dried, chives leaves are extensively used for culinary purposes for flavoring diverse dish such as potatoes, omelets and several other dishes with eggs and salads (CHARLES, 2013). It can also be sprinkled in soups, baked potatoes, and mashed potatoes or served raw in the decoration of dishes. In China, chives is often served with fish and are used to garnish several food items such as cookies, buns, pancakes, dumplings and also in many dairy and meat products (CHEN, 2006). In Brazil it is one of the plants most used as seasoning, and in combination with parsley it forms a condiment known as *cheiro-verde*.

It is usually served in small amounts and never as the main dish, negative effects are rarely encountered, although digestive problems may occur following over consumption (ŠTAJNER et al., 2011).

3.1 Chemicals and Phytochemical Constituents in Chives

According to USDA National Nutrient data base, chives contain both essential as well as non-essential vitamins and minerals (**Table 4)** (United States Department of

Agriculture- USDA, 2018). It is low in calories (30 kcal /100 g) and is rich in minerals, vitamins, lipids and amino acids (SINGH et al., 2018).

Table 4. Data adapted of nutritional and recommendation (%) content of chives (*Allium schoenoprasum*) from USDA Nutrient Database (2018).

Chives- *Allium schoenoprasum*, fresh **Nutritional value per 100 g (3.5 oz)** **Energy 30 kcal**	
Carbohydrates	
Total Carbohydrates	4.35 g
Sugars	1.85 g
Dietary fiber	2.5 g
Lipids	
Total Fat	0.73 g
Total fatty acids	0.146
Total monounsatured fatty acids	0.095 g
Total polyunsatured fatty acids	0.267 g
Phytosterols	9 mg
Protein	
Total Protein	3.27 g
Vitamins	**value per 100 g**
Vitamin A equiv.	4353 IU
Beta-Carotene	2612 µg
Lutein zeaxanthin	323 µg
Thiamine (B1)	0.078 mg
Riboflavin (B2)	0.115 mg
Niacin (B3)	0.647 mg
Pantothenic acid (B5)	0.324 mg
Vitamin B6	0.138 mg
Folate (B9)	105 µg
Vitamin C	58.1 mg
Vitamin E	0.21 mg
Vitamin K	212.7 µg
Minerals	**value per 100 g**
Calcium	6.2 mg
Iron	1.60 mg
Magnesium	42 mg
Manganese	0.373 mg
Phosphorus	58 mg
Potassium	296 mg
Sodium	3 mg
Zinc	0.56 mg

Data adapted from: USDA Nutrient Database (2018).
Units: g = micrograms; mg = milligrams; IU = International units.

Chives contain high amounts of vitamin A (4353 IU/100 g of plant), β-carotene (pro- vitamin A) (2612 IU/100 g of plant), and vitamin K (212.7 µg/100 g of plant) which may act as important co-factors (coenzymes), and therefore, its consumption may improve cell functions, since, it is considered that vitamins are essential for normal cell

functioning and possess antioxidants properties (DINICOLANTONIO et al., 2015; USDA, 2018).

Allium schoenoprasum L like most Allium species are highly rich in pungent sulphur containing volatile compounds, which attributes the unique flavour and aroma (BARAZANI et al., 2004; MNAYER et al., 2014; KIM et al., 2016). Apart from sulphur compounds, the plants of *Allium* genus contain many other important chemical constituents like, flavonoids, phenols, phytosterols, and carotenoids (VINA e CERIMELE, 2009; SINGH et al., 2018).

SANTOS et al. (2014) evaluated the content of carotenoids in four herbs, including chives. They observed that this plant showed higher levels of vitamin E (α-tocopherol) and ascorbic acid (vitamin C) 2.80 and 93.10 (mg/100g), respectively. VINÃ e CERIMELE (2009) observed 16.8 (mg/100g of fresh tissue) of total carotenoid contents in chives.

In relation to fatty acids, the results of study of chives seeds showed high amounts of oil (15.8%), that was composed of 10.1% saturated and 90.0% unsaturated fatty acids. Linoleic (69.1%) and palmitic (7.0%) acids were the most abundant unsaturated and saturated fatty acids, respectively (HU et al., 2006).

MNAYER et al. (2014) observed the following fatty acids in their research with chive essential oil (ethyl linoleate, ethyl palmitate, methyl linolenate, methyl palmitate and palmitic acid).

Although phystosterols are considered important in plants, being a component with important functional properties, however, studies on this subject in chives are scarce. Phytochemicals components of chives leaf extract revealed the presence of the following phystosterols concentrations of β-Sitosterol and Campesterol (25.09 and 7.21 mg/100g, respectively) in *Allium schoenoprasum* L (VLASE et al., 2013).

3.2 Phenolic Compounds

Phenolic compounds in plants are generally involved in protection against ultraviolet radiation or aggression by pathogens, parasites and predators (PRIECINA e KARLINA, 2013). These compounds have antiproliferative and tumour arresting effects (KUCEKOVÁ et al., 2011). Flavonoids are phenolic compounds isolated from a wide variety of plants, and are valuable for their multiple properties; they might act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening. Within the subgroups of flavonols and flavones, the flavonol quercetin is the most frequently occurring compound in foods. Other frequent components are kaempferol, isorhamnetin, and the flavones apigenin and luteolin (CAO et al., 2010).

According to literature, the most common structures of identified phenolic compounds in chives are given in **Figure 4**.

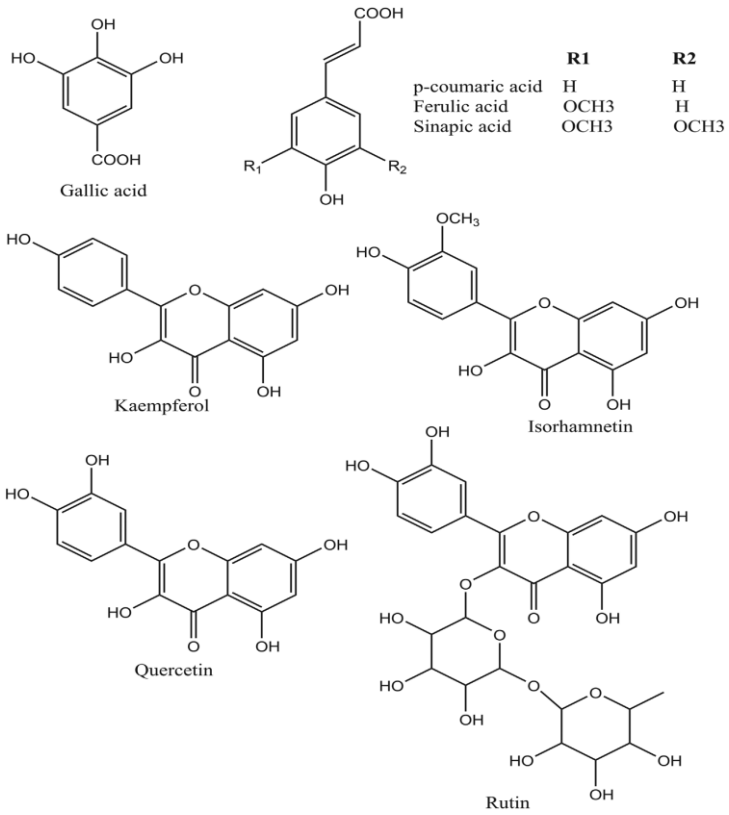

Figure 4 - Structures of identified phenolic acids and flavonoids in *Allium schoenoprasum* (SINGH et al., 2018).

The analysis of extracts from the leaves of chives determined the presence of 19 polyphenolic compounds (µg/100 g leaves). Samples non-hydrolyzed contain *p*-coumaric acid (149.59)/, ferulic acid (188.06), sinapic acid (44.91), isoquercitrin (363.78), quercetol (58.38) and kaempferol (129.83). On the other hand, the hydrolysed samples presented *p*-coumaric acid (163.71), ferulic acid (542.33), sinapic acid (44.91), quercetol (200.48) and kaempferol (1563.46) (PARVU et al., 2010; VLASE et al., 2013). The amounts of all polyphenols were higher in hydrolysed samples, suggesting that these substances are present both as unbonded and bonded glycosides and/or esters (PARVU et al., 2010). Isoquercitrin and rutin were also identified in *A. schoenoprasum*, as well contain glycosides of kaempferol and quercetol (VLASE et al., 2013).

PARVU et al. (2010) found the main polyphenolic compounds present in chives: *p*-coumaric acid, ferulic acid, isoquercitrin and rutin. The health properties of these natural plants depend on the contents of bioactive compounds, mainly polyphenolics and substances with antioxidant effects (CHUN et al., 2005; BEATO et al., 2011).

BERETTA et al. (2017) observed the presence of chorogenic acid (1.1), coumaric acid (0.4), ferulic acid (10.6), caffeic acid (0.18) (mg/100g dm), respectively. Although chives exhibited the most potent antioxidant in their research, the phenolic profiles varied greatly, suggesting that individual phenolic compounds differ in antioxidant strength. BALASUNDRAM et al. (2005) proposed that the antioxidant capacity of phenolic compounds depends on their chemical structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings.

TRICHOPOULOU et al. (2000) studied the presence of flavonoids in 7 wilds edible greens, which contain a rich mixture of polyphenolic antioxidants from the Mediterranean diet. The flavonoid content of chives were myricetin (< 0.03 mg/100g), quercetin (10.04 mg/100g), kaempferol (12.5 mg/100g), isorhamnetin (8.5 mg/100g), luteolin (0.3) and apigenin (< 0.07 mg/100g). Among the others edible wild greens in the study, chives presented the higher value of content of Kaempferol. Apart from enzymatic components, kaempferol was also identified as one of the antioxidant compound from chives (NUUTILA et al., 2003). Other study showed aerial parts comparable antioxidant capacity with quercetin and dl-α-tocopherol (SOURI et al., 2004).

CAO et al. (2010) investigated both the composition and content of flavonoids in 100 edible vegetables and fruits commonly consumed in China. They evaluated the effect of seas on factors on flavonoid amounts in foods and observed in chives, collected in spring, high contents of total flavonoids (103.8 mg/kg), especially kaempferol (59 mg/kg) as well as the following amounts (21.7, 4 and 19.1 mg/kg) of luteolin, quercetin, and isorhamnetin, respectively.

ALEZANDRO et al. (2011) evaluated commercial spices and industrial ingredients seeking flavonoids content for functional foods development. The phenolic compounds found in chives are summarized on **Table 5**.

Table 5. Flavnoids and hydroxycinnamic contents from A, B and C brands of chives (mg/100g f.w.)

	Apigenin	Luteolin	Kaempferol	Quercetin	Total	Hydroxycinnamic acids
Chives Brand A	6.89	nd	13.33	32.2	52.4	14.86
Chives Brand B	12	nd	8	5.88	26	21
Chives Brand C	0.11	nd	0.56	50	51	1.1

Data adapted from ALEZANDRO et al., 2011.

MORAVČÍKOVÁ et al. (2012) investigated the content of polyphenols in edible flowers and their effect on proliferation activity of human hepatocellular carcinoma cells, they reported that *Allium schoenoprasum* contained gallic acid (29.88µg/g), coumaric acid (30.07µg/g), ferulic acid (131.43µg/g) and rutin (3.00µg/g).

Concentrations (%) of compounds identified in ethanol extracts analyzed using CG-MS reported the following phenolic contents in chives: sinapic acid, *p*- coumaric acid, ferulic acid, caffeic acid, kaempferol, quercetin, isovanillic acid, (0.10, 2.72, 15.60, 0.56, 4.60, 0.93, and 0.12), respectively (TIVERON et al., 2012).

LÓPEZ-GARCÍAet al. (2013) found great levels of Ferulic acid (FA) (887.44), Coumaric (207.29), and Gallic (201.76) µg/g dry matter, receptively, as major constituents of the methanolic extracts of chives flowers. FA has many biological activities such as improvement of microcirculation, elimination of oxygen-free radicals, anti-inflamatory properties and suppression of carcinogens (BASKARAN et al., 2010; KUMAR e PRUTHI, 2014; SGARBOSSA et al., 2015).

Flavonoids have a wide range of biochemical and pharmacological effects, including antiinflammatory, antioxidant, and antiproliferative actions, which may protect the body from various diseases, such as cancers and cardiovascular diseases (WANG et al., 2014; ZHANG et al., 2015; GRIFFITHS et al., 2016, KIM et al., 2017). Moreover, a recent systematic review and metaanalysis of cohort study provided strong

evidence for the recommendation of consuming flavonoids-rich food to reduce risks of mortality from all causes as part of a healthy diet among general adults (LIU et al., 2017).

It has been established that phenolic compounds are the major plant compounds with antioxidant capacity and this activity is due to their redox properties. Phenolic compounds are a class of antioxidant agents which can adsorb and neutralize the free radicals (ASIF, 2015; NIMSE et al., 2015; JULIET e SIVAKKUMAR, 2018). Probably due to the presence of appreciable amounts of these compounds, in addition to antioxidant enzymes, and lower contents of free radicals, chives might be able to grow under diverse climatic conditions (ZDRAVKOVIĆ-KORAĆ et al., 2010).

3.3 Antioxidants Properties of Chives

The antioxidant capacity of the *Allium* species is related to the presence of a variety of compounds sulfur-containing, and their precursors, in addition to other bioactive compounds such as polyphenol (BARAZANI et al, 2004; NENCINI et al., 2007).

ZHENG e WANG (2001) observed the antioxidant capacity in chives and found 1.05 mg (GAE/ g of fresh weigh) of total phenolic, and 9.15 (µmol of TE/g of fresh weigh) ORAC.

ALEZANDRO et al. (2011) observed 3.05 of total phenolic contents (*Folin-Ciocalteu* reducing capacity) (g catechin equivalent/ 100 g (f.w.) of dehydrated spices, and 3.22 DPPH (Scavenging ability) (mmoles Trolox equivalents/100g).

Previous results showed that the leaves, stalks and roots of the *Allium schoenoprasum* cultivated plant exhibited antioxidant capacity (ŠTAJNER et al., 2004, 2006, 2008). Moreover, ŠTAJNER et al. (2011) indicated that the crude extract of *Allium schoenoprasum* tissue culture exhibited antioxidant and scavenging abilities in all investigated plant parts, especially in the roots. According to their results, the tissue culture plants exhibited the highest activities in the roots in contrast to the cultivated plants where highest activities were observed in the leaves (**Table 6**).

Table 6. Free radical - scavenging capacity, total antioxidant capacity, inhibition of generation and total phenol content in *Allium schoenoprasum* tissue culture organs.

Investigated parameter	Plant organ		
	Root	Stalk	Leaf
DPPH RSC (%)	24.33	10.43	13.71
FRAP	82.00	25.66	52.66
NO inhibition (%)	52.65	4.61	17.77
Total phenols (mg/100 g)	53.52	33.16	52.65

Data adapted from ŠTAJNER et al. (2011)

TIVERON et al. (2012) performed a study to observe the antioxidant capacity of vegetables commonly consumed in Brazil. According to the results they found in chives high values [130.8 µmol (Fe^{2+}/g DW), 25.8 (µmol Trolox/g DW), 28.8%, 1.24, and 8.2 (µmol Trolox/g DW)] for FRAP, ABTS, β-carotene, Rancimat (protection factor), and DPPH, respectively.

PARVU et al. (2014) presented the following results regarding the content of total phenolics: 68.5 g of gallic acid equivalent/g (plant) in relation to the antioxidant capacity *in vitro*, which the results were: (6.72 g/mg DPPH) and (132.8 g Trolox eq/g plant). Whereas, MNAYER et al.(2014) found 6.76 GAE (mg/g) total phenolic contents (TPCs) of chive essential oil, and 5.59 DPPHIC50% inhibitions, and LENKOVA et al. (2016) observed 1591 (mg/kg) TPCs, and 76.57% DPPH. Their results on the value of antioxidant capacity ranged from 12.29 to 76.57%.

SEAL (2014) evaluated the antioxidant activities of seven wild edible plants e.g. *Allium schoenoprasum*, testing different solvents (benzene, chloroform, acetone and methanol). Their results showed ranged from 20.49 to 62.46 (GAE mg/g dry material), 0.57 to 6.86 (Rutin equivalent mg/g dry material), 3.08 to 16.51 (Quercetin equivalent mg/g dry material), 6.87 to 13.41 (Ascorbic acid equivalent (AAE) mg/g dry material), and 0.28 to 0.41 IC50 mg/g dry material), respectively of the total phenolic content flavonoid and flavonol contents, reducing power, and DPPH free radical scavenging activity. The methanol extract showed the best result in most analyzes. The authors concluded that this plant exhibited different extent of antioxidant activity, thus, chives can be considered a valuable source for the formulation of nutraceutical supplements.

In a recent research, BERETTA et al. (2017) observed potent antioxidant values chives 2088.2 (µmol TE per 100 g dm). In their study, chives among other plants, showed to be the most potent antioxidant species, had significantly higher values of catechin, kaempferol, ferulic and chlorogenic acids than all other *Allium* vegetables. The results suggest that antioxidant capacity in *Allium* depends not only on the chemical structure, but also on the mass fraction of each phenolic compound and on the combinations and interactions among them.

3.4 Potential of Chives for Health

Allium schoenoprasum L was used in traditional medicine to treat anemia, to cleanse the blood, and to stimulate digestion (GRZESZCZUK et al., 2011, CHARLES, 2013). In Moroccan folk medicine was used as a cure against arterial hypertension, diabetes (ZIYYAT et al., 1997). In Indonesia, *Allium schoenoprasum* L is used as a traditional drug for antihypertensive actions (AMALIA et al., 2008). In East Asia, is used to relieve cold, flu and lung congestion (CHARLES, 2013). Chives have a beneficial effect on the circulatory system by lowering the blood pressure, and they have antimicrobial activity, especially antifungal, and antioxidant properties, is also useful to relieve sunburn and sore throat (RATTANACHAIKUNSOPON e PHUMKHACHORN, 2008; VLASE et al., 2013; PARVU et al., 2014). Various other pharmacological activities of *Allium* species have been described including, anti-inflammatory, anticancer, anti-HIV, anticoagulant, neuroprotection, immunomodulation, antitubercular and anti-allergy (RATNAKAR e MURTHY, 1995; KYO et al., 1998; HODGE et al., 2002; BENKEBLIA, 2004; TEPE et al., 2005; ARIGA e SEKI, 2006; KAISER et al., 2009, VENKATESH, 2018). The pharmacological effects are due to diallyl sulfides (diallyl monosulfide, diallyl disulfide, diallyl trisulfide, and diallyl tetrasulfide), flavonoids, vitamin C, and carotenoids (VLASE et al., 2013).

Oxidative stress is one of the major factor involved in progression of various acute and chronic disorders including diabetes, cardiovascular, neurodegenerative and renal disorders (GANGULY e ALAM, 2015; GHOSH e BANERJEE, 2015; DAVALLI et al., 2016; RATLIFF et al., 2016; LAWSON et al., 2017; LOTFY et al., 2017). The

imbalance between oxidants (reactive oxygen species) and endogenous antioxidants, potentially leading to lipids, proteins, enzymes degradation causing cell death (MATÉS et al., 1999; SEIFRIED et al., 2007; LOBO et al., 2010; LUSHCHAK et al., 2014, GASCHLER et al., 2017; GHOSH et al., 2017; ISLAM et al., 2017; LAURINDO et al., 2018).

AMALIA et al. (2008) validated the traditional claim of chives as antihypertensive drug. The bulb extract was tested and showed reduction on the blood pressure in Wistar rats by increasing blood nitric oxide levels and thereby vasodilation. Showing it possess similar effects to standard vasodilator (isosorbidedinitrate).

The anti-inflammatory effects of three concentrations of chive extracts (25, 50 and 100%) were evaluated *in vivo* and *in vitro* for their ability to inhibit phagocytosis, the total oxidative status, total antioxidant response, and oxidative stress index. The pure extract (100% concentration) achieved the highest inhibitory activity on phagocytosis and oxidative stress. The phytochemical analyses of chives suggest that anti-inflammatory and antioxidant effects are due to the presence of the polyphenols (PARVU et al., 2014).

Recently, for the first time, significant *in vitro* antiplatelet activity induced by aqueous extracts was report of this plant. The results exhibited significant antiaggregatory effects, as compared to their negative controls (BERETTA et al., 2017). In their study, the species total organosulfur and phenolic content, and the HPLC profiles of 11 phenolic compounds were characterized and used to investigate the relationship between these compounds and antiplatelet and antioxidant activities. The results showed significantly positive correlations between the in vitro antiplatelet activity and total organosulfur, and phenolic (TP) content, as well as between the antioxidant capacity and TP and total organosulfur content in chives. In addition, their results suggest that both organosulfur and phenolic compounds contribute similarly to *Allium* antiplatelet activity, whereas phenolics, as a whole, are largely responsible for antioxidant activity, with broad variation observed among the contributions of individual phenolic compounds.

The actual medical management of the treatment of kidney stones is expensive or not, with no side effects. Thus, the search of anti-calculi drugs from natural sources has been assumed greater importance. Due to the variety of phytochemical compounds and various minerals present in chives, this plant has been evaluated as anti-calculi activity. The highest effect of dissolving the calcium oxalate crystal in kidney was in the dried chives leaves infuse. The study concluded that chive leaves could be an alternative to calculi disease treatment (HARO et al., 2017).

Along the same line of research anti-calculi activity, a study with *Allium schoenoprasum* L. was able to dissolve calcium kidney stones. According to the author, the effect was probably due to the presence of compounds such as alkaloids, flavonoids, glycosides, steroids, tannins and minerals such as potassium, calcium, magnesium and sodium (HUTABALIAN, 2017).

During the past decades have been published diverse experimental studies shown that polyphenols possess anti-carcinogenic properties (BAGCHI et al., 2004; YANG et al., 2009; LAI e ROY, 2004; LALL et al., 2015; ROLEIRA et al., 2015; SHAHIDI e AMBIGAIPALAN, 2015; ÇAKIR e GÜLSEREN, 2017; SANTINO et al., 2017, JULIET e SIVAKKUMAR, 2018). These studies strengthen the fact that chives possess anticancer activity. In the case of chives, the activity was independent of the concentration (25, 50, 75 and 100 µg/mL) applied, demonstrated the impact of phenolic compounds present in this herb inhibiting the proliferation of HaCaT cells. Thus, the

results were similar for all of the four concentrations, thereby demonstrating that even in lower concentration, chives achieved great results, reaching values of about 80% decreased of cell viability, probably, due to the high concentration of ferulic acid present in this herb (KUCEKOVÁ et al., 2011).

HSING et al. (2002) studied the association between incidence of prostate of cancer and consumption of *Allium* vegetables (garlic, scallions, onions, chives, and leeks) in Chinese population (238 cases in male subjects with confirmed prostate cancer). The trial observed that men who took *Allium* vegetables (including chives) more than 10 g daily presented significantly lower risk of cancer than those who consumed less than 2.2 g/day. Similarly effects were reported on the consumption of *Allium* vegetables including chives, lowering the risk of stomach and esophageal cancer in Chinese population (YOU et al., 1989; GAO et al., 1999; SETIAWAN et al., 2005; ZHOU et al., 2011).

Another study investigated the content of polyphenols in edible flowers wild chives and their effect on proliferation activity of human hepatocellular carcinoma cells. Anti-proliferative effect was evaluated *in vitro* using following concentrations of polyphenols 100, 75, 50 and 25 μg/ml. The results indicated that even low concentrations of edible flowers' polyphenols inhibited cell proliferation significantly. This effect indicates possible employment of studied edible flowers for prevention as well as for treatment of cancers. Whereas, where anti-proliferation activity of the cells incubated in the presence of chives extract had remarkably lower proliferation compared with control group. As shown in the research, high amount of polyphenols (especially ferulic acid) were found in chives (MORAVČÍKOVÁ et al., 2012).

SHIRSHOVA et al. (2013) developed a comprehensive chemical study of cultivated chives. Their results showed a wide range of biologically active substances and trace elements involved in the antioxidant and anticarcinogenic protection system of humans. The experiment for the evaluation of the antitumor potential of these substances showed inhibition on the growth of subcutaneously grafted Ehrlich carcinoma in male BDF mice at the stage of intense tumor development.

TIMITÉ et al. (2013) studied four isolated compounds from chives for anticancer activity in human colon cancer cell lines. Laxogenin 3-O-α-l-rhamnopyranosyl-(1–2)-β-d-glucopyranoside was reported as inactive, while deltonin was highly active against both cell lines.A phytochemical analysis of the whole plant of *Allium schoenoprasum*, has led to the isolation of four spirostane-type glycosides (1–4), and four known steroidal saponins. Therefore, the isolated compounds were tested for cytotoxic activity against the HCT 116 and HT-29 human colon cancer cell lines.

Chives flowers showed high antiproliferative activity 72%-57%, disclosing inhibition power and affecting HaCaT cell growth. The antiproliferative activity may be related to the polyphenolic content differences, along with synergistic effects. It has been substantiated that flavonoids have roughly higher reactivity than phenolic acids. This might explain why the phenolic extract of chives with 20.26 μg/g of rutin and two hydroxycinnamic compounds, couramic and ferulic acid presented high antiproliferative rates (LÓPEZ-GARCÍA et al., 2013). In fact coumaric and ferulic acids together are efficient modulators of NF-κB activity compared with their effect separately. Moreover, gallic acid has three hydroxyls on its phenyl ring. Generally, for benzoic and phenylpropanoids an increase in the number of hydroxyl groups results in a higher antioxidant activity. Compounds with two or three hydroxyl groups on the phenyl ring of phenolic acids present high antioxidant capacity (CAMPBELL et al.,

2006; KOWALCZYK et al., 2010; HOLE et al., 2012), consequently, greater anti-cancer power.

In addition to all the researches evaluating the antioxidant capacities of chives *in vitro* and *in vivo* presented, studies also reported other ability of chives in different areas.

BARAZANI et al. (2004) tested the potential of chives to accumulate and tolerate cadmium in aqueous Hoagland medium at 50lM and 250lM was tested under continuous growth or several successive harvests of shoots. According to the authors *A. schoenoprasum* presented high tolerance to Cd as well as to other toxic heavy metals and point to chives phytoremediation potential. Cd, like other environmental stresses, induces also antioxidant responses in plants.

Other use of chives is related to ornamental and in decorative arrangements due to the beautiful ball like lavender coloured umbels (ZDRAVKOVIĆ-KORAĆ et al., 2010). Moreover, chives also possess insect repelling properties which make them useful in gardens to control pests (ŠTAJNER et al., 2011).

LÓPEZ-GARCÍA et al. (2013) studied the potential of chives as atelocollagen matrix and the results showed to be perfectly apt for keratinocyte cell growth and proliferation. Therefore, this approach strengthens knowledge about the use of atelocollagen, and allows consideration of these materials as potential candidates for tissue engineering and would healing applications.

4. FINAL CONSIDERATIONS

In this review were compiled all information available on literature of parsley (*Petroselinum crispum*) (Mill.) Nym. and chives (*Allium schoenoprasum* L.), taking into account their botanical description, phytochemistry and chemical composition, antioxidant properties, in addition traditional pharmacological uses, and the potential on medicinal tests. Considering the current concerns about adverse effects of synthetic antioxidants in human health, it is observed that these aromatic herbs may be presented as an accessible source of natural antioxidants. From the available literature, it is evident that these herbs have both great potential in food science, and medical area.

The high content in phenolic compounds, the antioxidant capacities and their potential importance in the prevention of several chronic diseases require reconsideration of these aromatic herbs. Ongoing research will help elucidate the role of antioxidants benefits of these traditional herbs, in addition to the use of the mixture of parsley and chives (*cheiro -verde*) on inhibition of fish lipid oxidation.

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CAPÍTULO II

CHEMICAL COMPOSITION, IDENTIFICATION OF BIOACTIVE COMPOUNDS BY UPLC-ESI-*MS* AND ANTIOXIDANT CAPACITIES *in vitro* AND *in vivo* OF PARSLEY (*Petroselinum crispum*) (Mill.) Nym., CHIVES (*Allium schoenoprasum* L.) AND BRAZILIAN *CHEIRO-VERDE*

Em preparação para envio a Funcional Foods

ABSTRACT

Herbs with long tradition of use such as chives (*Allium schoenoprasum* L.) and parsley (*Petroselinum crispum*) (Mill.) Nym. are widely used in culinary dishes, by popular medicine and in diverse testes in the medical area and food science. This study presents the chemical composition, bioactive compounds, identification of phenolic compounds by UPLC-ESI-MS, and evaluation of antioxidant activities *in vitro* and the first *in vivo* test using the aromatic herbs, parsley and chives, and their mixture (Brazilian *cheiro-verde*) and yeast *Saccharomyces cerevisiae*. The results indicate that these herbs can be considered as a low energy food, being a good source of fiber and minerals. The phytosterols determined were: brassicasterol, β-sitosterol, stigmasterol, campesterol, as well as the presence of the oxidized 7-ketostigmasterol. As major fatty acids identified in these herbs (g/100g of oil) were (C16:0), (C20:1 *n*9), (C18:2 *n*6) and (C18:3 *n*3). The samples showed a higher levels of total phenolic (mg GAE/g), total flavonoids (mg of quercetin equivalent (QE)/g), and total carotenoids (µg/g). The identification of twenty four phenolic compounds in (80:20) methanol/water extract present in all of the three studied samples showed as main compounds were (protocatechuic acid, luteolin, kaempferol, quercetin, and ferulic acid). The aromatic herbs showed great antioxidant properties *in vitro*; the evaluation of DPPH radical scavenging activity ranged from 51.59 to 54.92%; ABTS from 5.12 to 8.29 (µmol TE/g); and FRAP from 6.33 to 8.32 (µmol TE/g). In the *in vivo* test with *S. cerevisiae*, the bioactive compounds from the aromatic herbs extracts evaluated showed antioxidant action the yeasts, reducing the effects of oxidative stress caused by hydrogen peroxide. Thus, the protective effect of the extracts *in vivo* converges with the results obtained in the *in vitro* tests, corroborating to indicate their use in combating radicals formed in food processing.

Keywords: UPLC-ESI-MS. Bioactive Compounds. Natural Antioxidants *in vitro* and *in vivo.*

RESUMO

Ervas com longa tradição de uso como a cebolinha (*Allium schoenoprasum* L.) e salsa (*Petroselinum crispum* (Mill.) Nym.) são amplamente utilizadas em pratos culinários, pela medicina popular e em diversos testes na área médica e ciência dos alimentos. Este estudo apresenta a composição química, compostos bioativos, identificação de compostos fenólicos por UPLC-ESI-MS e a avaliação de atividades antioxidantes *in vitro* e é o primeiro estudo utilizando ervas aromáticas salsa e cebolinha, e sua mistura (cheiro-verde brasileiro) em testes *in vivo* com a levedura *Saccharomyces cerevisiae*. Os resultados indicam que essas ervas podem ser consideradas como um alimento de baixa caloria energética, sendo uma boa fonte de fibras e minerais. Os fitoesteróis determinados foram: brassicasterol, β-sitosterol, estigmasterol, campesterol, bem como foi observado à presença do óxido de fitoesterol (7-cetostigmasterol). Como principais ácidos graxos identificados nestas ervas (g/100g de óleo) foram encontrados (C16: 0), (C20:1 *n*9), (C18:2 *n*6) e (C18:3 *n*3). As amostras apresentaram consideráveis teores de conteúdo fenólico total (mg GAE/g), flavonoides totais (mg de quercetina equivalente (QE)/g) e carotenóides totais (µg/g). A identificação de vinte e quatro compostos fenólicos no extrato (80:20) metanol/água presente em todas as três amostras estudadas mostrou-se como principais os seguintes compostos: ácido protocatecuico, luteolina, kaempferol, quercetina e ácido ferúlico. As ervas aromáticas também apresentaram um bom desempenho em relação às propriedades antioxidantes *in vitro*; DPPH variou de 51,59 a 54,92%; ABTS de 5,12 a 8,29 (µmol TE/g) e FRAP de 6.33 a 8.32 (µmol TE/g). No teste *in vivo* com *S. cerevisiae*, os compostos bioativos dos extratos de ervas aromáticas avaliados mostraram ação antioxidante, reduzindo os efeitos do estresse oxidativo causado pelo peróxido de hidrogênio. Assim, o efeito protetor dos extratos *in vivo* converge com os resultados obtidos nos testes *in vitro*, corroborando para indicar a utilização das mesmas no combate, como inibidores ou sequestradores dos radicais formados no processamento de alimentos.

Palavras-chave: UPLC-ESI-MS. Compostos bioativos. Antioxidantes naturais *in vitro* e *in vivo.*

1 INTRODUCTION

In recent years, the potential of diverse plants and their extracts have attracted the researchers attention due their effectiveness as natural antioxidants in food preservation (GYAWALI e IBRAHIM, 2014; CALO et al., 2015; CALEJA et al., 2016; MATUMOTO-PINTRO et al., 2017; MUNEKATA et al., 2017; DE OLIVEIRA, 2018). Especially, because most of these are sources of secondary metabolites such as polyphenols and show great potential to be used as antioxidant/antimicrobial and diverse other biological activities (EMBUSCADO, 2015; BARBIERI et al., 2017; BERETTA et al., 2017; NILE et al., 2018; JIANG e XIONG, 2016; TIAN et al., 2018).

Other compounds present in the plantae kingdom, besides vitamins, minerals, and phenolic compounds, there are the bioactive lipids, including phytosterols and polyunsaturated fatty acids (PUFAs), which are considered essential and recognized as functional foods (CHEN et al., 2013; VLASE, 2013). Studies have found an association between the consumption of certain bioactive lipids and the improvement of human health, preventing or delaying treatment of diverse chronic diseases, such as cancer, cardiovascular disease, osteoporosis, and immune disorders (JONES e ABUMWEIS, 2009; WOYENGO et al., 2009; CHEN et al., 2013; VLASE, 2013; ZHANG et al., 2013; CHAWLA et al., 2016; ROCHA et al., 2016; YI et al., 2016; ZHANG et al., 2013; JURADO-RUIZ et al., 2017; EGERT et al., 2018; MOREAU et al., 2018).

In addition to determination and identification of chemical compounds, current researches have evaluated the potential of natural extracts *in vivo* model activities; which are also required for bioactive purposes (GRANATO et al., 2018). The yeast *Saccharomyces cerevisiae* is used in this type of studies, due to its biochemical and molecular similarities with human cells, relevant for studies on mechanisms that respond to oxidative stress (WU et al., 2011; SÁ et al., 2013; STINCO et al., 2015; CHANAJ-KACZMAREK et al., 2015; ODRIOZOLA-SERRANO et al., 2016; JARDIM et al., 2017; MENG et al., 2017).

Among the aromatic herbs, chives and parsley are widely used in culinary, alternative medicine, medicinal tests against diverse diseases, and also in the food science area. Chives (*Allium schoenoprasum* L.) family of *Amaryllidaceae,* also known as green-onions, adapts to different environments and can be harvested all year long (PARVU et al., 2014). In addition to the use as medicinal purpose son the circulatory system by lowering the blood pressure, antifungal and antioxidant properties; this plant is extensively used for culinary purposes throughout the world (RATTANACHAIKUNSOPON e PHUMKHACHORN, 2008; PARVU et al., 2014).

Parsley (*Petroselinum crispum)* is a biennial herb belonging to the family *Apiaceae*. It is an important aromatic herb originally from the Mediterranean and has been used worldwide since antiquity in culinary, for its attractive and aromatic leaves in food as well as in medicine (DÍAZ-MAROTO et al., 2003; SOYSAL, 2004; PARTHASARATHY et al., 2008; FARZAEI et al., 2013). Parsley has attracted the attention of researchers due its chemical composition, where studies have shown the potential effects of the bioactive compounds present in this herb as natural antioxidant, hepatoprotective, neuroprotective, antidiabetic, analgesic, spasmolytic, immunosuppressive, anti-coagulant, anti-ulcer, laxative, estrogenic, diuretic, hypotensive, antibacterial, and antifungal have been previously reported in various studies (FARZAEI et al., 2013). The combination of parsley and chives, popularly known as *cheiro-verde,* is commonly used as fresh chopped seasoning and garnishing in

gastronomy, moreover, the use of both herbs enhances the flavor in typical dishes of Brazilian cuisine.

Although, researches have been carried studies on determination of chemical compounds and antioxidant potential in *Allium schoenoprasum* L. and *Petroselinum crispum*, on the other hand, limited investigations have been conducted on bioactive lipids such as phytosterols and fatty acids; also *in vivo* studies using the yeast *Saccharomyces cerevisiae*. In this context, the aim of this work was to determine the nutritional composition, phytosterols, fatty acids contents, bioactive compounds, identification of phenolic compounds by UPLC-ESI-MS, and also the antioxidant potential by different *in vitro* and *in vivo* tests of these aromatic herbs and their mixture.

2 MATERIALS AND METHODS

2.1 Samples

The organic parsley (*Petroselinum crispum* (Mill.) Nym. *var. neapolitanum*) and chives (*Allium schoenoprasum* L.) were obtained from the Experimental Agricultural Station of EMBRAPA - Agrobiologia and Universidade Federal Rural do Rio de Janeiro (UFRRJ) in Seropédica – Rio de Janeiro, Brazil. The voucher samples of *Petroselinum crispum* (RBR 37741) and *Allium schoenoprasum* L. (RBR 37740) were deposited at the RBR herbarium, by Dr Marcelo da Costa Souza, Department of Botany/Institute of Biological and Health Sciences (UFRRJ). After harvesting, the fresh herbs were selected, kept under refrigeration, protected from daylight, until further analysis. The *cheiro-verde* samples consisted in the mixture of 50% of parsley and 50% of chives.

2.2 Chemicals

Phytosterol standards, including brassicasterol, campesterol, stigmasterol, β-sitosterol and 7-cetostigmasterol were obtained from Sigma-Aldrich (St. Louis, MO, USA); and the standards used in the antioxidant capacity assays were: ((±)-6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) (Trolox), 2,4,6-tris-2,4,6-tripyridyl-2-triazine (TPTZ), gallic acid. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). 2,2’-azinobis (3-ethyl-benzo-thiazolione-6-sulphonate) (ABTS) was obtained from Fluka Chemie (Buchs, Switzerland). Methyl undecanoate was obtained from Sigma (St. Louis, MO, USA) and a total of 37 saturated, mono-unsaturated and polyunsaturated fatty acids standards (SupelcoTM FAME Mix 18919, Bellefonte, PA, USA) were used. The purities of the standards ranged from 95% to 99%. Sodium methoxide was purchased from Sigma (St. Louis - MO, USA). HPLC grade acetonitrile and *n*-propanol were obtained from Mscience (Darmstadt, Germany), and all other analytical grade solvents were obtained from Vetec (Sigma, São Paulo, Brazil).

2.3 Nutritional Composition

The moisture, protein and ash contents were determined according to AOAC (2010).The lipids were extracted and determined according to BLIGH e DYER (1959), with modifications proposed by SALDANHA et al. (2008).

Dietary fiber content was determined according to SILVA e QUEIROZ (2002).

2.4 Minerals

Potassium (K) were analyzed by nitric digestion (Digester MARS) in microwave and determined by flame photometer (Analyser, 910M) according to ELEMENT, 2007. Calcium (Ca) and magnesium (Mg) were determined by atomic absorption spectrophotometry (AA6200, Tokyo, Japan). For phosphorus (P) analysis, was followed the method according to MALAVOLTA (1997).

2.5 Phytosterols analysis (HPLC-PDA /UPLC-APCI-MS)

Phytosterols were extracted according to SALDANHA et al. (2006) with modifications to the initial weight of the samples (500 mg). The HPLC system consisted of a Waters liquid chromatography (Waters, Milford, MA, USA) equipped with on-line PDA (Waters 2998) and refractive index (RID-Waters, 2414) detectors, a rheodyne injector with a 20 µl loop, a tertiary solvent delivery system (Waters 600), oven heated column at 32 ^{o}C and software (Empower 2). The analytical column was an X-Terra® MSC18 (3.5µm × 4.6 × 250mm) Waters column, (Ireland). Mobile phase acetonitrile/isopropanol (85:15), 0.8 ml/min of flow and reading at PDA: 210nm. Quantification was done by external standardization, with a standards concentration range from 0.1 to 1.8 mg/ml.

In order to confirm the phytosterol structures, samples of parsley, chives and *cheiro-verde* were analyzed by UPLC-APCI-MS. The chromatographic analyses were performed on a UPLC Acquity chromatograph coupled to a TQD Acquity mass spectrometer (Micromass-Waters Manchester, England), with an APCI source. An X-Terra® MSC18 (3.5µm × 4.6 × 250 mm) Waters columnwas used. The solvent acetonitrile/isopropanol (85:15) with a flow rate of 0.8 mL. min^{-1} and 10 µL of the samples were injected into the UPLC.

Ionization was performed in the APCI positive ion mode and the optimization conditions were adopted from (ISHIDA, 2014). The ionization parameters were: full scan m/z 100-500, capillary voltage 4000 V; corona current 10 µA; drying gas flow 5 mL/min; drying gas temperature 350 ^{o}C; fragmentor voltage 200 V; nebulizer pressure 60 psi; vaporizer temperature 500 ^{o}C.

Phytosterol standards were analyzed individually, and data were collected in the selected ion monitoring (SIM) mode. Under the proposed chromatographic conditions were observed retention times of the five analysed sterols were: 8.0 min for 7 Ketostigmasterol, 10.0 min for Brassicasterol, 15.3 min for campesterol, 15.6 for stigmasterol and 17.0 min for β-sitosterol.

2.6 Fatty Acid Composition

The lipids were converted into methyl esters by transesterification catalyzed with sodium methoxide following the procedure according to ZHU et al. (2011). The

fatty acids were determined using a gas chromatograph (Shimadzu GC 2010, Tokyo, Japan), equipped with a split injector (1:50), a flame ionization detector, and a workstation. The chromatographic separation was performed in a fused silica CP-SIL 88 capillary column 100 m × 0.25 mm i.d., with 0.20 µm film thickness (Chrompack, Middelburg, The Netherlands). The chromatographic conditions were: initial temperature, 100 °C (5 minutes) followed by 5°C/minute up to 160 °C (zero minute), 8 ºC/minute up to 230 °C (12 minutes); injector and detector temperatures were 250 °C and 280 °C, respectively. The hydrogen was the carrier gas at flow rate of 1 mL/min. and nitrogen as the make-up gas at 30 mL/minute. Retention times of FAME standards were used to identify the chromatographic peaks of the samples, and the quantification was done by internal standardization (C11). The results were calculated in mg per 100 g of lipids (AOCS, 1989).

2.7 Bioactive Compounds

2.7.1 Preparation of extracts

The extractions were carried out according to KUŹMA et al., 2014 with some modifications; it was used (500 mg) of the fresh parsley, chives and *cheiro-verde*. The extracts were done in triplicate and used for all analyzes. The absorbance was read in a Nova 2000 UV spectrophotometer (São Paulo, Brazil).

2.7.2 Determination of total phenolic contents

The total phenolic was determined with *Folin-Ciocalteu* reagent, according to (SWAIN e HILLIS, 1959) with small alterations. The results were expressed as gallic acid equivalent (mg GAE/100g dry weight of sample). Total phenolics were measured using a standard curve by gallic acid equivalent, ranging from 12.5 to 100 µmol/L. The regression coefficient was higher than 0.99.

2.7.3 Total Flavonoids

The total flavonoid content was determined according to (SCAPIN, 2016). A 250 µL aliquot of the extract was mixed with 1250 µL of distilled water and 75 µL sodium nitrite solution (5%). After 5 minutes, 150 µl of 10% aluminum trichloride solution was added, the mixture allowed to stand for another 5 minutes, followed by the addition of 500 µL of sodium hydroxide solution (1 M) and 775 µL of distilled water. The mixture was homogenized and the absorbance measured in a spectrophotometer (NOVA 2000 UV) at 510 nm. The total flavonoid content was determined using a standard quercetin curve with concentrations ranging from 0 to $4x10^{-4}$ g/mL (r=0.9901). The results were expressed in mg of quercetin equivalent (EQ) per gram of sample.

2.7.4 Carotenoids

The carotenoids were analyzed according to the methodology proposed by LICHTENTHALER (1987), which determines the levels of carotenoids and chlorophyll

a and *b* simultaneously. For extraction, 2 g the leaves of parsley and chives were macerated and added 0.2 g of calcium carbonate and 7 ml of acetone 80%. The extract was filtered to a 25 mL volumetric flask and the volume swelled with the solvent. The absorbance was measured in a spectrophotometer (Model NOVA 2000 UV) at 470 nm for total carotenoids, 647 nm and 663 nm for chlorophyll a and b, respectively. The total concentrations of chlorophyll *a* and *b*, and carotenoids were determined according to equations 1, 2 and 3, respectively. The results were expressed as µg/g sample.

$$\text{Chlorophyll } a \text{ (Ca)} = [12.25\ A_{663.2} - 2.79\ A_{646.8}] \quad (1)$$

$$\text{Chlorophyll } b \text{ (Cb)} = [21.50\ A_{646.8} - 5.10\ A_{663.2}] \quad (2)$$

$$\text{Total carotenoids} = [100\ A_{470} - (1.82Ca - 104.96\ Cb)/198] \quad (3)$$

2.8 Phenolic Compounds Identification using UPLC-ESI-MS

Chromatographic analyses of samples were performed using a UPLC Acquity chromatographer coupled with a TQD Acquity mass spectrometer (Micromass-Waters, with electrospray ionization (ESI) in the negative ion mode. The conditions were capillary = 3.00 kV, cone = 30 V, source temperature = 150°C, desolvation temperature = 350 °C, with data acquisition between m/z 100 and 900. MS/MS of selected peaks were acquired via CID with and collision energy = 30 V. The chromatographic conditions were: a C18 BEH Waters Acquity (2.1 mm x 50 mm x 1.7 µm) column at 30ºC; mobile phase A (0.1% formic acid) and B (HPLC grade methanol) (phase A) at a flow rate of 0.2 mL/min with a linear gradient starting at 5% B and increasing to up 100% methanol in 7.5 min, before holding until 9 min, and then returning to the initial conditions, followed by column re-equilibration until 10 min; injection of 5 µL.

2.9 *In vitro* antioxidant capacity

2.9.1 DPPH radical-scavenging activity

Antioxidant capacity was determined by scavenging the radical 2,2-diphenyl-1-picryhydrazyl (DPPH) according to RUFINO et al. (2007). In this study 0.1 ml of extract was mixed with 3.9 ml of $6x10^{-5}$M DPPH solution. After 60 min, the absorbance of samples, standards and blanks (methanol) was determined at 517 nm. Antioxidant capacitywas expressed as a percentage of DPPH radical-scavenging activity (IC 50% DPPH). The quantification was performed by external standardization with calibration curve concentrations ranging from 5 – 65 µol/L. The regression coefficient was higher than 0.99.

2.9.2 Free radical scavenging ability

The ABTS test was carried out according to RUFINO et al. (2007). An aliquot of 30 µl of the extracts was added to 3.0 ml of radical cation $ABTS^+$ solution formed by the chemical reaction with potassium persulfate. The absorbance was determined at 734 nm after 6 min, at 30 ºC. The free radical scavenging ability was expressed as µmol Trolox equivalent (TE)/g of sample and as a percentage of ABTS radical-scavenging

activity (% ABTS). The quantification was performed by external standardization with calibration curve concentrations ranging from 10–300 µmol/L. The regression of coefficient was higher than 0.99.

2.9.3 Ferric Reducing Power Antioxidant

The FRAP (Ferric Reducing / Antioxidant Power) method was used as described by RUFINO et al. (2006). The reaction was started by adding 90 µl of extract, with 270 µl of distilled water and 2.7 ml of reagent FRAP. The mixture was heat at 30ºC, for 30 min. The absorbance was measured at 595 nm for the FRAP assay. The free radical scavenging ability was expressed as µmol of Fe^{+}/g of the sample. The quantification was performed by external standardization with calibration curve concentrations ranging from 5–80 µol/L. The regression of coefficient was higher than 0.99.

2.10 *In vivo* Antioxidant Activity

2.10.1 Microorganism and culture conditions

Saccharomyces cerevisiae strain BY4741 (MATa genotype; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) (synthetic, Euroscarf, Frankfurt, Germany) were used for the *in vivo* tests. They were stored in 2% YPD solid medium and kept under refrigeration. Cellular inoculum was performed with fresh cells in a previously prepared liquid medium and autoclaved at 121 °C for 15 minutes.

2.10.2 Cell growth

For 24 hours before each experiment, fresh shoots were prepared in 2% YPD solid medium (2.0% glucose, 2.0% peptone, 1.0% yeast extract, and 2% of agar) and kept in an oven at 30 °C. Cellular inoculum was performed in sterile liquid medium and the concentration of the cell suspension was measured by reading the optical density (OD) at 570 nm.

2.10.3 Determination of extract toxicity

The cells were incubated with the herbs extracts in the same conditions cited above for 2 hours. Then, the cells were washed twice with distilled water and resuspended in sodium phosphate buffer (50 mM, pH 6.0). A solution of hydrogen peroxide (H_2O_2, 1 mM) was added to the suspension and maintained at 28 °C/160 rpm for 1 hour. Then, cells were plated (40 µg) on solidified 2% YPD medium and incubated at 30 °C for 48 hours and then colonies were counted. The results were compared with the results of positive control, cells without the treatment with natural extracts exposed only to the H_2O_2 solution, and negative control, cells only in phosphate buffer.

2.10.4 Determination of cells viability

After cells growing on a liquid 2% YPD medium, cells (20 mg) at the first exponential phase growing (around 1 mg/mL) were incubated with the extracts at 10µg/mL and 100 µg/mL (28 °C/160 rpm), for 1 hour and 3 hours. Cell survival was determined by plating cells (40 µg) on solidified 2% YPD medium. The plates were incubated at 30 °C for 48 hours and then colonies counted. The results were compared with positive control, cells exposed to stress with a hydrogen peroxide 1 mM (28 °C/160 rpm), and negative control cells, without any treatment (28 °C/160 rpm).

2.11 Statistical Analyses

All the analyses were carried out in triplicate. A one-way analysis of variance (ANOVA) was applied to the data. The means were compared using the *Tukey* multiple comparisons test, with $P < 0.05$. The software used was Origin 8.0 for Windows.

3 RESULTS AND DISCUSSION

3.1 Nutritional Composition of Chives, Parsley and *Cheiro-Verde* Samples

The overall macronutrient composition of the herbs is presented in **Table1**. Herbs have high water content, with the total moisture varying from 87.50±1.10 to 94.70±0.45 g/100g. Chives had higher moisture content (94.70±0.45g/100g). These findings are in agreement with those reported by SANTOS et al. (2014) in a previous study of chives and parsley (92.7 and 85.2 g/100 g (f.w.) product), respectively.

Table 1.Water, protein, fat, ash and fiber composition of parsley, chives and *cheiro-verde* (g/100g).

Components	Parsley	Chives	*Cheiro-verde*
Water	89.00±0.30^{b}	94.70±0.45^{a}	87.50±1.10^{b}
Protein	1.30±0.10^{b}	2.70±0.17^{a}	2.90±0.31^{a}
Fat	0.60±0.04^{a}	0.20±0.00^{b}	0.30±0.07^{b}
Ash	1.30±0.26^{a}	0.50±0.08^{b}	1.40±0.00^{a}
Fiber content	7.80±0.10^{c}	10.30±0.10^{a}	9.10±0.10^{b}

Data are expressed as mean values ± standard deviation (n=4).
Different letters in the columns indicate significant differences $P<0.05$.

Significant differences were observed in protein content, varying from 1.30 ± 0.10 to 2.90 ± 0.31 g/100 g. C*heiro-verde* and chives showed higher protein levels than parsley.As expected, very low fat levels were observed in all samples, varying from 0.20±0.09 to 0.60±0.04 g/100g. The parsley fat content was significantly greater than the *cheiro-verde* and chives.The highest fiber levels were observed in chives (10.30 ± 0.10), followed by *cheiro-verde* and parsley. Lower levels were determined by SANTOS et al. (2014) in parsley and chives samples, equivalent to 5.0 and 2.2 g/100g of fiber, respectively.

The mineral contents of parsley, chives, and *cheiro-verde*, expressed as mg/kg on a dry basis, are shown in **Table 2**.

Table-2. Mineral contents (mg/kg) of parsley, chives, and *cheiro-verde.*

Mineral contents (mg/kg)	Parsley	Chives	*Cheiro-verde*
K	540±0.06[a]	380±0.06[c]	440±0.04[b]
P	8320±0.43[a]	6390±0.61[b]	6280±0.63[b]
Ca	6.00±0.20	6.10±0.60	6.30±0.60
Mg	2.10±0.10	2.70±0.30	2.40±0.10

Data are expressed as mean values ± standard deviation (n=3).
Different letters in the columns indicate significant differences (P<0.05).

The data indicate that these aromatic herbs contain essential dietary minerals, such as potassium, calcium, magnesium and phosphorous. Parsley showed higher K content (540.00 ± 0.06 mg/Kg) than *cheiro-verde* and chives. The results presented for chives in this study were superior to the K content compared to those found by TRICHOPOULOU et al. (2000) that observed levels equivalent to 268 mg/kg.

The contents of Ca and Mg did not differ statistically among samples. Ca content varied from 6.00 ± 0.20 to 6.30 ± 0.60 mg/Kg, and Mg content ranged from 2.10 ± 0.10 to 2.70 ± 0.30 mg/Kg of samples. For the phosphorous content, parsley showed the highest values and *cheiro-verde* the lowest. These results agree with those reported by SANTOS et al. (2014) regarding the higher P content in parsley (163.00 mg/100g) than in chives (77.4 mg/100g) leaves.

Differences of results for the macronutrients and mineral contents with other researchers are expected due to different locations, varieties, conditions of growth and the post harvesting processes.

3.2 Phytosterols

In the present study, the phytosterols determined were: brassicasterol, β-sitosterol, stigmasterol, campesterol, as well as the presence of the oxidized 7-ketostigmasterol.

The results are expressed in mg/100g of sample, on a dry basis (**Table 3**).

Table 3. Phytosterol composition (mg/100 g of sample, dry basis) of parsley, chives and *cheiro- verde.*

Herbs/Fitos	Parsley	Chives	*cheiro-verde*
Brasicasterol	77.60±1.80[a]	29.50±2.30[c]	63.20±6.00[b]
Campesterol	27.20±0.30[b]	19.50±3.90[c]	42.50±3.80[a]
Stigmasterol	15.20±1.00[b]	42.20±4.00[a]	46.10±6.80[a]
β-Sitosterol	51.90±2.50[c]	65.70±0.90[a]	58.30±7.60[b]
7-Ketostigmasterol	11.60±1.10[b]	16.30±1.20[b]	26.60±4.80[a]
Total sterol	**183.50±1.34[b]**	**173.20±2.46[c]**	**236.70±5.80[a]**

Data are expressed as means values ± standard deviation (n=3).
Different letters in the columns indicate significant differences (P<0.05).

The total phytosterol contents demonstrated significant differences among the samples (p<0.05), with superior results in *cheiro-verde* (236.70 ± 5.80) followed by parsley (183.50 ± 1.34) and chives (173.20 ± 2.46), suggesting a synergism for the mixture of parsley and chives.

The main phytosterol presented in analysed samples was Brassicasterol, which ranged from 29.50 ± 2.30 to 77.60 ± 1.80. The highest value was observed in parsley, followed by *cheiro-verde* and chives. Stigmasterol contents varied from 15.20 ± 1.00 to 46.10 ± 6.80 mg/100g. Similar values were observed for chives and *cheiro-verde* while the lowest value was found in parsley samples.

The campesterol contents differed among the analyzed herbs. The highest value was observed in *cheiro-verde* (42.50 ± 3.80), followed by parsley and chives. These results indicate that a synergism effect may have occurred for that compound in the sample containing both herbs. The β-Sitosterol levels in these herbs ranged from 51.90 ± 2.50 to 65.70 ± 0.90. Chives presented the highest concentration (65.70 ± 0.90 mg/100g) in all sample in the present study. On the other hand, VLASE et al. (2012) found lower concentrations of β-Sitosterol and campesterol (25.09 and 7.21 mg/100g), respectively, in chives. In a study of detection for simultaneous analysis of phytosterols and cholesterol in plant foods ITO, MEIKO et al (2017) observed the presence of stigmasterol, campesterol and β-Sitosterol (0.77, 0.64 and 0.45 mg/g tissue) in fresh parsley.

Although they are of great importance in human health, due the ability of plant sterols and stanols (phytosterols/phytostanols) to reduce serum low-density lipoprotein (LDL)-cholesterol level, there are few studies characterizing these compounds in fresh herbs (BOLDIZSÁR et al., 2013). PIIRONEN et al. (2003) studied the content of plant sterols in vegetables and observed the presence of brassicasterol, campesterol, stigmasterol, and sitosterol (2, 12, 115, and 136 mg/kg^{-1}dw) in parsley. BOLDIZSÁR et al. (2013) reported the presence two phytosterols (stigmasterol and β-sitosterol) in parley fruits (PFr) and parsley leaves (PLe) samples. The concentrations of stigmasterol and β-sitosterol in PFr were 3.36 and 2.77 μmol/g, and in PLe samples were 2.77 and 2.76 μmol/g, respectively.

Different aspects may influence the contents of phytosterols in plants, such as genetic factors, growth and post-harvest conditions and the separation of the sample taken for analysis (PIIRONEN e LAMPI, 2004).

The levels of 7-Ketostigmasterol in the samples ranged from 11.60 ± 1.10 t o 26.60 ± 4.80. *Cheiro-verde* presented the highest content of this product compared to parsley and chives. Moreover this is the first time that 7-ketostigmasterol has been reported in these herbs. According to OTAEGUI-ARRAZOLA et al. (2010) the hydroxylases and other enzymes could be involved in the oxidation of the phytosterols, yielding endogenously phytosterol oxidation products or POPs. Thus justifying the presence of 7-ketstigmasterol in the studied herbs. MARQUES et al. (2008) in a study on the lipid composition of trimming residues of tagasaste (*Chamaecytisusproliferus spp. palmensis*) observed the presence of oxidized 7- Stigmasterol; however, it was not quantified.

The phytosterols are recognized as functional foods, due their ability to reduce cholesterol absorption, by displacing cholesterol from mixed micelles in the small intestine so that cholesterol absorption is partially inhibited (ROZNER e GARTI, 2006; JONES e ABUMWEIS, 2009, ROCHA et al., 2016; YI et al., 2016); due to structural similarity to cholesterol (YANKAH, 2006; GYLLING e SIMONEN, 2015; CHAWLA et al., 2016; MOREAU et al., 2018).

The results of the present research showed that there was a synergy between the mixture of parsley and chives, since the highest values of total phytosterols were observed in *cheiro-verde* samples. Thus, these results obtained study are very promising, as the levels of the phytosterol compounds were relatively higher.

3.3 Fatty acid Composition

The fatty acids found in parsley, chives and their mixture are shown on **Table 4**.

As major components across samples, the main fatty acids identified in these herbs (g/100g of oil) were (C16:0), (C20:1 *n*-9), (C18:2 *n*-6) and (C18:3 *n*-3) respectively.

Significant differences among the samples were observed for the fatty acid profiles, total fatty acids and fatty acid ratios. It was found that parsley contains a high concentration of C20:2 *n*-6 (25.86 ± 1.76), in contrast chives did not present practically this fatty acid. Other important variability in the fatty acid composition of the studied herbs is the presence of *trans* fatty acids in parsley (C18:1 *n*-9*t* and C18:2 *n*-6*t*) and absence in chive samples.

Furthermore, the content of total of saturated fatty acids (SFA), MUFA, PUFA and the ratio of *n*3/*n*6, varied in two species and their mixture, which suggested that each species had its own fatty acid pattern. Parsley presented the highest MUFA contents (14.18), ∑ *n*6 (43.16) and also for ∑*Trans* (10.86). On the other hand, chives presented higher levels of ∑SFA (18.81), ∑PUFA (67.45) as well as the ∑*n*3 (43.39).

Table 4. Fatty acid composition (mg/100 g of oil, dry basis) of parsley, chives and *cheiro- verde.*

Fatty acids	**Aromatic herbs**		
g/100g	**Parsley**	**Chives**	***Cheiro - verde***
C10:0	2.09±0.13^{a}	--	0.75±0.03^{b}
C12:0	0.88±0.14^{a}	0.24±0.02^{b}	0.09±0.0^{c}
C14:0	0.34±0.35^{b}	0.88±0.19^{a}	0.67±0.09^{ab}
C15:0	0.10±0.01^{c}	0.28±0.00^{a}	0.19±0.00^{b}
C16:0	10.18±0.72^{c}	15.60±1.30^{a}	12.2±0.22^{b}
C17:0	0.08±0.04^{c}	0.18±0.00^{a}	0.10±0.06^{b}
C18:0	0.50±0.11^{c}	0.99±0.07^{a}	0.76±0.07^{b}
C20:0	0.14±0.10^{a}	0.17±0.01^{a}	0.15±0.01^{a}
C21:0	0.03±0.01^{a}	0.01±0.00^{a}	0.02±0.00^{a}
C22:0	0.07±0.01^{c}	0.18±0.01^{a}	0.14±0.00^{b}
C23:0	--	0.28±0.02^{a}	0.11±0.02^{b}
C15:1	0.02±0.00^{a}	--	--
C16:1	0.85±0.36^{a}	0.82±0.02^{a}	0.64±0.08^{b}
C17:1	--	0.03±0.01^{a}	0.04±0.00^{a}
C18:1 *n*9*t*	7.94±0.90^{a}	--	4.05±0.35^{b}
C18:1 *n*9*c*	0.91±0.13^{b}	1.08±0.09 ^{a}	1.06±0.00^{a}
C20:1 *n*9	4.46±0.41^{b}	7.58±0.84^{a}	7.54±0.36^{a}
C18:2 *n*6*t*	2.92±0.35^{a}	0.02±0.00^{c}	1.65±0.08^{b}
C18:2 *n*6*c*	14.30±0.90^{c}	23.61±2.01^{a}	20.71±0.32^{b}
C18:3 *n*3	22.80±1.30^{c}	43.14±2.50^{a}	32.70±0.72^{b}
C20:2 *n*6	25.86±1.76^{a}	0.37±0.02^{c}	9.37±0.61^{b}
C20:3 *n*3	0.07±0.00^{a}	0.07±0.00^{a}	0.05±0.00^{b}
C20:4 *n*6	0.08±0.00^{a}	0.06±0.00^{a}	0.07±0.00^{a}
C20:5 *n*3	0.11±0.01^{b}	0.18±0.01^{a}	0.13±0.01^{b}
∑SFA	14.41^{b}	18.81^{a}	15.18^{b}
∑MUFA	14.18^{a}	9.51^{c}	13.38^{b}
∑PUFA	66.14^{ab}	67.45^{a}	64.68^{b}
∑ *n*3	22.98^{c}	43.39^{a}	32.88^{b}
∑ *n*6	43.16^{a}	24.06^{c}	31.80^{b}
*n*3/*n*6	0.53^{c}	1.80^{a}	1.03^{b}
∑Trans	10.86^{a}	0.02^{c}	5.71^{b}

Mean± standard deviation (n=4). Different letters in the rows indicate significant difference (P<0.05). ∑SFA (total saturated fatty acids); ∑Trans (total transfatty acids); ∑MUFA (total monounsaturated fatty acids); ∑PUFA (total polyunsaturated fatty acids); DHA (docosahexaenoic acid).

PARRY et al. (2007) evaluate the fatty acid profile of cold-pressed parsley seed oil. The authors identified 92% of unsaturated fatty acids, most of which were oleic (C18:1 *n*-9) and linoleic acid (C18:2), followed by saturated acids: palmitic (C16:0) and stearic (C18:0). Their results for fatty acids profiles disagree with the data obtained in the present study.

The main dietary *n*-6 PUFAs are critical to many physiological functions, and their derivatives are involved in various molecular pathways on human health. The American Heart Association and many scientists advise consumption of at least 5 to 10% of energy as *n*-6 PUFA to improve heart health (CHEN et al., 2013). Many important aspects related to increased immunity, such as cytokine production, antibody formation, differentiation, cell proliferation, migration and antigen presentation, are regulated by the eicosanoids (LAYE et al., 2018; SIOEN et al., 2017).

Regarding the importance of fatty acids, there is evidence that the diets rich in α-linolenic acid, a plant derived omega-3 PUFA (polyunsaturated fatty acid), have been associated with decrease of incidence of several chronic diseases (ZHANG et al., 2013; JURADO-RUIZ et al., 2017; EGERT et al., 2018). The data obtained showed that the herbs evaluated have a positive health potential regarding PUFAs, as they presented high levels of *n*-6 in parsley and *n*-3 in chives. Overall, the presented results of *cheiro-verde* suggested that the combination of the two aromatic herbs is an option of having the essentials PUFAs concentrations in a mixture of both samples.

3.4 Bioactive Compounds

The **Table 5** shows the total levels determined for the bioactive compounds evaluated in the aromatic herbs.

Table 5 - Content of phenolics, flavonoids and total carotenoids, and chlorophyll a and b of parsley (*Petroselinum crispum*), chives (*Allium schoenoprasum* L.) and *cheiro-verde.*

	Parsley	**Chives**	***Cheiro-verde***
Total Phenolic Content (mg GAE/g)	8.34 ± 0.30^{a}	5.71 ± 0.16^{b}	6.09 ± 0.42^{b}
Total Flavonoids (mg (QE)/g)	8.89 ± 0.34^{b}	10.98 ± 0.51^{a}	10.94 ± 0.53^{a}
Total Carotenoids (µg/g)	205.95 ± 0.17^{a}	111.35 ± 0.16^{c}	189.65 ± 0.16^{b}
Chlorophyll *a* (µg/g)	23.59 ± 0.00^{a}	11.93 ± 0.00^{c}	17.76 ± 0.14^{b}
Chlorophyll *b* (µg/g)	5.20 ± 0.00^{c}	16.56 ± 0.00^{a}	10.87 ± 0.04^{b}

Data are expressed as means values ± standard deviation (n=9). Different letters in the same rows indicate significant difference (P<0.05). GAE- Gallic acid equivalent; QE- quercetin equivalents.

Total phenolic compounds content (TPC) of organic parsley, chives and *cheiro - verde* were investigated in order to contribute to the knowledge of antioxidant proprieties of the select botanical materials and their mixture. Data shows that TPC of the herbs varied from 5.71 ± 0.16 to 8.34 ± 0.30 mg GAE/g of sample (**Table 5**). The results showed a positive effect on the mixture of parsley and *chives-verde* presented great results compared to the other two culinary herbs.

The literature showed different results for TPC in parsley samples. ZHENG e WANG (2001) found lower values (1.05 mg GAE/g) than our study in parsley. On the other hand, LUTHRIA et al. (2006) studied the influence of experimental conditions of phenolic compounds extracted of parsley and reported higher levels (12.1 mg GAE/g) than the present work.

TRIFUNSCHI e ARDELEAN (2012) evaluated the contents of phenolic in *Petroselinum crispum* leaves in different extracts. The contents observed in ethanol extract (54.20 mg/g) were higher than chloroform and methanol extracts (15.20 and 35.60 mg/g), respectively. TANG et al. (2015) also tested different extraction systems, showing values ranging from 9.63 to 42.31 mg GAE/g. KARACA e VELIOGLU (2014) reported (23.9 mg GAE/g) in the methanolic extract.

PEREIRA e TAVANO (2014) studied the natural antioxidant potential of parsley in cooked beans and found 5.40 (mg GAE/g). SHEHATA et al. (2014) evaluated the effect of temperature and time of extraction on total polyphenol and observed (29.90, 32.50, 42.50, and 52 mg/100g fresh weigh), in cold water, and in hot water for 10, 30, and 60 min, respectively. VIOLETA NOUR et al. (2017) found 3.61 (mg GAE/g), higher levels than OPARA e CHOHAN (2014), ie 8.9 mg GAE/g, but lower than those presented by SHAN et al. (2005) ie 6.36 mg GAE/ g fw or ČÍŽ et al. (2010) 5.99 mg GAE/g in parsley.

Different results were reported by other studies of TPC of chives. ZHENG e WANG (2001) and ALEZANDRO et al. (2011) found lower levels of total phenolic (1.05 mg GAE/ g of fresh weigh and 3.05 g catechin equivalent/100 g (f.W.)), respectively, than that presented in this study. While DENG et al. (2013) found higher levels of TPC (7.38 mg GAE/g FW). On the other hand, MNAYER et al. (2014) reported similar (6.76 mg GAE/g) values of TPC in chives. VINÃ e CERIMELE (2009) observed 84.4 (mg catechin/100 g fresh tissue), and LENKOVA et al. (2016) observed 1591 (mg/kg) of TPCs.

The differences between the obtained results in this work from others researches could be explained by the different of assay systems used, especially due types of extraction methods, as well as the use of different parts of plants such as leaves, stems, bulbs, seeds. Also the phenolic compounds and antioxidant activities depend on the variety, location, conditions of growth of the plant and post harvesting processes.

Chives and *cheiro-verde* presented similar values of total flavonoids, but higher contents than parsley ($p<0.05$) (**Table 5**).

Different results were shown on others researchers regarding the contents of total flavonoids in parsley and chives. TRIFUNSCHI e ARDELEAN (2012) observed difference between the contents of flavonoids in parsley leaves ethanol extract (42.10) mg/g, which were higher than chloroform and methanol extracts (4.50 and 25.12mg/g), respectively.

CHANDRA et al. (2014) observed higher values 14.35 (mg quercetin acid equivalent (QE)/g DW) of total flavonoids in parsley than found in this study. PEREIRA e TAVANO (2014) exhibited 4.25 (mg CE/g) total flavonoids. SHEHATA et al. (2014) also evaluated the effect of temperature and time of extraction on total flavonoids in parsley and observed (163.30, 207, 276, and 276 mg/100g fresh weigh), in cold water, and in hot water for 10, 30, and 60 min, respectively. Hence, the authors concluded that parsley leaves have the potential to be suitable source of flavonoids. VIOLETA NOUR et al. (2017) presented high total flavonoid content (260.55 mg QE/100 g) in methanolic extract, value in good agreement with the high content of myricetin (151.03 mg /100 g) and quercetin (71.33 mg/100g) recorded in parsley.

In chives, CAO et al. (2010) evaluated the effect of season factors on flavonoid amounts in foods; therefore, they observed contents of total flavonoids (103.8 mg/kg), collected in spring. ALEZANDRO et al. (2011) evaluated flavonoids on 3 different brands of commercial spices for development of functional foods, and observed values ranging from 26 to 52.4(mg/100g f.w.) in chives.

This study showed great contents of total carotenoids determined for parsley, chives, and *cheiro-verde*, ranging from (111.35 ± 0.16 to 205.95 ± 0.17) μg/g sample (**Table 5**). *Cheiro-verde,* once again, behaved effectively, showing to have good results on the mixture of the two herbs.

Other authors evaluated the difference on the total carotenoid contents of parsley. PARRY et al. (2006) found 40.49 (μmol/kg) of total carotenoids, and KAMEL (2013) found 40.00 mg/kg carotenoids in parsley leaves blended in water in a study of the effect of microwave drying process on some bioactive compounds of *Petroselinum crispum.* KUŹMA et al. (2014) observed 31.28 (mg/100 g dry matter) total carotenoids in parsley. Whereas, according to LISIEWSKA e KMIECIK (1997) superior carotenoid contents were observed (54.3 mg/100 d.m.).

According to PARRY et al. (2006), *Petroselinum crispum* may serve as natural source of carotenoids due to the presence of a conjugated double bond system which acts neutralizing the radicals present in the medium, thus present excellent antioxidant properties. Carotenoids were shown to be associated with a low risk of several human chronic disorders including age-related macular degeneration and certain cancers, matching the wide use of this vegetal species in folk (popular) medicine (KARIMI et al., 2012; FARZEI et., 2013).

VINÃ e CERIMELE (2009) observed 16.8 (mg/100g of fresh tissue) of total carotenoid contents in a study of quality of chives during storage.

The sum of chlorophyll (*a* plus *b*) observed in the aromatic plants were very similar, ranging from 28.49 to 28.79 (μg/g), in parsley and chives, respectively. A content of chlorophyll (*a* and *b*) reached (0.185 mg/g dry matter) in parsley (KUŹMA et al., 2014). The value examined by LISIEWSKA e KMIECIK (1997) was very superior to the previous findings 3.93 mg/g d.m.VINÃ e CERIMELE (2009) reported different value 73 (mg/100g of fresh tissue) of total chlorophyll content for chives.Whereas, KMIECIK e LISIEWSKA (1999) reported a higher chlorophyll (*a* plus *b*) content in fresh chive leaves, which corresponded to 121 mg/100 g of fresh matter.

Chlorophyll pigments are very susceptible to many factors, including e.g. temperature, pH, oxygen, light or enzymes activity. During processing they are undergoing changes, the extent of which is determined by the character of plant material and processing conditions (MINGUEZ-MOSQUERA et al., 2002).

Carotenoids, for example, are excellent singlet oxygen abstractors, as well as ascorbic acid which is not a direct sequester of lipophilic radicals, has a synergistic effect when in the presence of tocopherols in the removal of peroxides radicals (CAROCHO e FERREIRA, 2013).

3.5 Identification of Phenolic Compounds of Aromatic Herbs

Compounds were characterized based on their mass spectra, using the precursor ion, fragment ions, and comparison of the fragmentation patterns with molecules described in the literature. The assigned identification of twenty four phenolic compounds in (80:20) methanol/water extract present in parsley, chives and Brazilian *cheiro-verde*, respectively; are given in **Table 6**, where the compounds are numbered according to their retention times in the total ion chromatograms (TICs).

Table 6- Identification of phenolic compounds presents in parsley, and chives.

Parsley				References
RT	**M-H (*m/z*)**	**Fragments (ms^2/ms^3)**	**Assigned identification**	
0.72	179	178.3 (100); 59.2 (70); 163.9 (60)	Caffeic acid	El Sayed et al., 2016
2.18	353	353.1(50); 101.1(100); 113 (80); 131 (50); 191.2(50); 220.7 (50)	5-Caffeoylquinic acid	Martini et al., 2017; Vallverdú-Queralt et al., 2014
2.49	301	301.4(50); 124.9 (100)	Hesperetin	Justesen et al., 1998 Vallverdú-Queralt et al., 2014
2.58	153	153.1 (100); 151.7 (85); 69.2 (70)	Protocatechuic acid	Martinii et al., 2017;
2.78	285	283.4 (100); 139.2 (80); 227.8 (80)	Luteolin	Stan et al., 2012 Vlase et al., 2013
2.90	299	298.8 (20); 137 (100)	*p*-hydroxycinnamic acid	Dias et al., 2014
3.17	289	200.5 (50); 231 (50); 281.8 (50)	(+) Epicatechin	Vallverdú-Queralt et al., 2014
3.39	593	593.2 (60); 299.1 (100)	Diosmetin-apiosylglucoside	Luthria 2008 Kaiser *et al.*, 2013 Sęczyk *et al.*, 2016
3.48	431	430.9 (90); 137.1 (100)	Apigenin -7-glucoside - comoin	Vallverdú-Queralt et al., 2014
3.61	475	161.2 (100); 283.5 (100); 149.2 (80)	Luteolin glucoronic acid	Dias et al., 2014
3.77	463	463.5 (100); 119 (100); 149 (70)	Quercitin-3-O-glucoside	Martinii et al., 2017; Vallverdú-Queralt et al., 2014 Zhang et al, 2015.
3.79	337	191.4 (100); 119.1 (70)	4-Coumaroylquinic acid	Martini et al., 2017
3.93	325	119.1 (100); 163.1 (100)	*p*-Coumaric acid 4-O-hexoside	Kaiser et al., 2013
4.20	609	607.4 (40); 315.3 (40)	Rutin	Vallverdú-Queralt et al., 2014
4.40	367	179.6 (100); 370.7 (80); 101.2 (70)	Feruloylquinic acid	Martini et al., 2017
4.87	607	606.7 (100); 269.1 (50); 565.2 (50)	Apigenin-apiosylglucoside-malonyl	Luthria 2008
5.30	163	119.1 (100)	*m*-hydroxycinnamic acid	El Sayed et al., 2016
5.68	269	269.2 (70); 117.3 (100); 160.7 (75)	Apigenin	Justesen et al., 1998 Stan et al., 2012
5.73	447	285.1 (100); 283.4 (90); 447.2 (80)	Kampferol-3-O glucoside	Vallverdú-Queralt et al., 2014
6.53	193	177.3 (100); 149 (80); 187 (80)	Ferulic acid	Vlase et al., 2013 El Sayed et al., 2016
Chives				
RT	**M-H (*m/z*)**	**Fragments (ms^2/ms^3)**	**Assigned identification**	
2.65	153	153.6 (100); 151.7 (85); 69.9 (90)	Protocatechuic acid	Martini et al., 2017;
3.50	431	436.9 (100); 119.3 (90); 118.3 (70)	Coumaric acid-glucoside	Zhang et al, 2015
3.77	609	285.1 (100); 446.4 (50); 607.6 (50)	Luteolin-diglucoside	El Sayed et al., 2016
4.06	289	289 (40); 267.3 (100); 266.5 (80)	Cathechin	Deng et al., 2013 Vallverdú-Queralt et al., 2014
4.33	609	607.8 (50); 606.6 (30); 444 (30)	Rutoside	Vlase et al., 2013
4.43	609	607 (50); 606. (40); 444 (30)	Kaempferol	Parvu et al., 2010; Vlase et al., 2013
4.67	447	447.4 (40); 284.2 (100); 255.1 (40)	Quercitin	Vlase et al., 2013)
5.64	285	285.2 (100); 285.4 (80); 284.6 (70)	Luteolin	Stan et al., 2012 Vlase et al., 2013
6.53	193	133.8 (100); 177.5 (90); 192.5 (60)	Ferulic acid	Vlase et al., 2013 El Sayed et al., 2016

RT- Retention time; $[M-H]^-$ Main ion, MS^2- secondary ions; Polyphenols identified by UPLC-ESI-MS.

Parsley presented higher number of phenolic compounds (20) compared to chives (9). According to the TIC (total ion chromatograph) chromatogram, the compounds with the highest intensities in parsley were: quercitin-3-O-glucoside, caffeic acid, hesperetin, luteolin, *p*-hydroxycinnamic acid and apigenin.

In agreement with our study, apigenin was found as major compound observed at high concentration in most researches with parsley. In addition, several studies have identified similar compounds present in parsley, as well as other bioactive phytochemicals, particularly flavonoids (apennine, malonyl-Apennine, luteolin, crisoeriol, cosmosiin, quercetin, kaempferol, *p*-coumaric acid, myricetin and isorhamnetin) (JUSTESEN et al., 1998; MATTILA, et al., 2000; FEJES et al., 2000; JUSTESEN e KNUTHSEN, 2001; GEBHARDT et al., 2005; YILDIZ et al., 2008; HUBER et al.,2009; CAO et al., 2010; PARVU et al. 2010; GADI et al., 2012; PÁPAY et al., 2012; STAN et al., 2012; FARZAEI et al., 2013).

CHAVES et al. (2011) also isolated and identified the following flavonoids: apigenin, apigenin-7-O-glucoside or cosmosiin, apigenin-7-O-apiosyl-(1→2)-O-glucoside or apiin, and coumarin 2'', 3''-dihydroxyfuranocoumarin or oxypeucedanin hydrate in aqueous parsey extract. On the other hand, researchers identified flavonoids, such as flavones (apigenin and luteolin), and flavonols (quercetin and kaempferol) (STAN et al., 2012); quercetin in methanolic parsley extracts (VERMA e TREHAN, 2013). This fact may be due to the difference of soil, climate, time of year, and post harvest, in addition to the use of difference solvents during extraction.

VIOLETA NOUR et al. (2017) also reported diverse phenolic compounds found in parsley such as: gallic acid, catechinhidrate, vanillic acid, chlorogenic acid, caffeic acid, siringic acid, epicatechin, coumaric acid, ferulic acid, sinapic acid, salicylic acid, rutin, ellagic acid, myricetin, *trans*-cinnamic acid, and quercetin.

The compounds identified and the main ion (*m/z*), in chives (**Table 7**) were the following, respectively: protocatechuic acid (153), coumaric acid-glucoside (431), cathechin (289), rutoside (609), kaempferol (609), quercetin (447), luteolin (285), and ferulic acid (193).

Others researches reported similar profile found in this study, for eg., coumaric acid, ferulic acid rutin and gallic acid (KUCEKOVA et al. 2011); catechin, and gallic acid (DENG et al., 2013); *p*-coumaric, ferrulic acid, sinapic acid, isoquercetrin, quercetol, kaempferol, and rutin were also identified in *A. schoenoprasum*, as well as glycosides of kaempferol and quercetol (PARVU et al., 2010; VLASE et al., 2013). TRICHOPOULOU et al. (2000) studied the presence of flavonoids (myricetin, quercetin, kaempferol, isorhamnetin, luteolin, and apigenin) in chives, which contain a rich mixture of polyphenolic antioxidants from the Mediterranean diet. The same was reported by ALEZANDRO et al. (2011) that presented besides luteolin, kaempferol, quercetin, and as well as hydroxycinnamic acids. They also observed the presence of apigenin in chives, compound predominantly found in parsley.

CAO et al. (2010) evaluated the effect of season factors on flavonoid and observed in chives, collected in spring, the presence of kaempferol, luteolin, quercetin, and isorhamnetin. MORAVČÍKOVÁ et al. (2012) showed that *Allium schoenoprasum* also contain gallic acid, coumaric acid, ferulic acid, and rutin. TIVERON et al. (2012) identified in ethanol extracts the following phenolic contents in chives: sinapic acid, *p*-coumaric acid, ferulic acid, caffeic acid, kaempferol, quercetin, and isovanillic acid. LÓPEZ-GARCÍAet al. (2013) found ferulic, coumaric, and gallic acids as major constituents in methanolic extracts of chives flowers. BERETTA et al. (2017) observed the presence of phenolic acid such as chorogenic, coumaric, ferulic, and caffeic acid,

respectively. Although chives exhibited the most potent antioxidant in their research, the phenolic profiles varied greatly, suggesting that individual phenolic compounds differ in antioxidant strength.

In general, aromatic plants are complex matrices regarding their content on phenolic compounds. Their composition are influenced by many factors, including soil, variations in cultivar, growing location, irrigation, agricultural practices and processing, and climatic conditions (CAO et al., 2010). Moreover, soil cultivation of crops may also result in year-to-year variability in the composition of phytochemicals and in total yield (BOURGAUD et al., 2001). Therefore, the structural identification of phenolic compounds can be affected by the chemical structure of the studied analytes, the selected methods of extraction, the composition/nature of the aromatic plant and storage conditions (COSTA et al., 2015).

In addition, BALASUNDRAM et al. (2005) proposed that the antioxidant capacity of phenolic compounds depends on their chemical structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings.

The *cheiro-verde* samples presented a mix of compounds related with both herbs. Some compounds (protocatechuic acid, luteolin, kaempferol, quercetin, and ferulic acid) were observed in all of the three studied samples. Ferulic acid presents great biological activities related to improvement of microcirculation, elimination of oxygen-free radicals, anti-inflamatory properpities and suppression of carcinogens (BASKARAN et al., 2010; LIN et al., 2010; KUMAR e PRUTHI, 2014; SGARBOSSA et al., 2015). In addition, quercetin was observed in all samples, it has a great potential chemopreventive activity and is a major bioflavonoid in the human diet (PANCHE, DIWAN e DIWAN, 2016).

As found in parsley, the *cheiro-verde* extract presented caffeic acid, a hydroxycinnamic acid, as well as the flavones such as luteolin and apigenin, which has been employed as natural antioxidant for inhibiting oxidation in fish lipids (MEDINA et al., 2012). Furthermore, flavanones, also called dihydroflavones, are related with diverse health benefits due their free-radical scavenging abilities (PANCHE, DIWAN e DIWAN, 2016). The health properties of these aromatic plants depend on the contents of bioactive compounds, mainly polyphenolics and substances with antioxidant effects (CHUN et al., 2005; BEATO et al., 2011).

This study identified important compound phenolics present in the aromatic herbs, parsley and chives, as observed in literature.

3.6 *In vitro* antioxidant of Chives, Parsley and *Cheiro-Verde*

The results of the antioxidant capacities of the studied aromatic herbs are shown on **Table 8**.

Table 7**.** Antioxidant capacity of parsley, chives, and *cheiro-verde.*

Antioxidant activity	**Parsley**	**Chives**	***Cheiro-verde***
DPPH (%)	54.67±0.63	51.59±0.52	54.92±0.48
ABTS (µmol TE/g)	8.29±0.25^{a}	5.21±0.33^{b}	5.12±0.11^{b}
FRAP (µmol TE/g)	8.32±0.16^{a}	6.33±0.00^{b}	6.69±0.00^{b}

Data are expressed as means values ± standard deviation (n=9). Different letters in the same rows indicate significant difference (P<0.05).

The DPPH assay is widely used to investigate the free radical scavenging activities of several natural compounds. The evaluation of DPPH of the herbs presented great values of radical scavenging activity, ranging from 51.59 ± 0.52 to 54.92 ± 0.48%, presenting no significant difference (P<0.05) among samples.

The results found in our study of DPPH scavenging effect in parsley were similar to that shown by ZHANG et al. (2006) (51%). On the other hand, very lower levels were observed in parsley by other authors; CHANDRA et al. (2014) reported 8.69% of DPPH scavenging activity and, SHEHATA et al. (2014) evaluated the effect of temperature and time of extraction on DPPH scavenging activity in parsley and observed (3.30, 2.79, 2.5 and 2.0 % free radical scavenging activity), in cold water, and in hot water for 10, and 30 min, respectively. JIA et al. (2012) observed the antioxidant capacity (37.8 % free radical-scavenging activity) in parsley and their effect on the oxidative stability of food during storage, suggesting that parsley can be used to suppress lipid oxidation.

For chives samples, similar findings were observed by GONÇALVES et al. (2015) that identified 55.2% of free radical-scavenging activity of DPPH, and LENKOVA et al. (2016) found superior values (76.57% DPPH) in chives. In contrast, MNAYER et al. (2014) observed much lower result (5.59) IC_{50} (concentration mg/mL for 50% inhibition. ALEZANDRO et al. (2011) evaluated dehydrated spices and observed 3.22 (mmoles Trolox equivalents/100g) of DPPH (scavenging ability). TIVERON et al. (2012) performed a study to observe the antioxidant capacity of vegetables commonly consumed in Brazil, observing 8.2 (µmol Trolox/g DW) DPPH in chives. VINÃ e CERIMELE (2009) observed 327.2 (µmol DDPH/100 g fresh tissue).

The use of different solvents to obtain the extracts, make it difficult to compare the results among studies. In addition, the values of the antioxidants activities are often given in different unities.

The results of the ABTS antioxidant evaluation presented significant differences among samples (P<0.05). Parsley showed the highest value for ABTS equivalent to 8.29 ± 0.25 µmol TE/g, while c*heiro-verde* and chives presented similar results.

An assay of free radical scavenging activity from herbs and spices commercialized in Brazil, MARIUTTI et al. (2008) obtained 4.97 and 6.2 TEAC (mM/g spice) in chives and parsley, respectively.

CHOHAN et al. (2008) determined the antioxidant capacity using the $ABTS^{\bullet+}$ radical cation assay, expressed in Trolox equivalent/g dry weight. The analyses of parsley samples showed 59 ± 5.7 µmol/g and increased to 172 ± 5.7 µmol/g in microwaved parsley soup. The authors suggested that this increase in the values may have occurred due to the liberation of polyphenolic compounds as a consequence of exposure to heat.

TIVERON et al. (2012) and DENG et al. (2013) observed higher values in chives (25.8 µmol Trolox/g DW and 15.71 TEAC µmol Trolox/g), respectively, than that presented in this study.

The results of FRAP antioxidant evaluation are expressed in µmol TE/g. Significant differences were identified amongst the samples. Parsley demonstrated a superior activity (8.32 ± 0.16) to chives and *cheiro-verde*.

No data of FRAP for species of chives in particular, *Allium schoenoprasum* L., was found in the literature expressing the same unit and standard to compare with the results of the presented study. However, RAMKISSOON et al. (2013) reported a value of 0.942 (mg ascorbic acid equivalent (AAE)/mL) in parsley; and TIVERON et al.

(2012) observed in chives high values (130.8 µmol Fe $^{2+}$/g DW) FRAP, and DENG et al. (2013) found 18.01 FRAP (µmol Fe (II)/g FW).

The use of bioactive compounds in different commercial sectors such as pharmaceutical, food and chemical industries indicates the need of the most appropriate and standard method to extract these active components from plant materials. Along with conventional methods, numerous new methods have been established but untill now no single method is regarded as standard for extracting the bioactive compounds from plants. The efficiencies of conventional and non-conventional extraction methods mostly depend on the critical input parameters; understanding the nature of plant matrix; chemistry of bioactive compounds and scientific expertise (AZMIR et al., 2013).

However, for a spice to act as health promoter does not depend only of the phytochemicals content, but also the way of preparation and the amount consumed. Similarly to what occurs with other plants, the composition and concentration of active ingredients of spices extracts can differ widely depending on the origin, species, variety, genetic control, the development stage during harvesting, stimuli provided through, for example, climate, exposure to microorganisms, insects and type of post-harvest processing.

All these features provide specific sensory properties in a particular way in every aromatic plant, it can determine in some species the concentration of a particular active ingredient, making it difficult to generalize the results of the composition of these types of materials. Also antioxidant activities depend on the variety, location, conditions of growth of the plant, post harvesting processes and extraction conditions (NACZK e SHAHIDI, 2004; LUTHRIA et al., 2006).

The results of antioxidant capacity of parsley, chives and *cheiro-verde* showed great values, comparing to the literature, in all the analyses. In this way, the bioactive compounds present in these culinary herbs, in addition of providing nutrients, are capable of adding flavors, as well as presented great potential as natural antioxidants that can be used as new sources in the food industry.

3.7 *In vivo* Antioxidant Activity

In vivo analyzes were performed using strains of *Saccharomyces cerevisiae* and hydrogen peroxide (H_2O_2), as oxidative agent. Hydrogen peroxide (H_2O_2) is the most abundant oxygen reactive species *in vivo* and is constantly produced as a co-product of aerobic metabolism (CHANAJ-KACZMAREK et al., 2015; JARDIM et al., 2017). In this way, *S. cerevisiae* strains incubated with extracts of parsley, chives and *cheiro-verde* were submitted to oxidative stress through the presence of hydrogen peroxide, in order to observe the antioxidant effect of the natural extracts.

Due to biochemical and molecular similarities with human cells, *S. cerevisiae* is considered a model organism in the studies and understandings of cellular responses to oxidative stress. In addition to being a simple and non-pathogenic microorganism, it has a short generation time under favorable conditions and does not require high costs for its growth and maintenance (CHANAJ-KACZMAREK et al., 2015; JARDIM et al., 2017).

The yeast cells were analyzed during the first exponential phase of growth, since in this period the cells do not yet have a fully developed intracellular defense mechanism, which are more sensitive to the oxidative environment. The toxicity tests

aim to verify the potential that a substance or extract has to cause the death of the cells, through possible irreparable damages in their cellular structures and metabolism. When comparing the effect of the extract with the action of a highly toxic agent (H_2O_2), the yeast cells were tolerant to exposure to the extracts, mainly in the time of 1 hour.

When comparing control cells with cells treated with the oxidative agent, the presence of H_2O_2 led to a reduction of 60 and 90% of survival compared to the non-stressed control.

In **Figure 1**, the results revealed that with 1 hour to exposure of the cells of chives and *cheiro-verde* extracts there was no toxicity, however there was low toxicity with the use of the parsley extract. After 3 hours there was an appearance of toxicity of chives and *cheiro- verde* extracts at 100 µg/mL, but the toxicity remained constant for the extracts of parsley. Compared with the action of a highly toxic agent (H_2O_2) on yeast cells, they were tolerant to exposure to the extracts, mainly in the time of 1 hour. From this, to evaluate the antioxidant potential of the extracts; it was used two concentrations (10 µg/mL and 100 µg/mL) incubated for 2 hours, possibly revealing a low or no toxicity to this cell type.

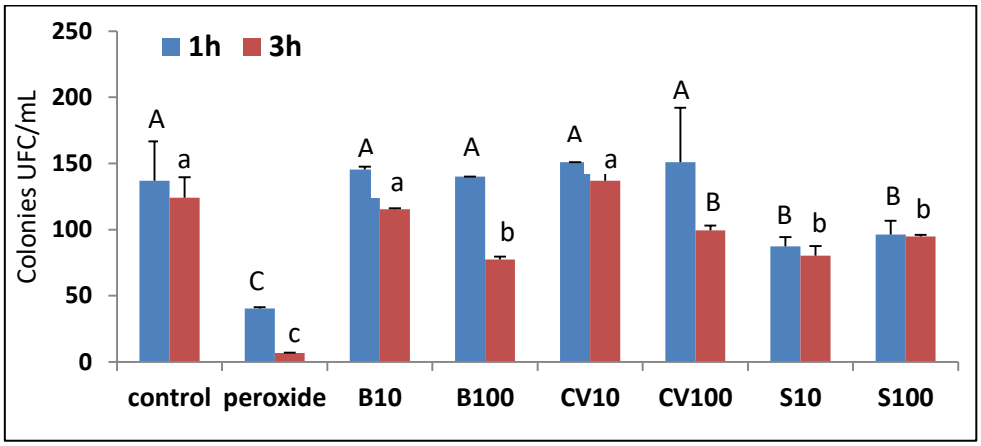

Figure 1. *S. cerevisiae* cells tolerance exposed to aqueous extracts of chives (B), *cheiro- verde* (CV) and parsley (S) at concentrations of **10µg/mL** and **100µg/mL** for **1h** and **3h**, respectively. Different lowercase letters differ by Tukey's test (p <0.05) and the different upper case letters differ by Tukey's test (p <0.05).

Once the toxicity test was carried out, the next step was to verify the protective action of the extracts in cells under oxidative stress with hydrogen peroxide. It is important to note, furthermore, that washing with distilled water removes the extract from the supernatant medium, thus allowing only the action of the compounds that have probably been absorbed by the yeast to be evaluated. **Figure 2** shows the percentage of cell survival for the different treatments.

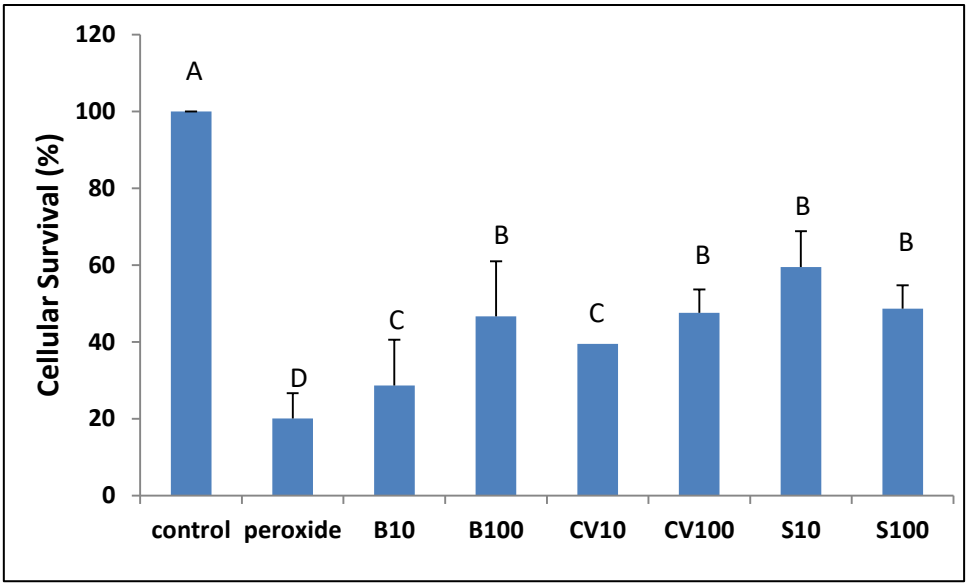

Figure 2. The antioxidant potential determined by the viability of cells under oxidative stress (H_2O_2 1.0 mM) preincubated with aqueous extracts of chives (B), *cheiro verde* (CV) and parsley (S) at concentrations of 10µg/mL and 100µg/mL for 2h.

According to the **Figure 2**, it is possible to observe that the cells were drastically affected by direct exposure to hydrogen peroxide, presenting a percentage of cellular survival of 20.03. However, the exposure of the pre-incubated cells with extracts of the aromatic herbs, increased cell viability compared to cells under oxidative stress. Even without reaching the level of the control experiment (cell suspension in phosphate buffer), there was antioxidant protection of extracts regardless of the concentration used, an increase in cellular survival was observed from 20.03 to 60% in parsley (S) at (100 µg/mL) being the highest value observed. Meanwhile, chives (B) at (10 µg/mL) presented the lowest protective effect. However, in general, all the extracts were efficient regarding the antioxidant action.

Tests of antioxidant capacity *in vivo* with yeast *Saccharomyces cerevisiae*, using extracts of parsley and chives are rare. No work was found in the literature. However, different natural extracts and natural compounds have been evaluated for toxicity and antioxidant capacity in experiments involving *S. cerevisiae* (SÁ et al., 2013; BAYLIAK et al., 2014; OPREA et al., 2014; FRASSINETTI et al., 2015; STINCO et al., 2015; LINGUA et al., 2016; JARDIM et al., 2017).

The effect of propolis alcoholic extract was evaluated on *S. cerevisiae* cells, which did not present yeast toxicity in concentrations between 25 and 100 µg/mL (SÁ et al., 2013). Furthermore, the pretreatment with the 25 Ug/mL natural extract prior to exposure to 2 mM H_20_2 for 1 h was able to increase the percentage of cell survival from approximately 8 to 27%.

STINCO et al. (2015) used *S. cerevisiae* as cellular model to evaluate the antioxidant effect of hydrophilic fractions of orange juice. Cells were exposed to oxidative stress by the presence of H_20_2 (0.75 mM) for 1h, so that pretreatment with juice resulted in a 23-38% increase in cell survival.

Grape extracts (*Vitis vinifera* L.) were also evaluated by exposing the yeast cells in question to H_20_2 (2 mM) for 1 h, leading to a 14 to 20% increase in cell survival when compared to treatment in which there was no prior exposure to extracts (LINGUA et al., 2016).

The oxidative stress generated by the presence of hydrogen peroxide induces the occurrence of oxidative damages in biomolecules such as lipids, proteins and DNA, which lead to the development of innumerable degenerative pathologies (PIMENTEL et al., 2012; LINGUA et al., 2016; JARDIM et al., 2017). On the other hand, phenolic compounds and other bioactive components present in natural extracts such as the aromatic herbs studied can act to protect against the action of oxidants.

GRANATO et al. (2018) emphasizes that, although, *in vitro* tests are useful in the determination of bioactive compounds, however, based solely in colorimetric methods are not sufficient for antioxidant capacity measurements. Thus, it is required *in vivo* models for bioactive purposes, or, at least, methods that employ distinct mechanisms of action (i.e., single electron transfer, transition metal chelating ability, and hydrogen atom transfer). In this regard, better understanding and application of *in vitro* and *in vivo* screening methods should help design of future research studies on 'bioactive compounds'.

4 CONCLUSIONS

The characterization and quantification of the chemical components present in parsley, chives and *cheiro-verde* revealed a high content of bioactive compounds due to phytosterols composition, fatty acids profile, phenolic compounds and the antioxidant properties. The antioxidant capacity revealed that the parsley presented better effects than chives and *cheiro-verde* regarding antioxidant potential. The bioactive compounds present in the aromatic herbs extracts evaluated, presented also antioxidant action in yeasts, among the three samples evaluated, parsley presented the highest effect reducing oxidative stress caused by hydrogen peroxide *S. cerevisiae* cells. Thus, the protective effect of the extracts *in vivo* converges with the results obtained in the *in vitro* tests, proving that, although parsley presented the best antioxidant results in the *in vitro* and *in vivo* analyzes, however, the use of the mixture (*cheiro-verde*) also demonstrated a synergistic effect with good results in the antioxidant activities, corroborating to indicate their use in combating radicals formed in food processing.

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CAPÍTULO III

IMPACT OF AIR FRYING ON CHOLESTEROL AND FATTY ACIDS OXIDATION IN SARDINES: PROTECTIVE EFFECTS OF AROMATIC HERBS

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ABSTRACT

The high temperatures used to fry fish may induce thermo-oxidation of cholesterol, forming cholesterol oxidation products (COPs). COPs have been associated to coronary heart diseases, atherosclerosis and other chronic diseases. Air fryers are an alternative thermal process technology to fry foods without oil, and are considered a healthier cooking method. This study is the first to evaluate the formation of COPs and the degradation of polyunsaturated fatty acids (PUFAs) in air-fried sardine fillets. Furthermore, we evaluated the effect of fresh herbs added as natural antioxidants to sardines subjected to air frying. Parsley (*Petroselinum crispum*), chives (*Allium schoenoprasum* L) and a mixture of both herbs (*cheiro-verde*) were added in quantities of 0, 2 and 4%. Air frying significantly decreased the content of essential PUFAs, and increased the levels of COPs from 61.2 μg/g (raw) to 283 μg/g ($p < 0.05$) in the control samples. However, the use of herbs as natural antioxidants proved to be effective reducing such levels of COPs in most samples. The addition of 4% of *cheiro-verde* in air fried sardines presented the best protective effect against lipid oxidation.

Keywords: Air frying. Cholesterol oxides. Natural antioxidants

RESUMO

As altas temperaturas utilizadas para fritar peixes podem induzir a termo-oxidação do colesterol, formando produtos de oxidação de colesterol (POCs). POCs têm sido associados a doenças coronarianas, aterosclerose e outras doenças crônicas. Fritadeiras a ar são uma tecnologia alternativa de processo térmico para fritar alimentos sem óleo e são consideradas um método de cozimento mais saudável. Este estudo é o primeiro a avaliar a formação de COPs e a degradação de ácidos graxos poliinsaturados (PUFAs) em filés de sardinha ao ar. Além disso, avaliamos o efeito de ervas frescas adicionadas como antioxidantes naturais às sardinhas submetidas à fritura com ar. Salsa (*Petroselinum crispum*), cebolinha (*Allium schoenoprasum* L) e uma mistura de ambas as ervas (cheiro-verde) foram adicionadas em quantidades de 0, 2 e 4%. Air frying diminuiu significativamente o teor de PUFAs essenciais, e aumentou os níveis de POCs de 61,2 μg/g (cru) para 283 μg/g ($p<0,05$) nas amostras de controle. No entanto, o uso de ervas como antioxidantes naturais provou ser eficaz na redução de tais níveis de POCs na maioria das amostras. A adição de 4% de cheiro verde nas sardinhas processadas por *air-fryer* apresentou o melhor efeito protetor contra a oxidação lipídica.

Palavras-chave: Air-fryer. Sardinhas. Óxidos de colesterol. Antioxidantes naturais.

1 INTRODUTION

Frying, which is extensively employed in homes, restaurants and industry, consists in dehydrating food immersed in hot oil. However, during frying, numerous reactions with oxygen, due to the high temperatures and the release of water, take place and consequently a succession of physical and chemical changes occur in the food products (TERUEL et al., 2015).

Air frying circulates hot air uniformly around the food instead of immersing it in hot oil and also reduces the fat contents in the fried food. This process consists of the direct contact between the food and a dispersion of oil droplets in hot air in the air-fryer (ANDRÉS et al., 2013; SANSANO et al., 2015). Thus, the product is progressively dehydrated in a fixed frying temperature of 180 °C, while the crisp characteristic appears on the fried products (HEREDIA et al., 2014) with minimum variations in food quality. Air fryers are available on the market as an alternative to fry foods without oil, and are considered a convenient method of cooking; however, there are no studies regarding degradation of lipids by this thermal process.

Brazilian sardines (*Sardinella brasiliensis*) are an important source of fish commercialized in Brazil and are largely consumed due to their excellent nutritional quality and low cost. They contain proteins of high biological value, in addition to their lipid composition, especially *n*-3 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic (EPA 20:5 *n*-3) and docosahexaenoic acids (DHA 22:6 *n*-3). The regular consumption of n-3 PUFAs is associated with health benefits (CASAS et al., 2014; FAO, 2016; ZOCK et al., 2016).

However, some authors have demonstrated that sardines also contain high levels of cholesterol (SALDANHA et al., 2008; SCHERR et al., 2015). These two types of compounds, n-3 PUFAs and cholesterol, are not chemically stable and this instability is influenced by their chemical structure, the presence of oxygen, light, metal ions and the processing techniques (heat) used, among other factors (DANTAS et al., 2015; BARRIUSO et al., 2017).

The temperature used and the time required to prepare fish and fish products are perhaps the main factors that contribute to the degradation of cholesterol, forming cholesterol oxidation products (COPs) (DANTAS et al., 2015; LEAL-CASTAÑEDA et al., 2016). These substances are structurally similar to cholesterol and are normally present in small quantities formed by endogenous metabolic processes. However, the exogenous cholesterol oxides formed during the processing of food can be potentially harmful to health (DANTAS et al., 2015) and these COPs are associated with the development of cardiovascular and neurodegenerative diseases, besides inflammation, atherosclerosis, mutagenesis, carcinogenesis and cell death (DORIA et al., 2016; BARRIUSO et al., 2017).

In recent years, polyphenolic compounds present in herbs and spices have been used as an alternative to replace synthetic antioxidants (CAROCHO e FERREIRA 2013; FIGUEIRÊDO et al., 2015). They have also attracted attention as they can prevent lipid oxidation in fish and fish products (SANCHO et al., 2011; MAQSOOD et al., 2013; FRANK et al., 2014; MAQSOOD et al.,2014; FIGUEIRÊDO et al., 2015; MELEIRO et al., 2016; TARVAINEN et al., 2016; VAISALI et al., 2016). Aromatic herbs are used worldwide by various culinary cultures and the antioxidant effects of parsley (*Petroselinum crispum*) and chives (*Allium schoenoprasum* L.) have been evaluated and proven (ZHANG et al., 2006; JIA et al., 2012; SĘCZYK et al., 2015; TANG et al., 2015; SĘCZYK et al., 2016). The aromatic herbs, parsley and chives are

good sources of essential nutrients such as vitamins and minerals as well as antioxidant compounds. The phenolic compounds are the principal bioactive phytochemicals identified in these herbs in several studies, particularly flavonoids (apigenin, apennine, malonyl-apennine, luteolin, crisoeriol, cosmosiin, quercetin, kaempferol, p-coumaric acid, myricetin and isorhamnetin (PARVU et al., 2010; STAN et al., 2012; FARZAEI et al., 2013; VLASE et al., 2013); gallic acid, coumaric acid, ferulic acid and rutin in *Allium schoenoprasum* (KUCEKOVA et al., 2011). The combination of parsley and chives, also known as *cheiro-verde*, is an important fresh condiment commonly used in the Brazilian cuisine. It is usually finely chopped and then added to dishes such as soups, meats, fish and sauces for salads and others. However, there are no data in the literature about the use of this herbal mixture as a natural antioxidant.

Considering the high degradation of cholesterol and PUFAs when heating fish, with the consequent formation of COPs, it is necessary to re-evaluate the processing techniques for these foods, which are mainly affected by thermo-oxidation. The aim of the present study was to evaluate the impact of air frying on lipid degradation in sardine samples and the effects of adding aromatic herbs such as parsley, chives and the mixture of both herbs (*cheiro-verde*) as natural antioxidants to protect the cholesterol and PUFAs from thermal degradation.

2 MATERIALS AND METHODS

2.1 Samples and Sample Preparation

2.1.1 Sardines

Ten kilograms of fresh Brazilian sardines (*S. brasiliensis*) were obtained from Angra dos Reis, Rio de Janeiro, Brazil (Latitude: 23 ° 00 '24 "S and Longitude: 44 ° 19' 05" W), in March 2016, and transported in a refrigerated truck to UFRRJ at Seropédica, Rio de Janeiro, Brazil. After evisceration, the sardines were immediately washed, homogenized and separated into 8 lots. The sardines had an average weight of 88.34 ± 5.04 g and measured between 19 and 21 cm in length. One of them was analyzed on the same day the fish were acquired, corresponding to fresh (raw) sardines. The other samples were packed in polyethylene film and stored at 5 °C in a domestic refrigerator, until preparation the following day.

2.1.2 Herbs

Fresh parsley (*Petroselinum crispum*) and chives (*Allium schoenoprasum* L.) were obtained from the Experimental Agricultural Station of EMBRAPA (Seropédica, Rio de Janeiro-Brazil). The herbs were selected, washed and chopped (3 x 3 mm approximately). The moisture content of the fresh herbs was (94.70 % ± 0.45; 87.50 % ± 0.80 and 89.00 % ± 0.30) for chives, parsley and *cheiro-verde*, respectively. *Cheiro-verde* samples consisted of the combination of the mixture of the two herbs: 50 % (w/w) of parsley and 50 % (w/w) of chives. Two levels of parsley, chives and *cheiro-verde* were employed, selected according to the concentration used in the local cuisine (2 and 4% (w/w)). The total phenolic was determined with Folin-Ciocalteu reagent, according to SINGLETON e ROSSI (1965). The obtained results of these herbs were 8.34 ± 0.30, 5.71 ± 0.1 and 6.09 ± 0.42 mg GAE/g in parsley, chives and *cheiro-verde*, respectively.

2.1.3 Preparation of sardine samples

Each treatment consisted of 12 fillets of raw sardines; the average weight of the sardine fillets was 61.66 ± 3.53 g and the dimensions 12 x 7 ± 0.5 cm. The control treatment was samples air-fried without the addition of herbs; the other treatments were sardines air-fried with the addition of 2 and 4% of parsley, chives and *cheiro-verde*, respectively. The percentage of chopped herbs (g/100 grams) was calculated according to the weight of the lot. After this, the herbs were added homogeneously on both sides of the sardine fillets. An electric air-fryer was used for the thermal processing, (Air Fryer-Mondial, model: 4470-04, China); time and temperature were used according to the manufacturer's recommendations, simulating domestic use, with a nominal power of 1500 W. The samples were air-fried for 10 minutes; no oil was added in the air fryer chamber, with fixed heating temperature of 180°C, according to the specifications of the equipment. The internal temperature (75 ± 0.5 °C) was monitored using a digital calibrated thermometer (Traceable Long Stem, VWR, Friendswood, TX, USA) and the surface temperature of the processed sardines was approximately 78 ± 0.7 °C monitored by a laser thermometer (LASERGRIP 1080 infrared thermometer, ETEKCITY - Anaheim, CA, EUA). After air frying, the samples were ground and homogenized in a multi-processor (Walita, Brazil) to obtain a homogeneous mass. Convenient aliquots were taken for the analyses, which were carried out in triplicate.

2.2 Standards, Reagents and solvents

20α-hydroxycholesterol (20α-OH), 22S-hydroxycholestrol (22S-OH), 22R-hydroxycholesterol (22R-OH), 25-hydroxycholesterol (25-OH), 7-ketocholesterol (7-keto), 7β-hydroxycholesterol (7β-OH), 5,6α-epoxycholesterol (5,6α-Ep) and 5,6β-epoxycholesterol (5,6β-Ep) were acquired from Sigma Chemical Company (St. Luis, USA). Cholesterol (Chol), 25R-hydroxycholesterol (25R-OH) and 7α-hydroxycholesterol (7α-OH) were obtained from Steraloids (Wilton, NH, USA). Undecanoic methyl ester was purchased from Sigma (St. Louis, MO, USA) and the standard mixtures of fatty acids were purchased from Supelco TM 37 (FAME Mix 18919, Bellefonte, PA, USA). The purities of the standards ranged from 95 to 99%. HPLC grade *n*-hexane and 2-propanol were obtained from Vetec (Sigma, São Paulo, Brazil).

2.3 Analytical Procedure

2.3.1 Moisture and total lipids

The moisture was determined according to AOAC (2002). The lipids were extracted and determined according to BLIGH e DYER (1959).

2.3.2 Fatty acid composition

For the analysis of fatty acids was used an aliquot of 25 mg of lipids, that was submitted to saponification and methylation using BF3 in methanol (JOSEPH e ACKMAN, 1992). A GC instrument (GC 2010) from Shimadzu (Tokio, Japan) equipped with a split injector (1:50), fused silica CP-SIL 88 capillary column 100m x 0.25mm i.d., 0.2µm film thickness (Chrompack, Middelburg, The Netherlands), flame ionization detector and workstation was used to identify the different compounds. The chromatographic conditions were: initial temperature, 100 °C (5 minutes) followed by an increase of 5 °C/minute up to 160 °C (zero minutes), then 8 °C /minute up to 230 °C

(12 minutes); injector and detector temperatures were 250 °C and 280 °C, respectively. The equipment used hydrogen as the carrier gas at a flow rate of 1 mL/minute and nitrogen as the make-up gas at 30 mL/minute. Retention times of FAME standards were used to identify the chromatographic peaks of the samples, and the quantification was done by internal standardization, using undecanoic methyl ester as the internal standard. Factors for the conversion of fatty acid methyl esters to their corresponding triglycerides were used (CARPENTER et al., 1993).

2.3.3 Cholesterol and cholesterol oxides

2.3.3.1 HPLC-PDA-RI

Cholesterol and cholesterol oxides were obtained by direct saponification performed with continuous agitation (2g of the samples, 4mL of a 50 % aqueous solution of KOH and 6mL of ethanol) at 24 °C for 22h in the dark and the non-saponifiable matter was extracted 4 times (10 mL of hexane for each extraction, totaling 40 mL) (SALDANHA et al., 2006).

HPLC analysis was performed with a Waters device (Milford, MA, EUA), equipped with PDA/RID detectors, rheodyne injector with a 20 µl loop, a tertiary solvent delivery system (Waters 600), oven heated column at 32 °C and software (Empower 2). The column used was a CN Hyperchrome 250 mm × 4.3mm × 5.0 µm (Phenomenex, Colorado, USA). The mobile-phase was n-hexane: 2-propanol (97:3, v/v) at a flow rate of 1mL/min and an analysis time of 30 min. Quantification was done by external standardization, with concentrations ranging from 5.0 to 150.0 µg/mL for the oxides and from 0.1 to 2.0 mg/mL for the cholesterol, respectively. The epimeric 5,6-epoxides were quantified using a refractive index detector, because they do not absorb at UV wavelengths. The cholesterol and other oxides were quantified using PDA detector.

2.3.3.2 UPLC-APCI-MS

In order to confirm the cholesterol oxide structures, chromatographic analyses were performed on a UPLC Acquity chromatographer coupled to a TQD Acquity Mass Spectrometer (Micromass-Waters Manchester, England), with an APCI source configuration, with a triple quadrupole mass spectromter. A CN Hyperchrome 250 mm ×4.3mm×5.0 µm column (Phenomenex, Colorado, USA) was used. Isocratic mobile phase containing hexane: *n*-propanol (97:3), at a flow of flow 1 mL/min, oven temperature 32 °C, and 10 µL of the samples were injected into the UPLC. Ionization was performed in the APCI positive ion mode and the optimization conditions were adapted from (SALDANHA et al., 2006). The ionization parameters were: full scan m/z 100-500, capillary voltage 1500V; corona current 20 µA; drying gas temperature 350 °C; cone voltage 20V; and vaporizer temperature 150 °C.

2.4 Statistical Analysis

The analysis of variance (ANOVA) was employed on dependent variables and when significant differences were observed, the *Tukey* multiple range tests were applied. Multivariate analysis was used to describe the data from the quantification of cholesterol, fatty acids and COPs. Principal component analysis (PCA) was used to group similar samples and was performed on the standardized data to make sure all the

elements had the same influence over the results. Hierarchical Clustering on Principle Components (HCPC) was done to group samples with similar characteristics using the function HPCP in the FactoMineR. All analyses were performed using the software R version 3.2.4 (R Foundation for Statistical Computing, Vienna, Austria) and the FactoMineR package version 1.32.

3 RESULTS AND DISCUSSION

3.1 Effects of Air Frying on Moisture, Total Lipid and Cholesterol

The moisture, lipid and cholesterol contents in raw, hot air-fried control and sardines with addition of parsley, chives and *cheiro-verde* are shown in **Table 1**. The total lipids (g/100 g) and cholesterol (mg/100 g) were calculated on the dry basis.

Table 1 – Moisture (g/100 g), total lipids (g/100 g dry basis) and cholesterol (mg/100 g dry basis) levels in raw, air-fried sardines (control) and air-fried sardines with chives, parsley and *cheiro-verde* at two levels (2 and 4 %).

	Raw	**Control**	**Chives 2 %**	**Chives 4 %**	**Parsley 2 %**	**Parsley 4 %**	***Cheiro-verde* 2 %**	***Cheiro-verde* 4 %**
Moisture	75.2 ^{A} (0.3)	55.2 ^{D} (0.3)	58.0 $^{C;b}$ (0.5)	59.5 $^{B;a}$ (0.4)	53.7 $^{E;c}$ (0.3)	57.2 $^{C;b}$ (0.1)	54.8 $^{DE;c}$ (0.1)	57.7 $^{C;b}$ (0.2)
Total lipid	16.5 ^{A} (0.2)	13.4^{C} (0.7)	11.6 $^{D;c}$ (0.6)	14.6 $^{B;a}$ (0.6)	13.1 $^{C;b}$ (0.2)	11.4 $^{D;c}$ (0.4)	9.42 $^{E;d}$ (0.4)	8.4 $^{E;d}$ (0.2)
Cholesterol	237.2^{A} (3.7)	136.4 ^{E} (0.4)	150.6 $^{D;cd}$ (3.3)	208.2$^{B;a}$ (4.0)	188.9$^{C;b}$ (6.2)	158.3$^{D;c}$ (4.3)	138.0 $^{E;d}$ (4.8)	203.6 $^{B;a}$ (5.6)

Values represent means ± standard deviation (n=3). Capital letters in the same row indicate significant differences by the *Tukey* test ($p < 0.05$) between raw, control and sardines fried with chives, parsley and *cheiro-verde*, respectively. Values followed by different lowercase letters in the same row differ from each other, according to the *Tukey* test ($p < 0.05$) in a factorial design (herbs and concentrations factors).

The moisture content in raw sardines was 75.2 ± 0.3 g/100g. In the air-fried sardines, the moisture ranged from 55.2 ± 0.3 to 59.5 ± 0.4 g/100g. Air frying caused a significant loss of water in the control sardine samples, decreasing 20% in comparison to the initial levels. The lipid levels in raw sardines were 16.5 ± 0.2 g/100 g; while the control sardine samples presented 13.4 ± 0.7 g/100 g of lipids and the air-fried samples with the addition of herbs ranged from 8.4 to 14.6 g/100 g. The lipid contents decrease significantly after air fried processing, and the losses varying from 15.6 to 49%. The heating of sardine fillets implies peripheral dehydration and decrease of lipids by dripping (GARCIA-ARIAS et al., 2003; SALDANHA et al., 2008).

The cholesterol levels ranged from 237.2 ± 3.7 to 136.4 ± 0.4 mg/100 g in the raw and control samples, lower values than the obtained by Saldanha et al., 2008 with 342 ± 2.7 to 219.0 ± 1.6 mg/100 g in raw and grilled Brazilian sardines, respectively. Air frying affected the cholesterol contents, producing a significant loss of this compound, approximately 34.9% in control samples compared to the raw sardines. Other authors have found similar effects in microwave, roasted, fried and grilled fishes (CANDELA et al., 1997; ECHARTE et al., 2001; OZOGUL et al., 2015). The losses of cholesterol contents in heated sardines could be attributed to oxidative process (SALDANHA et al., 2008).

Among air-fried sardines with the herbs, the cholesterol levels varied from 138.0 ± 4.8 to 208.2 ± 4.0 mg/100 g. The addition of herbs showed a protective effect on the

air-fried samples, however, sardines with 4% chives presented the highest protective effect (34.8%), followed by 4% *cheiro-verde* (31.9%). Thus, the use of herbs at the levels applied in the present study protected cholesterol from thermal degradation, and the phenolic compounds present in the plants are probably responsible for this effect.

3.2 Effects of Air Frying on Fatty Acids

The fatty acid compositions in sardines expressed as g/100 g of oil are presented in **Table 2.**

Table 2 – Fatty acid composition (g/100 g of oil) of raw, hot air-fried (control) and hot-air fried sardines with the addition of chives, parsley and *cheiro-verde* at two levels (2 and 4%).

Fatty acids	Raw	Control	Chives 2 %	Chives 4 %	Parsley 2 %	Parsley 4 %	*Cheiro-verde* 2 %	*Cheiro-verde* 4 %
C12:0	0.22 ^{BC}	0.15 ^{CD}	0.23 $^{ABC;a}$	0.24 $^{AB;a}$	0.32 $^{A;a}$	0.11 $^{D;b}$	0.07 $^{D;b}$	0.11 $^{D;b}$
C14:0	16.05 ^{A}	15.14 ^{A}	14.44 $^{A;a}$	14.37 $^{A;a}$	15.83$^{A;a}$	10.30 $^{B;b}$	7.84 $^{C;c}$	10.35 $^{B;b}$
C15:0	2.23 ^{BC}	2.58 ^{ABC}	2.94 $^{A;a}$	2.71 $^{AB;ab}$	2.64 $^{AB;abc}$	2.21 $^{BC;bc}$	2.00 $^{C;c}$	2.25 $^{BC;c}$
C16:0	37.49^{D}	42.53 ^{BC}	44.63 $^{AB;ab}$	45.92 $^{A;a}$	44.55 $^{AB;ab}$	39.78 $^{CD;c}$	42.31 $^{BC;bc}$	40.82 $^{C;c}$
C17:0	1.35 ^{B}	1.95 ^{A}	1.94 $^{A;a}$	1.79 $^{AB;a}$	1.88 $^{A;a}$	1.97 $^{A;a}$	1.74 $^{AB;a}$	1.96 $^{A;a}$
C18:0	5.49 ^{C}	10.60 ^{A}	7.20 $^{BC;b}$	7.04 $^{BC;b}$	7.35 $^{BC;b}$	8.75 $^{AB;b}$	8.81 $^{AB;a}$	9.21 $^{AB;a}$
C20:0	0.41 ^{AB}	--	0.48 $^{A;a}$	0.44 $^{A;a}$	0.44 $^{AB;ab}$	0.37 $^{B;b}$	--	0.09 $^{C;c}$
C21:0	1.11 ^{A}	0.18 ^{C}	0.50 $^{B;a}$	0.43 $^{B;a}$	0.51 $^{B;a}$	0.17 $^{C;b}$	0.16 $^{C;b}$	0.19 $^{C;b}$
C22:0	0.17 ^{B}	--	0.23 $^{A;a}$	0.02 $^{B;b}$	0.20 $^{AB;ab}$	0.20 $^{AB;ab}$	--	--
C23:0	--	0.01 ^{B}	--	0.02 $^{B;b}$	0.02 ^{b}	0.18 $^{B;b}$	0.95 $^{A;a}$	0.20 $^{B;b}$
C14:1	0.66 ^{A}	0.03 ^{D}	0.26 $^{B;a}$	0.26 $^{B;ab}$	0.26 $^{B;ab}$	0.15 $^{C;b}$	0.03 $^{D;c}$	0.15 $^{C;b}$
C15:1	0.25 ^{A}	0.23 ^{A}	0.25 $^{A;a}$	0.27 $^{A;a}$	0.25 $^{A;a}$	0.18 $^{AB;ab}$	0.12 $^{B;b}$	0.19 $^{AB;ab}$
C16:1	7.03 ^{AB}	7.43 ^{A}	6.14 $^{CD;b}$	6.57 $^{BC;a}$	5.76 $^{DE;bc}$	5.25 $^{EF;d}$	4.96 $^{F;d}$	5.40 $^{EF;d}$
C17:1	0.48 ^{A}	0.37 ^{BC}	0.42 $^{AB;a}$	0.40 $^{ABC;ab}$	0.37 $^{BC;ab}$	0.35 $^{BC;ab}$	0.32 $^{C;b}$	0.39 $^{BC;ab}$
C18:1 *n*9*t*	--	0.22 ^{B}	--	0.28 $^{A;a}$	0.17 $^{B;b}$	0.17 $^{B;b}$	0.09 $^{C;c}$	0.19 $^{B;b}$
C18:1 *n*9*c*	6.35 ^{C}	9.90 ^{A}	7.09 $^{C;c}$	8.74 $^{B;b}$	7.01$^{C;c}$	8.46 $^{B;b}$	9.15 $^{AB;a}$	8.59 $^{B;b}$
C20:1 *n*9	0.19 ^{A}	0.06 ^{B}	0.07 $^{B;b}$	0.06 $^{B;b}$	0.07 $^{B;b}$	0.12 $^{AB;a}$	0.13 $^{AB;a}$	0.11 $^{AB;a}$
C22:1 *n*9	--	0.04 ^{BC}	0.05 $^{ABC;abc}$	0.03 $^{CD;c}$	0.04 $^{C;bc}$	0.09 $^{A;a}$	0.08 $^{A;a}$	0.08 $^{AB;ab}$
C24:1 *n*9	0.57 ^{B}	1.28 ^{A}	0.41 $^{BC;b}$	0.19 $^{C;c}$	0.43 $^{BC;b}$	1.32 $^{A;a}$	1.25 $^{A;a}$	1.41 $^{A;a}$
C18:2 *n*6*t*	0.34 ^{C}	0.59 ^{A}	0.33 $^{C;b}$	0.32 $^{C;b}$	0.29 $^{C;b}$	0.46 $^{B;a}$	0.42 $^{B;a}$	0.45 $^{B;a}$
C18:2 *n*6*c*	2.22 ^{AB}	1.48 ^{C}	2.36 $^{AB;a}$	1.45 $^{C;b}$	1.90 $^{BC;ab}$	2.62 $^{A;a}$	2.40 $^{AB;a}$	2.56 $^{AB;a}$
C18:3 *n*6	--	--	--	--	--	0.58 $^{B;b}$	0.84 $^{A;a}$	0.81 $^{A;a}$
C18:3 *n*3	2.19 ^{A}	0.79 ^{C}	1.51 $^{B;b}$	1.28 $^{BC;b}$	1.45 $^{B;b}$	2.56 $^{A;a}$	2.29 $^{A;a}$	2.45 $^{A;a}$
C20:3 *n*6	--	--	--	--	--	0.36 $^{B;b}$	0.46 $^{AB;ab}$	0.48 $^{A;a}$
C20:3 *n*3	0.94 ^{A}	0.53 ^{B}	0.34 $^{C;a}$	0.27 $^{C;a}$	0.40 $^{BC;a}$	0.37 $^{BC;a}$	0.35 $^{C;a}$	0.36 $^{BC;a}$
C20:4 *n*6	0.26 ^{A}	0.24 ^{AB}	0.23 $^{AB;a}$	0.12 $^{ABC;ab}$	0.14 $^{ABC;ab}$	0.06 $^{C;b}$	0.14 $^{ABC;ab}$	0.10 $^{BC;ab}$
C20:5 *n*3	3.11^{A}	0.76^{E}	1.14 $^{DE;d}$	1.55$^{CD;cd}$	1.18$^{DE;d}$	3.04$^{A;a}$	1.92 $^{C;c}$	2.49 $^{B;b}$
C22:6 *n*3	8.11^{A}	1.14^{E}	1.77 $^{DE;d}$	3.98 $^{C;c}$	2.67 $^{D;d}$	7.55 $^{A;a}$	5.36 $^{B;b}$	5.90 $^{B;b}$
∑SFA	64.52 ^{B}	73.15^{A}	72.59 $^{A;a}$	72.98 $^{A;a}$	73.84 $^{A;a}$	64.05 $^{B;b}$	63.89 $^{B;b}$	65.19 $^{B;b}$
∑MUFA	15.53 ^{CD}	19.56^{A}	14.69 $^{DE;b}$	16.80 $^{B;a}$	14.36 $^{E;b}$	16.09 $^{BC;a}$	16.13 $^{BC;a}$	16.51 $^{B;a}$
∑PUFA	17.17 ^{A}	5.53^{E}	7.68 $^{D;d}$	8.97 $^{D;c}$	8.03 $^{D;cd}$	17.60 $^{A;a}$	14.18$^{C;b}$	15.60 $^{B;b}$
∑*n*3	14.35^{A}	3.22^{F}	4.76 $^{E;f}$	7.08$^{D;d}$	5.70 $^{E;e}$	13.52 $^{A;a}$	9.92 $^{C;b}$	11.20 $^{B;b}$
∑*n*6	2.82^{AB}	2.31^{C}	2.92 $^{BC;b}$	1.89 $^{C;b}$	2.33 $^{C;b}$	4.08 $^{A;a}$	4.26 $^{A;a}$	4.40 $^{A;a}$
∑Trans	0.34 ^{D}	0.81^{A}	0.33 ^{E}	0.60$^{BC;ab}$	0.46 $^{D;c}$	0.63 $^{B;a}$	0.51 $^{CD;bc}$	0.64 $^{A;a}$

Values are means (n = 6). Different capital letters in the same row indicate significant differences among raw, control and sardines with added chives, parsley and *cheiro-verde* by the Tukey test ($p < 0.05$). Different lowercase letters in the same row differ according to the Tukey test ($p < 0.05$) to a factorial design (herbs and concentrations factors).

The main fatty acids found in sardines were palmitic (C16:0), myristic (C14:0), stearic (C18:0), docosahexaenoic DHA (C22:6 *n*3) and palmitoleic (C16:1 *n*7). The air fryer caused an increase of the total saturated (SFA) and monounsaturated fatty acids (MUFA) contents in the thermal processed samples, between 11.8 and 20.6%, respectively. Similar results were reported by (LEAL-CASTAÑEDA et al., 2017) in fish oil after microwave heating; (GLADYSHEV et al., 2006) in boiled and roasted humpback salmon and (GARCIA-ARIAS et al., 2003) in boiled and grilled sardines.

Air frying in sardines caused a significant decrease of PUFAs, approximately 70.2% in control sardines, with losses of essential fatty acids; there was a higher degradation of the *n*-3 series (80.7%), mainly DHA 85%. These results are in agreement with those reported by other authors in sardines after heating (GARCÍA-ARIAS et al., 2003; SALDANHA et al., 2008) and in other fish samples: hake (SALDANHA e BRAGAGNOLO, 2007); in salmon, mackerel, sardine and tuna, using different cooking methods (microwaving, boiling, and grilling) (MOUSSA et al., 2014). Other authors also reported the influence of thermal processing (boiling, frying, and microwave) on fatty acid contents in kutum roach (*Rutilus frisii kutum*) samples (HOSSEINI et al., 2014).

The amount of PUFA highly decreased after the heating treatment as a likely consequence of the development of oxidative reactions. PUFAs are very susceptible to oxidation and are easily incorporated into the chain mechanism of lipid peroxidation (HSIEH e KINSELLA, 1989; SALDANHA e BRAGAGNOLO, 2008), and tend to decrease with elevated temperatures. During the air fryer processing, sardines are subjected to heating and oxygen present in the chamber, and these two factors can accelerate the oxidative deterioration the PUFAs in sardines. Other studies observed a remarkable decrease in the PUFA contents in sardine samples after heating (CANDELA et al., 1997; GARCIA-ARIAS et al., 2003; SALDANHA et al., 2008). On the other hand, the addition of herbs to the air-fried sardines showed a protective effect against fatty acid degradation. The sardines with 4% parsley obtained the highest protective effect of PUFAs in relation to the control samples (216%). The effectiveness of herbal protection decreased in the following order: 4% parsley > 4% *cheiro-verde* > 2% *cheiro-verde* > 4% chives > 2% parsley > 2% chives. This fact is probably due the presence of the phenolic compounds in these plants, acting as natural antioxidants.

The aromatic herbs, parsley and chives, are good sources of bioactive compounds (PARVU et al., 2010; FARZAEI et al., 2013; VLASE et al., 2013). The flavonoids are the most dominant compound present in parsley (PÁPAY et al., 2012), and they are known to act as antioxidants. Diverse studies showed a potential role in the extracts of phenolic compounds derived from these herbs as natural antioxidants (ZHANG et al., 2006; WONG e KITTS 2006; FARZAEI et al., 2013; VLASE et al., 2013; PARVU et al., 2014).

In addition, results from others researches indicate a potential role in the extracts of phenolic compounds derived from parsley as natural antioxidants suggesting that this culinary herb can be used as an alternative to synthetic antioxidants, especially in fat-based foods, whereas parsley can be used to suppress lipid oxidation (GUERRA e LAJOLO 2005; JIA et al., 2012).

The antioxidant activity of chives is also related to the presence of a variety of compounds sulfur-containing and their precursors (VLASE et al., 2013), in addition to

other bioactive compounds such as polyphenols (STAJNE e VARGA, 2003; ŠTAJNER et al., 2006; BEZMATERNYKH et al., 2014).

The diverse results from other studies indicate a potential role in the extracts of phenolic compounds derived from these herbs as natural antioxidants (ZHANG et al., 2006; WONG e KITTS 2006; FARZAEI et al., 2013; VLASSE et al., 2013; PARVU et al., 2014).

Thermal degradation in lipids is a complex process and it is well established that during and after high temperature processing, chemical and biochemical reactions can occur, resulting in the loss of quality and damage to PUFAs, which could lead to primary and secondary lipid oxidation products (BORAN et al., 2006; SELMI e SADOCK 2007; SALDANHA et al., 2008). Thus, this important loss of PUFAs in the control samples, showed the high impact of air frying on sardines. In contrast, the addition of herbs to most of the treatments protected these fatty acids from degradation.

3.3 Effects of Air Frying on COPs

In the present study, the main oxides determined in raw sardines were: 5,6α-EP, 25-OH, 7-keto, 7β-OH and 7α-OH. The presence of oxidized cholesterol in the non-heated marine products suggests that this may be characteristic of the metabolism of the fish (OSADA et al., 1993). After thermal processing, the COPs found in the control samples and the sardines with herbs were: 5,6α-EP, 5,6β-EP, 20α-OH, 22R-OH, 22S-OH, 25-OH, 25R-OH, 7-keto, 7β-OH and 7α-OH (**Table 3**).

Table 3 – Cholesterol oxides (µg/g dry basis) of raw, air-fried sardines without herbs (control) and with the addition of chives, parsley and *cheiro-verde* at two levels (2 and 4%).

Cholesterol Oxides	Raw	Control	Chives 2%	Chives 4%	Parsley 2%	Parsley 4%	"*Cheiro-verde*" 2%	"*Cheiro-verde*" 4%
5,6α	21.6 ^{D} (0.9)	68.8 ^{A} (0.6)	43.6 $^{B;a}$ (0.1)	-	22.4 $^{D;c}$ (0.7)	22.4 $^{C;b}$ (1.0)	12.0 $^{E;d}$ (2.0)	-
5,6 β	--	63.4 ^{A} (1.2)	30.8 $^{B;a}$ (3.4)	13.0 $^{D;c}$ (0.0)	-	-	7.6 $^{E;d}$ (0.9)	24.0 $^{C;b}$ (3.1)
20 α	--	3.8 ^{D} (0.2)	7.9 $^{B;b}$ (0.9)	9.3 $^{B;b}$ (1.0)	11.2 $^{A;a}$ (0.5)	5.8 $^{C;c}$ (0.0)	1.5 $^{E;d}$ (0.0)	0.2 $^{EF;d}$ (0.0)
22R	7.2 ^{C} (0.3)	1.1 ^{E} (0.0)	10.4 $^{B;b}$ (1.5)	2.1 $^{DE;d}$ (0.2)	12.5 $^{A;a}$ (0.5)	6.1 $^{C;c}$ (0.1)	6.4 $^{C;c}$ (0.4)	3.3 $^{D;d}$ (0.0)
22 S	--	26.2 ^{B} (3.6)	50.3 $^{A;a}$ (2.1)	1.8 $^{F;e}$ (0.2)	6.3 $^{E;d}$ (0.6)	12.9 $^{C;b}$ (1.5)	10.8 $^{D;c}$ (0.5)	20.0 $^{C;b}$ (1.0)
25 OH	8.3 ^{C} (0.5)	23.8 ^{A} (0.4)	5.8 $^{D;c}$ (0.2)	0.8 $^{E;d}$ (0.0)	8.0 $^{C;b}$ (0.5)	8.4 $^{C;b}$ (0.5)	0.7 $^{E;d}$ (0.1)	13.9 $^{B;a}$ (0.3)
25 R	--	28.5 ^{B} (0.1)	28.4 $^{B;b}$ (1.2)	21.6 $^{C;c}$ (1.3)	36.1 $^{A;a}$ (3.1)	11.6 $^{D;d}$ (0.7)	34.0 $^{A;ab}$ (0.9)	12.3 $^{D;d}$ (0.7)
7-keto	7.1 ^{E} (1.0)	64.9 ^{A} (0.6)	56.2 $^{B;a}$ (2.9)	20.7 $^{D;c}$ (2.8)	39.3 $^{C;b}$ (1.2)	3.3 $^{EF;d}$ (0.4)	1.6 $^{F;d}$ (0.1)	4.4 $^{EF;d}$ (0.4)
7β-OH	8.3 ^{A} (0.0)	0.8 ^{EF} (0.0)	1.4 $^{D;c}$ (0.2)	2.6 $^{B;a}$ (0.3)	1.7 $^{C;b}$ (0.0)	1.0 $^{E;d}$ (0.1)	0.7 $^{F;e}$ (0.0)	1.1 $^{E;d}$ (0.0)
7α-OH	8.7 ^{A} (0.1)	1.6 ^{B} (0.1)	1.1 $^{C;a}$ (0.1)	0.7 $^{DE;bc}$ (0.2)	0.5 $^{DE;cd}$ (0.0)	0.3 $^{F;e}$ (0.0)	0.7 $^{D;b}$ (0.0)	0.4 $^{EF;d}$ (0.0)
Total	**61.2** ^{E} **(2.8)**	**282.9** ^{A} **(6.8)**	**235.9** $^{B;a}$ **(12.6)**	**72.6** $^{DE;c}$ **(6.0)**	**138.0** $^{C;b}$ **(7.1)**	**71.8** $^{D;c}$ **(4.3)**	**76.0** $^{D;c}$ **(4.9)**	**79.6** $^{D;c}$ **(5.5)**

Values represent means ± standard deviation in triplicates. Different capital letters in the same row indicate significant differences among all treatments, by the *Tukey* test ($p < 0.05$). Different lowercase letters in the same row indicate differences, according to the *Tukey* test ($p < 0.05$) to a factorial design (herbs and concentrations factors).

Air frying resulted in an increase (78.3%) of the total COPs, from 61.2 to 282.9 µg/g in raw and control samples, respectively. Despite the convenience of the heat processing using an air-fryer without the use of oil, the data obtained in this study demonstrates the high impact on air-fried sardines (control treatment), forming high levels of cholesterol oxidation products. As described in the literature the influence of cholesterol oxides on human health deserves some emphasis, because the exogenous cholesterol oxides formed during processing of foods can be potential harmful to health, affecting the cellular metabolism, changing the membrane composition and proprieties, being more cytotoxic than cholesterol and are associated with the development of cardiovascular and neurodegenerative diseases, among others, which can cause deleterious effects such as inflammation, atherosclerosis, mutagenic and carcinogenic, and cell death (THANAN et al., 2014; LAPARRA et al., 2015; BARRIUSO et al., 2016; DORIA et al., 2016; KULIG et al., 2016).

The amount of COPs formed in products depends on a number of parameters, such as the method of preparation and thermal treatment. There are no studies on the formation of cholesterol oxides in fish treated thermally using air fryer. However, the

increase of COPs after heating has been reported in other studies of fish and fish products in different types of thermal processes such as boiling, steaming and baking (HONG et al., 1996); pan-frying with oil (ECHARTE et al., 2001); frying (AL-SAGHIR et al., 2004); microwave, frying, grilling and roasted (ASTIASARAN et al., 2007); grilling (SALDANHA e BRAGAGNOLO, 2007; SALDANHA e BRAGAGNOLO, 2008); baked and fried (DEAN et al., 2009) and baking in electric or steam-convection ovens (FREITAS et al., 2015).

Large levels of COPs are formed when the food is subjected to direct heat (MORGAN e ARMSTRONG, 1992). Heating is a well-known lipid oxidation inducer, since high temperatures produce large amounts of free radicals due to the acceleration of propagation reactions and to the decomposition of lipid hydroperoxides (OTAEGUI-ARRAZOLA et al., 2010), accelerating the oxidative process.

In the cholesterol thermo-oxidation process, the prevailing compounds are those derived from C-7 carbon oxidation, although epoxidation occurs with the formation of the 5,6 epoxides (CHIEN et al., 1998). The 5,6α- and 5, 6β- epoxycholesterols were identified as products of cholesterol oxidation by air (GUARDIOLA et al., 1996). The processing parameters of sardines in air fryer, with higher temperature (180 ^{o}C) and air flow into the chamber provided the ideal conditions for the high levels of the 5,6-epoxides formation in processed sardine samples. Thus, the high levels of epoxides could be explained by the epoxidation of cholesterol molecules, due to the air frying of the sardine fillets. The increase of 7-Keto could possibly be due to dehydration of 7α- and 7β-hydroperoxides and/or dehydrogenation of 7α- and 7β-hydroxycholesterols.

Although thermal treatment by air frying significantly increased the levels of COPs in control samples, the addition of herbs minimized the cholesterol degradation process, inhibiting the formation of high levels of cholesterol oxides in most samples with herbs. The sardines with 4% parsley presented the highest protective effect (74.6 %) compared to control samples. In these samples, a decrease of 5,6α-EP, 5,6β-EP and 7-keto was also observed, probably due the bioactive compounds present in these aromatic herbs, acting as natural antioxidants, and inhibiting the free radical chain reaction and consequent epoxidation of cholesterol. The most effective antioxidants are those that act by disrupting the free radical chain reactions. The effects shown in the studied herbs could be probably attributed to the particular chemical structures present in polyphenols; the aromatic ring feature and highly conjugated system with multiple hydroxyl groups make these compounds good electron or hydrogen atom donors, neutralizing free radical and other reactive oxygen (ZHANG e TSAO, 2016). In contrast, sardines with 2% chives showed the highest level of COPs (235.9 µg/g), which was the lowest protective effect (16.6%) among the percentages and herbs tested in this work. The phenolic compounds present in parsley and chives have been shown to act as natural antioxidants in different research by different authors (JIA et al., 2012; PEREIRA e TAVANO 2014; PARVU et al., 2014; TANG et al., 2015; SĘCZYK et al., 2016).

As determined in the present study, TARVAINEN et al. (2016) reported similar findings using natural antioxidants to reduce cholesterol oxidation in baked salmon treated with rosemary and oregano extracts. Furthermore, VAISALI et al. (2016) showed that phenolic compounds (quercetin, rutin and caffeic acid) were effective in preventing the oxidation of sardine oil.

Even though the consumption of raw fish has grown in recent years (KAWAI et al., 2012), thermal processing is still one of the most commonly used forms of preparing fish. Moreover, despite the convenience of the heat processing using an air-fryer

without the use of oil, the data obtained in this study, demonstrates the high impact on air-fried sardines (control treatment), forming high levels of cholesterol oxidation products, which contrasts with the use of aromatic herbs that proved to be effective in most samples to inhibit such high levels of COPs formation. Due to the health hazards of synthetic additives, natural antioxidants are under intensive investigation to be used as safe alternatives to synthetic compounds. Thus, the use of plant bioactive compounds is being evaluated as potentially effective additives to prevent lipid oxidation in fish and fish products.

3.4 Correlation and Principal Component Analysis (PCA)

The PCA plot clearly showed the high cholesterol, EPA and DHA in sardines while these values decrease after air frying with concomitant increase of the COPs content in control samples (**Figure 1b**). The results showed that 7-Keto presented a strong correlation with total COPs and showed a negative correlation with EPA, DHA, n-3 and PUFA adequately represented by PC1 and PC2 (**Figure 1a).**

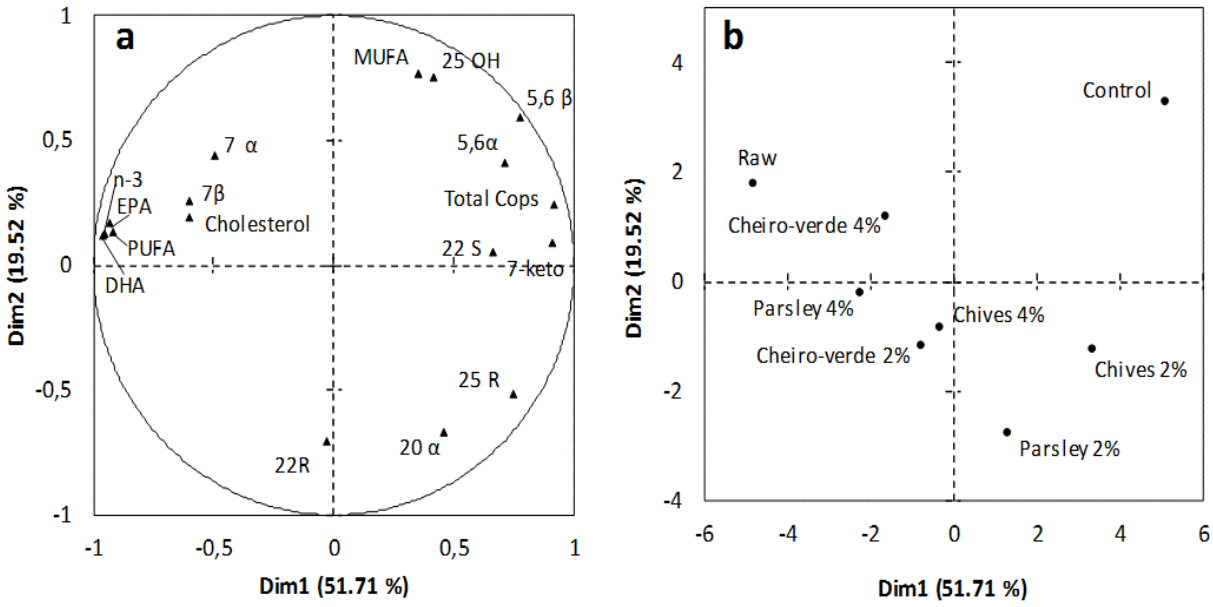

Figure 1. Principal components analysis (PCA) plots as a function of cholesterol oxides, cholesterol and fatty acids. (a) PCA loading plot for response variables (b) score plot for treatments of raw and air-fried sardines without herbs (control) and with addition of chives, parsley and *cheiro-verde* at two levels (2 and 4%).

The cut-off point of the HPCP suggested four groups (**Figure 2**). The raw and control samples formed two groups (Cluster 1 and 4, respectively) with one member in each group, as both treatments presented different results. On the other hand, the treatment of sardines with 4% parsley, 4% chives, 2% *cheiro-verde* and 4% *cheiro-verde* formed Cluster 2; while 2% parsley and 2% chives formed the Cluster 3 **(Figure 2a and 2b).**

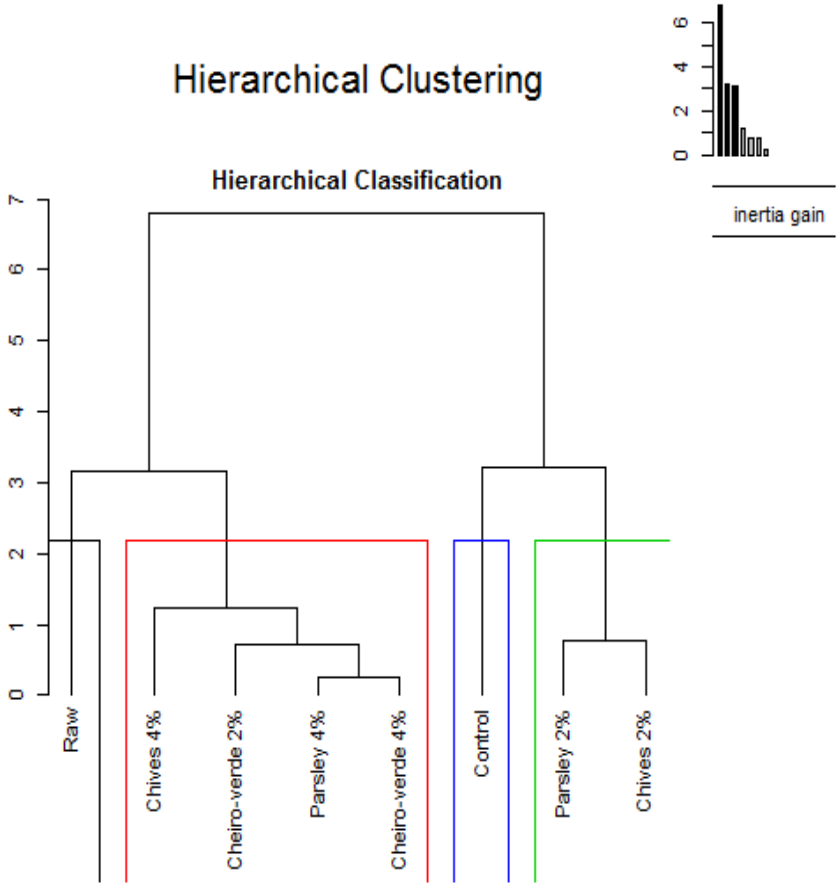

Figure 2. Hierarchical clustering on principal components of cholesterol oxides, cholesterol and fatty acids. The cut-off point of clusters was based on the inertia gain criterion.

Overall, the PCA analysis indicates that the addition of herbs protected the sardines during air frying, especially, the sample with 4% *cheiro-verde.* Thus, suggesting a probable synergistic effect between the antioxidant compounds present in both herbs.

4 CONCLUSIONS

The data obtained in this study showed that air frying presented a great impact on the lipid quality of sardines. In spite of the convenience of the air fryer, the results showed a significant decrease in PUFAs contents and high levels of COPs formation in the control treatment. On the other hand, the use of the aromatic herbs, parsley (*Petroselinum crispum*), chives (*Allium schoenoprasum* L) and their mixture (*cheiro-verde*) proved to be effective for most samples. The addition of 4 % *cheiro-verde* to the sardine samples presented the best protective effect on the lipid oxidation for air frying.

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Author Contributions

FS. Ferreira undertook most of the experimental work presented in this paper, T. Saldanha designed, supervised and organized the study, LM. Keller compiled the data, Davy W. H. Chávez did the statistical analysis, Alexandra C.H. F. Sawaya was responsible for the mass spectrometer analyses, Geni R. Sampaio and Elizabeth A. F. S. Torres supervised and organized the study. FS. Ferreira and T. Saldanha predominantly interpreted the results and drafted the manuscript with help from the other authors.

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CAPÍTULO IV

EFFECT OF PHENOLIC COMPOUNDS PRESENTS IN PARSLEY, CHIVES AND BRAZILIAN *CHEIRO-VERDE* ON THE INHIBITION OF CHOLESTEROL OXIDATION IN GRILLED SARDINES

Em preparação para a Food Chemistry

ABSTRACT

Nowadays, dietary oxidized lipids and health consequences are an important field of research. Therefore, the demand for natural antioxidants has increased the researchers' attention towards the discovery of natural sources. Thus, the culinary herbs parsley (*Petroselinum crispum*), chives (*Allium schoenoprasum* L) and their mixture (*cheiro-verde)* were selected to investigate their inhibitory effects on thermal oxidation against cholesterol oxides formation and changes in fatty acid composition of sardines (*Sardinella brasiliensis*) during grilling. The thermal treatment caused losses of cholesterol (39%), and polyunsaturated fatty acids (PUFAs) of grilled sardines changed from 32.2 in raw to 18.9 (g/100g of oil) in control samples. However, the addition of the aromatic herbs presented protective effect (84 and 80%) of PUFA and cholesterol (89 and 87%) contents in sardines added with 4% parsley and *cheiro-verde*, respectively. Similar results were observed with significant effect on inhibition of high levels of COPs formation (56 and 52%) in sardines added with 4 % parsley and *cheiro-verde*, respectively. The principal component analysis (PCA) indicates that the addition of the herbs (4% parsley and 4% *cheiro-verde*), were the most effective against lipid oxidation in sardines during grilling.

Keywords: Cholesterol oxidation products (COPs). Polyunsaturated fatty acids (PUFAs). Natural antioxidants. Parsley (*Petroselinum crispum*). Chives (*Allium schoenoprasum* L).

RESUMO

Atualmente, lipídios oxidados obtidos a partir da dieta e as consequências para a saúde tem sido um importante campo de pesquisa. Portanto, a demanda por antioxidantes naturais aumentou a atenção dos pesquisadores para a descoberta de novas fontes naturais. Assim, as ervas culinárias como a salsa (*Petroselinum crispum*), cebolinha (*Allium schoenoprasum* L) e a combinação das duas plantas, conhecida como (*cheiro-verde*) foram selecionadas para investigar seus efeitos inibitórios na oxidação térmica contra a formação de óxidos de colesterol e alterações na composição de ácidos graxos de sardinhas (*Sardinella brasiliensis*) grelhadas. O tratamento térmico causou perdas nos teores de colesterol (39%) e ácidos graxos poliinsaturados (PUFAs) nas sardinhas grelhadas, variando de 32,2 (crua) para 18,9 (g/100g de óleo) em amostras controle. Em contrapartida, a adição das ervas aromáticas apresentou alto efeito protetor (84 e 80%) dos teores de AGPI e colesterol (89 e87%) nas sardinhas acrescidas de 4% de salsa e cheiro verde, respectivamente. Resultados semelhantes foram observados com efeito significativo sobre a inibição de altos níveis de formação de POCs (56 e 52%) em sardinha adicionada com salsa a 4% e cheiro-verde, respectivamente. A análise de componentes principais indicou que a adição das ervas (4% de salsa e 4% cheiro-verde) foram mais efetivas contra a oxidação lipídica das sardinhas grelhadas.

Palavras-chave: Produtos de oxidação do colesterol (POCs). Ácidos graxos poliinsaturados (AGPIs). Antioxidantes naturais. Salsa (*Petroselinum crispum*). Cebolinha (*Allium schoenoprasum* L).

1 INTRODUCTION

The role of dietary fat in human health has been intensely debated and subject to research over the last few decades due its great importance regarding health and the emergence of diseases. Recently, studies have investigated the ingestion of oxidized lipids to the better understanding the effect in human health (MOUNT et al., 2015; BINDER et al., 2016; GARGIULO et al., 2017).

Lipid oxidation in foods can occur through auto-oxidation, heat, light and enzymatic oxidation pathways. Further, of the lipid classes in food those that contain polyunsaturated fatty acids (PUFAs) and cholesterol are more vulnerable to oxidation. Among the different forms of lipid oxidation in foods, especially in fish, the use of thermal processing with high temperatures is the most common (HUR, PARK e JOO, 2007).

Over the past decades, fish and fish products rich in n-3 polyunsaturated fatty acids (PUFAs) have received increasing attention due their beneficial effects on human health, reducing the risk of chronic diseases (OWEN et al., 2016) as well as protecting against neuronal disorders (DEBBABI et al., 2017). These benefits are attributed to the biological properties, mainly of eicosapentaenoic (EPA) and docosahexaenoic fatty acids (FAO, 2016).

On the other hand, high levels of cholesterol are also present in some fish, especially in sardines (SALDANHA e BRAGAGNOLO, 2008; SCHERR et al., 2015, FERREIRA et al., 2017). Considering the time and temperature required for fish preparation, this could be the main factors that may contribute to the degradation cholesterol to form cholesterol oxidation products (COPS) (DANTAS et al., 2015; LEAL-CASTAÑEDA, 2017).

COPs absorbed by the diet are more reactive and their effects are involved in various human health problems, such as the induction of proinflammatory, proapoptotic, and profibrogenic effects (HUR et al., 2014; KHATIB et al., 2014) leading to a dysfunction of the endothelial cells, and even to the fibrotic degeneration of the arterial wall (POLI et al., 2009). Moreover, COPs have been associated in the development of numerous chronic diseases such as atherosclerosis, neurodegenerative diseases, mutagenic and carcinogenic effects (MARWARHA et al., 2017; OLIVIER et al., 2017).

Thus, the incorporation of natural antioxidants in food during heating has attracted great attention for the prevention of lipid oxidation in fishes (MAQSOOD et al., 2010; SANCHO et al., 2011; MEDINA et al. 2012; CAROCHO e FERREIRA, 2013; MAQSOOD et al., 2014; FIGUEIRÊDO et al., 2015; VAISALI et al., 2016; TARVAINEN et al., 2016; XU et al., 2016; FERREIRA et al., 2017). Therefore, several culinary herbs are known to have beneficial effects for human health, which are attributed to the predominant phenolic compounds in these plant materials (VALLVERDÚ-QUERALT et al., 2014). Recently, has been growing awareness of the importance of the use of bioactive compounds widely distributed in plants as natural antioxidants in food matrices (PANCHE, DIWAN e CHANDRA, 2016; NILE et al., 2018; DE OLIVEIRA et al., 2018).

Parsley (*Petroselinum crispum*) is an important aromatic herb originated from the Mediterranean culture, and also has been utilized around the world in food as well in

medicine (FARZAEI et al., 2013). Chives (*Allium schoenoprasum* L) also known as green-onions are worldwide used as culinary herb in a variety of products due to their flavor, easy growth and long storage time (MNAYER et al., 2014). Several phytochemicals compounds were identified in parsley and chives and their effects as antioxidant was already successfully tested in foods (JIA, et al., 2012; SĘCZYK et al., 2015; PEREIRA e TAVANO, 2014; SĘCZYK et al., 2016; TANG et al., 2015).

Although the contribution of the use of the culinary herbs such as parsley, chives and the mixture of both herbs (*cheiro-verde*), have been successful tested in air-fried sardines as natural antioxidants inhibiting lipid oxidation process (FERREIRA et al., 2017), however, are necessary a determination and quantification of their phenolic profile and antioxidant capacities.

In this context, the aim of this study was to identify and quantify the main phenolic compounds present in parsley, chives and *cheiro-verde* and evaluate the protective effect of these phytochemicals against cholesterol and fatty acids degradation in grilled sardines.

2. MATERIALS AND METHODS

2.1 Reagents

Undecanoic methyl ester was from Sigma (St. Louis, MO, USA) and fatty acids standard mixtures were purchased from SupelcoTM 37 (FAME Mix 18919, Bellefonte, PA, USA).Cholesterol and other standards, including 20*α*-hydroxycholesterol (20 α-OH), 22S-hydroxycholesterol (22S-OH), 22R-hydroxycholesterol (22R-OH), 25-hydroxycholesterol (25-OH), 7-ketocholesterol (7-keto), 7*β*-hydroxycholesterol (7 β-OH), 5,6*α*-epoxycholesterol (5,6 *α*-Ep) and 5,6 *β*-epoxycholesterol (5,6 *β*-Ep) were from Sigma Chemical Company (St. Luis, USA). 25R-hydroxycholesterol (25R-OH) and 7*α*-hydroxycholesterol (7*α*-OH) were obtained from Steraloids (Wilton, NH, USA). The purities of the standards ranged from 95 to 99%. HPLC grade *n*-hexane and 2-propanol were obtained from Vetec (Sigma, São Paulo, Brazil).

2.2. Samples

2.2.1 Plant material – aromatic herbs

The organic parsley (*Petroselinum crispum*) (2 Kg), and chives (*Allium schoenoprasum* L.) (2 Kg) were donated from the Experimental Agricultural Station of EMBRAPA - Agrobiologia (Seropédica, Rio de Janeiro-Brazil). The voucher sample of *Petroselinum crispum* (RBR 37741) and *Allium schoenoprasum* L. (RBR 37740) were deposited at the Rural Federal University of Rio de Janeiro (UFRRJ) herbarium. The *cheiro-verde* samples consisted in the combination of both herbs, mixture of 50% of parsley and 50% of chives.

2.2.2 Sardines

The Brazilian sardines (*S. brasiliensis*) (10 Kg) were obtained from Angra dos Reis, Rio de Janeiro, Brazil (Latitude: 23°00'24"S and Longitude: 44°19'05"W) in March 2016, and transported on ice to UFRRJ at Seropédica, Rio de Janeiro, Brazil. After evisceration, the sardines were immediately washed, homogenized and separated into 8 lots. One of them was analyzed on the same day the fish were acquired, corresponding to fresh (raw) sardines. The other samples were packed in polyethylene film and stored at 5 °C in a domestic refrigerator, until preparation the following day.

2.3 *In vitro* Antioxidant Capacities and Quantification of Phenolic Compounds of Aromatic Herbs

2.3.1 Preparation of extracts

The extractions were carried out according to KUŹMA, DRUŻYŃSKA e OBIEDZIŃSKI (2014) with some modifications. 2 g of fresh parsley, chives and *cheiro-verde* was mixed with 50 mL of 80% solution of methanol and distilled water at room temperature under mechanical shaking for 1 h. The mixture was centrifuged at 3500 rpm for 1 min. and the supernatant was collected in a volumetric flask. The remaining residue was subsequently extracted two times each with 25 mL of 80% methanolic solution under mechanical shaking for 30 min. The extracts were done in triplicate.

2.3.2 DPPH radical scavenging activity

The DPPH assay was described by BRAND-WILLIAMS et al. (1995) with some modification as made by FUKUMOTO e MAZZA (2000). To a well in a 96 wells polystyrene microplate was added an aliquot of 20 – 120 µL of the extract and 200 µL of DPPH (1,1-diphenyl-2-picrylhydrazyl) in methanol (150 µM). The mixtures were shaken and left for 30 min at room temperature in the dark, after which the absorbance of the remaining DPPH was measured at 520 nm against a blank to eliminate the influence of herb extract. The plate was read in a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, USA). The DPPH scavenging activity was expressed as the inhibition of free radical DPPH in percent (IC%). I%= (A blank - A sample/ A blank) x 100, where a blank is the absorbance of the control reaction and A sample is the absorbance of the test extract.

2.3.3 ORAC

ORAC methodology was carried out according to the procedure described by OU et al. (2001) using a microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, USA) with 96 wells black microplate. Diluted samples in 75 mM phosphate buffer (50 µL) were incubated at 37 °C with 150 µL of fluorescein (93 mM). After 15 min, 50 µL of AAPH (221 mM) were added to the reaction medium. The microplates were incubated at 37 °C for 15 min prior to the addition of AAPH. For a period of 1 h, the fluorescence decay was measured at 37 ºC (λ excitation = 493 nm, λ emission = 515 nm) every minute. All extracts were analyzed in triplicate.

2.3.4 β-carotene/linoleic acid assay

The inhibition of β-carotene/linoleic acid peroxidation was evaluated as described by MILLER (1971). A stock solution of β-carotene/linoleic acid mixture was prepared by dissolving 20 mg of *β*-carotene in 1 mL of chloroform. Next, 28 µL this solution was mixed with 28 µL of linoleic acid and 200 mg of Tween® 40. The chloroform was evaporated under nitrogen and 140 mL of distilled water saturated with oxygen (30 min., 100 mL of O_2/min.) was added. After vigorous shaking, 5 mL of the reaction mixture were dispensed into the test tubes and 1 mL aliquot of the extracts was added. After shaking, the microplates were incubated at 50 °C and subsequent measurements were made every 15 minutes for 2 hours. The absorbance was read at 470 nm (model SpectraMax M5, Molecular Devices Inc). Results are expressed as percentage of peroxidation inhibition.

2.4 Quantification of Phenolic Compounds

The extracts of parsley and chives were prepared by decoction in hot water (for 10 minutes), cooled and then filtered at room temperature. The aqueous extract was lyophilized (lyophilizer Liotop K105). The quantification was performed using Prominence -Shimadzu HPLC, LC-20AT-SPD-M20A detector, CTO-20A oven, CBM-20A LC Solution controller - data acquisition. The analytical column used was a Betasil C18 Column (25 cm x 4.6 mm x 5 µm) – Thermo. The two mobile-phase were A: H_2O + 1% AcOH (90%), and B: MeOH (10%) at a flow rate of 1.0 ml/min, volume 20 Ul, pressure = 105 kgf/c, oven temperature 40 ^oC, PDA=200-500 nm (280 nm), and analysis time of 23 min. The pump gradient varied as follows: 10.00 Pumps B.Conc 55; 15.00 Pumps B.Conc 85; 18.00 B.Conc 85 Pumps; 19.00 Pumps B.Conc 65; 21.00 B.Conc 10 Pumps; 23.00 Controller Stop. The standards used were purchased from Sigma-Aldrich.

2.5 Preparation of sardine fillets

Each treatment consisted of 12 fillets of raw sardines. The control was considered the grilled sample without the addition of herbs; the other treatments were: grilled sardines with the addition of 2 and 4% of fresh parsley, chives and *cheiro-verde*.

The chives and parsley concentrations used in this study were selected in order to mimic the contents of the bioactive compounds present in the spices used to prepare fish and seafood traditional Brazilian dishes. The sardines were grilled in an electric grill (Mondial, CP-01, China); the time and temperature to grill the sardines were according to SALDANHA et al. (2008), until reaching an internal temperature of 75 ± 0.5 °C, monitored using a digital calibrated thermometer (Traceable Long Stem, VWR, Friendswood, TX, USA). The samples were ground and homogenized to obtain a homogeneous mass. Convenient aliquots were taken for the analyses, which were carried out in triplicate.

2.6 Analysis of sardine samples

2.6.1 Moisture and total lipids content

Moisture was measured and determined by the AOAC (2010). The lipids were extracted according to (BLIGH e DYER, 1959).

2.6.2 Fatty acid composition

The fatty acids composition of the sardine samples was determined after methyl esterification according to JOSEPH e ACKMAN (1992). The fames were analyzed using a gas chromatography (GC 2010) from Shimadzu (Tokio, Japan) equipped with a split injector (1:50), fused silica CP-SIL 88 capillary (column 100 m x 0.25 mm i.d., 0.2 µm film thickness) (Chrompack, Middelburg, The Netherlands), flame ionization detector and workstation was used. The chromatographic conditions were: initial temperature, 100 °C (5 minutes) followed by an increase of 5 °C/minute up to 160 °C (zero minutes), then 8 °C/minute up to 230 °C (12 minutes); injector and detector temperatures were 250 °C and 280 °C, respectively. The equipment used hydrogen as the carrier gas at a flow rate of 1 mL/minute and nitrogen as the make-up gas at 30 mL/minute. Retention times of FAME standards were used to identify the chromatographic peaks of the samples, and the quantification was done by internal standardization, using undecanoic methyl ester as the internal standard. Factors for the conversion of fatty acid methyl esters to their corresponding triglycerides were used (CARPENTER et al., 1993).

2.6.3 Cholesterol and Cholesterol Oxides

2.6.3.1 HPLC-PDA-RI

The simultaneous determination of cholesterol and cholesterol oxides were obtained by direct saponification (2 g of the samples, 4 ml of a 50% aqueous solution of KOH and 6 ml of ethanol) at 24 °C for 22 h in the dark and the non-saponifiable matter extracted 4 times with hexane. The hexane extract was dried, diluted with 1m of mobile phase and injected into the HPLC system (SALDANHA et al., 2006). For HPLC, a Waters (Milford, MA, EUA), equipped with PDA/RID detectors, rheodyne injector with a 20 µl loop, a tertiary solvent delivery system (Waters 600), oven heated column at 32 °C and software (Empower 2 2). The analytical column used was a CN Hyperchrome 250 mm × 4.3 mm × 5.0 µm (Phenomenex, Colorado, USA). The mobile-phase was n-hexane: 2-propanol (97:3, v/v) at a flow rate of 1ml/min and an analysis time of 30 min. The HPLC solvents were filtered through a 22 mm Millipore filter (Bedford, MA, USA) under vacuum prior to use. Quantification was done by external standardization, with a concentration ranged from 5.0 to 150.0 µg/ml for the oxides and from 0.1 to 2.0 mg/ml for cholesterol, respectively. The epimeric 5,6 epoxides were quantified using a refractive index detector, because they do not absorb at UV wavelengths. The other oxides were quantified using PDA detector.

2.6.3.2 UPLC-APCI-MS

In order to confirm the cholesterol oxides structures, samples were analyzed using UPLC-APCI-MS. The chromatographic analyses were performed on a UPLC

Acquity chromatographer coupled to a TQD Acquity Mass Spectrometer (Micromass-Waters Manchester, England), with an APCI source configuration, with a triple quadrupole). A CN Hyperchrome 250 mm × 4.3mm × 5.0 µm column (Phenomenex, Colorado, USA) was used. Isocratic mobile phase containing hexane: n-propanol (97:3), at a flow of flow 1 ml/min, oven temperature 32 °C, and 10 µL of the samples were injected into the UPLC. Ionization was performed in the APCI positive ion mode and the optimization conditions were adapted from SALDANHA et al. (2006). The ionization parameters were: full scan m/z 100-500, capillary voltage 1500V; corona current 20 µA; drying gas temperature 350 °C; cone voltage 20V; vaporizer temperature 150 °C.

2.7 Statistical Analysis

The analysis of variance (ANOVA) was employed in the completely randomized designing with two factors and their interactions. The first factor was the three different herbs (chives, parsley and *cheiro-verde*) and the second factor was the levels of the addition of herbs (2 and 4%). Additionally, when significant differences were observed, *Tukey* multiple range tests were applied. Multivariate analysis was used to describe the data from the quantification of cholesterol, fatty acids and COPs. Principal component analysis (PCA) was used to group similar samples and was performed on the standardized data to make sure all the elements had the same influence over the results. Hierarchical Clustering on Principle Components (HCPC) was done to group samples with similar characteristics using the function HPCP in the FactoMineR. All analyses were performed using the software R version 3.2.4 (R Foundation for Statistical Computing, Vienna, Austria) and the FactoMineR package version 1.32.

3 RESULTS AND DISCUSSION

3.1 *In vitro* Antioxidant Capacities of Aromatic Herbs

3.1.1 DPPH radical scavenging activity

Parsley extract exhibited the strongest DPPH, scavenging ability, higher than chives and *cheiro-verde* (**Table 1)**.

Table 1- Antioxidant capacities of aromatic herbs.

	Parsley	Chives	*Cheiro verde*
DPPH assay (%)	59.21±0.07[a]	32.15±0.02 [b]	34.34±0.03 [b]
ORAC assay (µmolar TE/g fresh weigh)	109.94±18.71[a]	31.49±1.09$_c$	63.92±4.39[b]
β-Carotene/Linoleic Acid assay (% inhibition)	40.74±1.95[a]	12.10±0.96[b]	42.66±4.03[a]

Values represent means ± standard deviation (n=3). Values followed by different lowercase letters in the same row differ from each other, according to the Tukey test ($p< 0.05$).

These results in parsley were similar to that shown by ZHANG et al. (2006) (51%). However, very lower levels were observed in parsley by other authors; CHANDRA et al. (2014) reported 8.69% of DPPH scavenging activity and, SHEHATA et al. (2014) evaluated the effect of temperature and time of extraction on DPPH scavenging activity in parsley and observed (3.30, 2.79, 2.5 and 2.0% free radical scavenging activity), in cold water, and in hot water for 10, and 30 min, respectively. JIA et al. (2012) observed the antioxidant capacity (37.8% free radical-scavenging activity) in parsley and their effect on the oxidative stability of food during storage, suggesting that parsley can be used to suppress lipid oxidation. PARRY et al. (2006) found lower levels (13.4% cold pressed parsley oil) than this work.

For chives samples, different results were reported in the literature. MNAYER et al. (2014) observed much lower levels (5.59) IC 50 (concentration mg/mL for 50% inhibition) than found in this work. In contrast, GONÇALVES et al. (2015) and LENKOVA et al. (2016) found superior values (55.2 and 76.57% DPPH) in chives.

The use of different solvents to obtain the extracts, make it difficult to compare the results among studies. In addition, the values of the antioxidants activities are often given in different unities.

3.1.2 β-Carotene/Linoleic Acid assay

The *β*-carotene bleaching method is usually used to evaluate the antioxidant capacity of compounds in emulsions accompanied with the coupled oxidation of *β*-carotene and linoleic acid (SHEHATA et al., 2014). Parsley and *cheiro-verde* presented higher value than chives ($p<0.05$) (**Table 1)**. These results are consistent with those of SHEHATA et al. (2014) who found similar values in parsley (40.90) for *β*-Carotene/Linoleic Acid assay (% inhibition). On the other hands, TIVEREON et al. (2012) reported higher results for parsley and chives (60.7 and 28.8% inhibition), respectively.

3.1.3 ORAC assay

The highest oxygen radical absorbance capacity (ORAC) values of 109.94 μmol Trolox equiv/g fresh weigh was found in parsley (**Table 1)**.

Similar results of ORAC levels were found in parsley 113.5 (μmol TE/g FW) by ČÍŽ et al. (2010). PARRY et al. (2006) found 1098 μmol TE/g in cold pressed parsley oil. ZHENG e WANG (2001) evaluated the antioxidant capacity and their results showed lower contents in parsley and chives (11.03 and 9.15 μmol of TE/g of fresh weigh) ORAC.

This assay is a sensitive method and reflects antioxidant properties even of such a low quantity of polyphenols. ORAC is a widely accepted measurement of free radical-scavenging capacity (PARRY et al., 2006).

3.2 Quantification of Phenolic Compounds

The quantification of 10 searched phenolic compounds (protocatechuic acid, apiin, *p*-coumaric acid, luteolin, apigenin, cathechin, ferulic acid, rutin, epicatechin, and hesperetin) present in parsley and chives are shown on **Table 2**.

Table 2- Quantification of phenolic compounds observed in parsley and chives (µg/mg sample).

Phenolic compounds	Parsley contents (µg/mg sample)	Chives contents (µg/mg sample)
Protocatechuic acid	0.22 ± 0.0	0.41± 0.0
Apiin	3.33± 0.0	-
p-Coumaric acid	0.08 ± 0.0	-
Luteolin	0.60 ± 0.0	-
Apigenin	0.80 ± 0.0	-
Cathechin	-	27.00 ± 0.0
Ferulic acid	-	0.47 ± 0.0
Rutin	-	1.28 ± 0.0
Epicatechin	39.81 ± 0.0	-
Hesperetin	0.26 ± 0.0	-

Values represent means ± standard deviation (n=3).

The results of this study showed high contend of epicatechin (39.81µg/mg sample) in parsley, followed by lower contents of apiin 3.34, and apigenin 0.80 (µg/mg sample). In agreement with the current study, the level of apigenin in parsley was found very low, only 4.4 mg/kg, and lower than the other contents (14.2, 18.5, 5, and 11.2 mg/kg) of luteolin, kaempferol, quercetin, and isorhamnetin, respectively (CAO et al., 2010). JUSTESEN et al. (1998) also observed hesperetin; whereas, KAISER et al. (2013) observed *p*-Coumaric acid in parsley.

Other researchers presented different reports related to the identification and quantification of phenolic compounds evaluated in parsley. JUSTESEN et al. (1998) reported high amount of apigenin (185 mg/100 g fresh weigh) in this herb, but lower levels in both kaempferol and luteolin (1.1 mg/100 g fresh weigh), respectively. MATTILA, et al. (2000) observed higher values (1484.2) for apigenin than isorhamnetin (36.4) and luteolin (21.7) µg/100 g dry weight (dw) in parsley, however the authors did not find kaempferol and quercetin. In agreement with their study, apigenin was found at high concentration in parsley 1521 (winter) and 1636 µg/g (summer), respectively (HUBER et al., 2009). The main flavonoids identified by JUSTESEN e KNUTHSEN (2001) in their study were: apigenin, isorhamnetin, kaempferol, luteolin, and quercetin. Among the studied fresh herbs, higher levels of flavonoids were found in parsley (apigenin 510-630 mg/100g). Others authors also identified different phenolic compounds in parsley. VIOLETA NOUR et al. (2017) reported the quantification of some phenolics found in this work such as coumaric acid, and epicatechin (1.21 and 2.67 mg/100 g fresh weigh), respectively. However, they observed other phenolic compounds, mainly: myricetin, *trans*-cinnamic acid, quercetin (151.03, 27.34, 71.33 mg/100 g fresh weigh), respectively.

These differences between the research results of the phenolic concentrations can be influenced by several factors, including climatic variation, different types of soil, and variations in cultivar, cultivation site, irrigation, agricultural practices and post-harvest processing (CAO et al., 2010).

Chives presented high contents of cathechin (27.00), and lower levels of rutin (1.28), ferulic acid (0.47) µg/mg sample, respectively. Different results were found in the literature of chives extracts. Deng et al. (2013) reported the following levels of cathechin (88.94 mg/100g), and observe the presence of gallic acid (8.82mg/100g). In other researches were identified the presence of*p*-coumaric acid (149.59), ferulic acid (188.06), sinapic acid (44.91), isoquercitrin (363.78), quercetol (58.38) and kaempferol

(129.83) µg/100 g leaves (PARVU et al., 2010; VLASE et al., 2013). BERETTA et al. (2017) observed the presence of chorogenic acid (1.1), coumaric acid (0.4), ferulic acid (10.6), caffeic acid (0.18) (mg/100g fm), respectively. Although chives exhibited the most potent antioxidant in their research, the phenolic profiles varied greatly, suggesting that individual phenolic compounds differ in antioxidant strength. In addition, BALASUNDRAM et al. (2005) proposed that the antioxidant capacity of phenolic compounds depends on their chemical structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings.

In general, aromatic plants are complex matrices regarding their content on phenolic compounds. Their composition can be affected by the chemical structure of the studied analytes, the selected methods, the composition/nature of the aromatic plant and storage conditions (COSTA et al., 2015). Moreover, different extraction techniques and solvents used may influence the detection and quantification of the phenolic compounds (DAI e MUMPER, 2010; KHODDAMI et al., 2013).

3.3 Moisture, Total Lipid and Cholesterol Contents of Raw and Grilled Sardines

Moisture, fat and cholesterol contents in raw, grilled control and grilled sardines with the addition of parsley, chives and *cheiro-verde* are presented in **Table 3**. The total lipids and cholesterol were calculated on dry basis.

Table 3 - Moisture (g/100 g), total lipids (g/100 g dry basis) and cholesterol (mg/100 g dry basis) contents in raw, grilled (control) and grilled sardines added with chives, parsley and *cheiro-verde* at two levels (2 and 4%).

	Raw	Control	Chives 2%	Chives 4%	Parsley 2%	Parsley 4%	*Cheiro-verde* 2%	*Cheiro-verde* 4%
Moisture	75.2^{A} (0.3)	58.3^{C} (0.6)	$65.4^{B;b}$ (0.8)	$68.0^{A;a}$ (0.3)	$63.3^{B;c}$ (0.4)	$63.0^{B;c}$ (0.5)	$63.0^{B;d}$ (0.1)	$63.3^{B;d}$ (0.0)
Total lipid	16.5^{A} (0.2)	11.5^{CD} (0.4)	$11.9^{C;b}$ (0.7)	$14.4^{B;a}$ (0.5)	$11.1^{CD;b}$ (0.6)	$10.6^{DE;bc}$ (0.5)	$10.10^{E;c}$ (0.1)	$9.7^{E;c}$ (0.4)
Cholesterol	237.2^{A} (3.7)	145.7^{D} (2.1)	$153.0^{D;c}$ (5.4)	$164.8^{C;b}$ (4.0)	$147.4^{D;c}$ (1.7)	$210.9^{B;a}$ (5.1)	$168.1^{C;b}$ (1.0)	$206.5^{B;a}$ (5.0)

Values represent means ± standard deviation (n=3). Capital letters in the same row indicate significant differences by the *Tukey* test ($p < 0.05$) between raw, grilled control and grilled sardines with chives, parsley and *cheiro-verde*, respectively. Values followed by different lowercase letters in the same row differ from each other, according to the *Tukey* test ($p < 0.05$) in a factorial design (herbs and concentrations factors).

The moisture levels ranged from 75.2 ± 0.3 to 58.3 ± 0.6 g/100 g in raw and grilled samples. Similar levels were determined by FERNANDES et al. (2014) (71.13 g/100 g) in raw sardines. Grilling affected the moisture contents significantly ($p < 0.05$).

The lipid contents in fresh sardines was 16.5 ± 0.2 g/100g, and this result was higher than the observed by other authors LUZIA et al. (2003); SALDANHA et al. (2008) (12.5 and 8.9 g/100g in dry basis), respectively. It is known that the lipid components of fish can vary according to the month of capture, the season, and the particular area of the sea (LUZIA et al., 2003). After the thermal processing of fish, occurs dehydration and loss of fat (GARCÍA-ARIAS et al., 2003). Lipid contents in grilled sardines ranged from 9.7 ± 0.4 to 14.4 ± 0.5 g/100g (**Table 3**). In grilled sardines, SALDANHA et al. (2008) found lower lipid content (8.2 g/100 g, dry basis). The chemical composition of sardines is closely related to nutrition, living area, fish size, catching season, month of the catching and sexual variation as well as other environmental conditions.

The cholesterol levels varied from 237.2 ± 3.7 in raw to 145.71 ± 2.09 mg/100, dry basis in grilled control samples (**Table 3**), lower than the determined by SALDANHA et al. (2008) in raw and grilled Brazilian sardines, with 342 and 282 mg/100 g (dry basis), respectively. Grilling affected the cholesterol content, producing a significant decrease in the evaluated samples. These results are in accordance with SALDANHA et al. (2008) and SALDANHA e BRAGAGNOLO (2010) in sardines and other authors (MÉNDEZ; GONZÁLEZ, 1997; OHSHIMA et al., 1996; OHSHIMA, 2002, OSADA et al., 1993; SALDANHA e BRAGAGNOLO, 2007) in grilled fishes.

The mean values of cholesterol content in grilled sardines added with the herbs ranged from 147.4 ± 1.7 to 210.9 ± 5.1 mg/100, dry basis. In this case, sardines added with 4% parsley showed the highest protective effect on cholesterol contents (89%), followed by 4% *cheiro-verde* (87%) (**Figure 1**). The cholesterol degradation was higher in control samples, than observed in the sardines added with aromatic herbs. This is due to high protective effect of phenolic compounds present in the aromatic herbs, mainly the sardine samples with addition of 4% parsley and *cheiro-verde*.

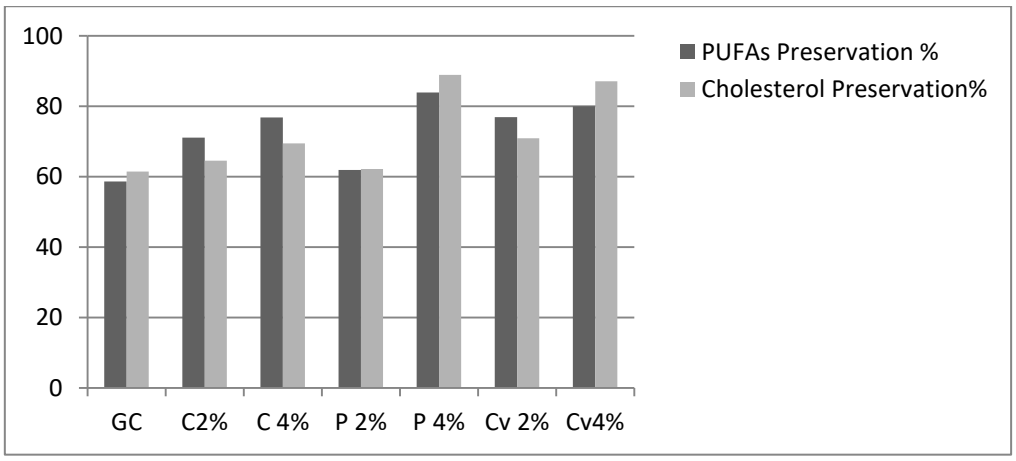

Figure1. Percentage of PUFAs and Cholesterol preservation after grilling in grilled control (GC); and sardines added with aromatic herbs: C2%, 2% chives; C4%, 4% chives; P2%, 2% parsley; P4%, 4% parsley; Cv2%, 2% *cheiro-verde* and Cv4%, 4% *cheiro-verde*.

3.4 Fatty Acid Composition of Raw and Grilled Sardines

The fatty acids contents in raw and grilled sardines are presented in **Table 4**, expressed in g/100 g of oil.

In the total fatty acids (FA) determined in raw samples, the main saturated fatty acids (SFA) were palmitic (C16:0), myristic (C14:0) and stearic (C18:0) acids, which palmitic was present in the highest amounts. The monounsaturated fatty acids (MUFAs), presented 19.1% of the total fatty acids, with the oleic acid (C18:1*n*9c) as the most abundant one, followed by the palmitoleic (C16:1). The percentage of polyunsaturated fatty acids (PUFAs) was 32.2%, consisting typically of docosahexaenoic (DHA, C22:6 *n*3) and ecoisapentanoic (EPA, C20:5 *n*3). These findings are in agreement with those obtained by other authors in sardine samples (SALDANHA et al., 2008; FERNANDES et al., 2014).

Table 4 - Fatty acid composition (g/100g of oil) of raw, grilled control (without herbs) and grilled sardines with the addition of chives, parsley and *cheiro-verde* at two levels (2 and 4%).

Fatty acids	Raw	Control	Chives 2%	Chives 4%	Parsley 2%	Parsley 4%	*Cheiro-verde* 2%	*Cheiro-verde* 4%
C12:0	0.11^{ABC}	0.09^{CD}	0.09$^{D;c}$	0.10$^{BCD;bc}$	0.13$^{A;a}$	0.10$^{BCD;bc}$	0.12$^{AB;ab}$	0.10$^{BCD;bc}$
C14:0	9.67^{AB}	8.91^{B}	8.85$^{B;c}$	9.48$^{AB;bc}$	10.84$^{A;a}$	9.54$^{AB;bc}$	10.46$^{AB;ab}$	9.92$^{AB;abc}$
C15:0	1.56^{F}	2.24^{BC}	2.59$^{A;a}$	2.15$^{CD;c}$	2.34$^{B;b}$	1.49$^{F;e}$	1.84$^{E;d}$	2.06$^{C;d}$
C16:0	37.15^{BC}	33.94^{CD}	33.60$^{D;c}$	36.14$^{CD;bc}$	41.57$^{A;a}$	39.83$^{AB;ab}$	41.81$^{A;a}$	36.40$^{CD;bc}$
C17:0	1.29^{C}	1.66^{BC}	2.16$^{AB;ab}$	1.81$^{BC;b}$	1.89$^{BC;b}$	2.71$^{A;a}$	1.88$^{BC;b}$	1.84$^{BC;b}$
C18:0	6.56^{E}	8.86^{ABCD}	8.94$^{ABC;ab}$	7.87$^{D;c}$	9.67$^{A;a}$	9.34$^{AB;a}$	8.52$^{BCD;bc}$	8.18$^{CD;bc}$
C20:0	0.53^{A}	0.48^{A}	--	0.04$^{C;cd}$	0.06$^{C;c}$	0.33$^{B;a}$	0.28$^{B;b}$	0.30$^{B;ab}$
C21:0	0.22^{AB}	0.21^{AB}	0.18$^{B;b}$	0.18$^{B;b}$	0.26$^{A;a}$	0.18$^{B;b}$	0.18$^{B;b}$	0.19$^{C;b}$
C22:0	0.29^{A}	0.22^{B}	0.22$^{B;a}$	0.09$^{C;b}$	0.04$^{D;d}$	0.19$^{B;a}$	0.17$^{B;a}$	0.09$^{C;b}$
C23:0	0.40^{A}	0.31^{B}	0.21$^{C;b}$	0.24$^{C;b}$	0.23$^{C;b}$	0.33$^{B;a}$	0.21$^{C;b}$	--
C14:1	0.46^{C}	0.40^{CD}	0.38$^{D;c}$	0.40$^{CD;c}$	0.63$^{B;b}$	1.27$^{A;a}$	0.21$^{E;d}$	0.43$^{CD;c}$
C15:1	0.17^{BC}	0.16^{C}	0.17$^{BC;bc}$	0.17$^{BC;bc}$	0.29$^{A;a}$	0.14$^{C;c}$	0.17$^{BC;bc}$	0.20$^{B;b}$
C16:1	5.74^{A}	4.66^{C}	5.40$^{AB;ab}$	5.14$^{BC;ab}$	5.73$^{A;a}$	5.45$^{AB;ab}$	5.06$^{BC;b}$	4.97$^{BC;b}$
C17:1	0.49^{A}	0.27^{C}	0.26$^{C;b}$	0.27$^{C;b}$	0.44$^{B;a}$	0.42$^{B;a}$	0.40$^{B;a}$	0.42$^{B;a}$
C18:1 *n*9*t*	0.31^{B}	0.48^{A}	0.17$^{C;a}$	0.21$^{C;a}$	0.22$^{C;a}$	0.21$^{C;a}$	0.18$^{C;a}$	0.19$^{C;a}$
C18:1 *n*9*c*	10.11^{A}	8.70^{BC}	8.42$^{C;ab}$	9.01$^{ABC;ab}$	9.67$^{AB;a}$	8.83$^{BC;ab}$	8.54$^{BC;ab}$	8.18$^{C;b}$
C20:1 *n*9	0.18^{E}	0.12^{E}	0.45$^{D;cd}$	0.51$^{C;c}$	0.88$^{A;a}$	0.42$^{D;d}$	0.78$^{B;b}$	0.75$^{B;b}$
C22:1 *n*9	0.64^{A}	0.45^{C}	0.38$^{D;d}$	0.44$^{CD;d}$	0.52$^{B;b}$	0.48$^{BC;b}$	0.62$^{A;a}$	0.64$^{A;a}$
C24:1 *n*9	1.03^{CDE}	0.88^{E}	0.95$^{DE;c}$	0.94$^{E;c}$	1.23$^{BC;b}$	1.39$^{AB;ab}$	1.18$^{BCD;bc}$	1.50$^{A;a}$
C18:2 *n*6*t*	0.37^{C}	0.58^{A}	0.45$^{B;a}$	0.45$^{B;a}$	0.41$^{BC;a}$	0.43$^{B;a}$	0.46$^{B;a}$	0.45$^{B;a}$
C18:2 *n*6*c*	2.34^{B}	2.30^{B}	2.59$^{AB;ab}$	2.39$^{B;a}$	2.95$^{A;a}$	2.93$^{A;a}$	2.58$^{AB;ab}$	2.67$^{AB;ab}$
C18:3 *n*6	2.91^{A}	1.77^{D}	2.96$^{A;a}$	3.00$^{A;a}$	1.77$^{D;d}$	2.10$^{C;c}$	2.83$^{A;a}$	2.52$^{B;b}$
C18:3 *n*3	2.31^{BC}	1.81^{D}	2.69$^{B;b}$	3.32$^{A;a}$	1.67$^{D;d}$	2.23$^{C;c}$	2.35$^{BC;bc}$	2.51$^{BC;bc}$
C20:2*n*6	--	0.25^{D}	0.38$^{AB;ab}$	0.40$^{A;a}$	0.31$^{CD;c}$	0.33$^{BC;bc}$	0.36$^{ABC;abc}$	0.34$^{BC;bc}$
C20:3 *n*6	--	--	0.11$^{C;c}$	0.26$^{B;b}$	--	0.23$^{B;b}$	0.41$^{A;a}$	0.41$^{A;a}$
C20:3 *n*3	1.21^{CD}	1.01^{E}	1.02$^{E;d}$	1.14$^{D;c}$	0.76$^{F;e}$	1.24$^{BC;b}$	1.49$^{A;a}$	1.3$^{B;b}$
C20:4 *n*6	0.28^{A}	0.21^{CD}	0.16$^{E;c}$	0.20$^{DE;bc}$	0.27$^{AB;a}$	0.25$^{ABC;a}$	0.23$^{BCD;ab}$	0.25$^{ABC;a}$
C20:5 *n*3	8.77^{A}	3.72^{F}	4.20$^{C;b}$	4.25$^{C;b}$	3.39$^{D;c}$	5.73$^{B;a}$	5.23$^{B;a}$	5.64$^{B;a}$
C22:6 *n*3	14.00^{A}	7.22^{F}	8.33$^{E;d}$	9.32$^{CD;bc}$	8.39$^{E;d}$	11.55$^{B;a}$	8.81$^{DE;cd}$	9.63$^{C;b}$
∑SFA	**57.78^{B}**	**56.93^{B}**	**56.84$^{B;b}$**	**58.10$^{B;b}$**	**67.03$^{A;a}$**	**64.04$^{A;a}$**	**65.47$^{A;a}$**	**59.08$^{B;b}$**
∑MUFA	**19.13^{A}**	**16.12^{C}**	**16.58$^{C;b}$**	**17.09$^{BC;b}$**	**19.61$^{A;a}$**	**18.61$^{AB;ab}$**	**17.14$^{BC;b}$**	**17.28$^{BC;b}$**
∑PUFA	**32.19^{A}**	**18.87^{E}**	**22.89$^{D;c}$**	**24.73$^{C;b}$**	**19.92$^{E;d}$**	**27.02$^{B;a}$**	**24.75$^{C;b}$**	**25.72$^{BC;ab}$**
∑*n*3	**26.29^{A}**	**13.76^{E}**	**16.24$^{D;d}$**	**18.03$^{C;bc}$**	**14.21$^{E;e}$**	**20.75$^{B;a}$**	**17.88$^{C;c}$**	**19.08$^{C;b}$**
∑*n*6	**5.90^{CD}**	**5.11^{E}**	**6.65$^{AB;ab}$**	**6.70$^{AB;ab}$**	**5.72$^{D;c}$**	**6.27$^{ABC;bc}$**	**6.87$^{A;a}$**	**6.64$^{AB;ab}$**
ω3/ω6	**4.46^{A}**	**2.69^{CD}**	**2.44$^{D;c}$**	**2.69$^{CD;bc}$**	**2.49$^{D;c}$**	**3.31$^{B;a}$**	**2.60$^{CD;bc}$**	**2.87$^{C;b}$**
∑Trans	**0.68^{B}**	**1.06^{A}**	**0.62$^{B;a}$**	**0.66$^{B;a}$**	**0.63$^{B;a}$**	**0.64$^{B;a}$**	**0.64$^{B;a}$**	**0.64$^{B;a}$**
∑EPA+DHA	**22.77^{A}**	**10.94^{G}**	**12.53$^{EF;de}$**	**13.57$^{DE;cd}$**	**11.78$^{FG;e}$**	**17.28$^{B;a}$**	**14.04$^{CD;c}$**	**15.27$^{C;b}$**

Values are means (n=6). Different capital letters in the same row indicate significant differences among raw, control and sardines with added chives, parsley and *cheiro-verde* by the *Tukey* test ($p < 0.05$). Different lowercase letters in the same row differ according to the *Tukey* test ($p < 0.05$) to a factorial design (herbs and concentrations factors).

After grilling a significant decrease ($P< 0.05$) was observed in control samples in the total of FA, mainly of PUFA and MUFA contents. The losses were between 41.4% in PUFA and 15.7% in MUFA, respectively. During grilling process, fish are subjected to heating and oxygen, and these two factors can accelerate the oxidative deterioration of fat, especially polyunsaturated fatty acids (SALDANHA e BRAGAGNOLO, 2008). In contrast, the content of *trans* fatty acids increased (36%) after grilling (**Table 4**). The same was observed in another study with grilled sardines (SALDANHA et al., 2008). The thermal processing can lead to hydrogenation reactions between the oil and water present in the sardine, which could generate *trans* fatty WEBER et al, 2008.

Fresh sardines showed the highest levels of n-3 fatty acids, being8.77 ± 0.21 and 14 ± 0.41 g/100 g for EPA and DHA, respectively. Grilling produced significant losses in these values, 48% for EPA and 37% for DHA. These results are in agreement with other studies (OHSHIMA et al., 1996; SALDANHA e BRAGAGNOLO, 2007; SALDANHA e BRAGAGNOLO, 2008; SALDANHA et al., 2008) that reported a decrease in the EPA and DHA levels in fish samples after heating.

Recent research investigated the effect of elevated temperature during cooking different methods (boiled, pan-frying and baking) to understand the changes in fatty acids profile and the potential of degradation of EPA and DHA in salmon. Oxidized products of n-3 and n-6 PUFAs increased in frying pan, whereas only those of n-3 PUFAs were elevated in baked salmon (LEUNG et al., 2018).

According to MAQSOOD, BENJAKUL e SHAHIDI (2013) the presence of high amounts of PUFAs makes fish muscle highly susceptible to lipid oxidation during cooking processes. The more double bonds present in the fatty acids the easier it is to remove hydrogen atoms and consequently to form a radical (LEUNG et al., 2018). Moreover, the lipid peroxidation is initiated by an attack towards a fatty acids side chain by a radical in order to abstract a hydrogen atom from a methylene carbon (CAROCHO e FERREIRA, 2013).

Although heating affected significantly the lipid contents in the sardines fillets, the addition of aromatic herbs showed a significant impact protecting the unsaturated fatty acid (PUFAs) contents after the thermal process, with 4% parsley and *cheiro-verde,* presenting higher protective effect (84 and 80%, respectively). The contents of PUFA were most preserved in the following order: 4% parsley > 4% *cheiro-verde*> 2% *cheiro-verde* > 4% chives > 2% chives > 2% parsley > control, suggesting that the addition level of 4 % of parsley and *cheiro-verde* presented the better effects against lipid oxidation in PUFA **(Figure 1)**. Among the sardines added with herbs, 4% parsley showed the highest levels of EPA and DHA. Therefore, this protective effect observed in sardines added with parsley in the current study is attributed to higher concentration of phenolic compounds presented in this herb (**Table 2**). It was also noticed that parsley exhibited higher antioxidant proprieties on the *in vitro* assays (DDPH %, ORAC, and β-Carotene/Linoleic Acid) compared to chives ($p<0.05$), and similar to the results of β-Carotene/Linoleic in *cheiro-verde* ($p<0.05$), shown on **Table 1**.

FERREIRA et al. (2017) evaluated the effect of air frying in sardine fillets, which significantly decreased the content of essential PUFAs in control samples. However, they also proved the effectiveness of the addition of aromatic plants acting as natural antioxidants. The results were very similar to the present study, showing the beneficial effects of the addition especially of 4% parsley and *cheiro-verde*.

Phenolic compounds present in chives and parsley as shown in **Table 2**, as well as observed in other studies (ZHANG et al., 2006; WONG e KITTS 2006; LUTHRIA,

2008; PARVU et al., 2010; PÁPAY et al., 2012; TIVERON et al., 2012; FARZAEI et al., 2013; VLASE et al., 2013; PARVU et al., 2014, VIOLETA NOUR et al. (2017) are suggested to act as antioxidant.

Thus, the addition of aromatic herbs proved to inhibit PUFAs degradation, since the presence of inhibitors such as phenolic compounds protected the sardine filets throughout the grilling process against the lipid oxidation.

3.5 Cholesterol Oxides of Raw and Grilled Sardines

It was possible to quantify and confirm the 10 structures of cholesterol oxides present in sardines by HPLC-PDA-RI and UPLC-APCI-MS analyses. Six COPs were determined in raw sardines: 5,6α-EP, 22(R)-OH, 25-OH, 7-keto, 7β-OH and 7α-OH (**Figure 2**).

The presence of oxidized cholesterol in the fresh sardines may be originated enzymatically in fish metabolism (OSADA et al., 1993). Therefore, the oxidation of cholesterol in food may occur intermolecular or intramolecular. In the intermolecular systems, hydrogen is extracted from the cholesterol by radical peroxides or oxides of polyunsaturated fatty acids (phospholipids) that are adjacent to the cell membrane. Whereas the intramolecular systems, the oxidation products of the fatty acids are esterified with cholesterol attack the cholesteryl portion of the cholesterol molecule (SMITH, 1987). Moreover, the molecule of cholesterol is prone to oxidation during prolonged storage and/or food processing, yielding a formation of oxysterols or cholesterol oxidation products (COPs) (VALENZUELA et al., 2003; XU et al., 2009).

In control samples, the levels of total of COPs increased significantly ($p<0.05$) after grilling (56%) from 61.2 ± 0.71 to 140.1 ± 3.9 (μg/g dry basis) in raw and the grilled samples, respectively. Nevertheless, SALDANHA et al. (2008), found lower amount of total COPs in raw and grilled sardines ranged from 19.4 to 41.6 (μg/g dry basis), respectively. This difference in raw fishes among these researchers could be due to the endogenous factors, as well as the location, and time of the year of capture. However, the ratios of COPs formation in grilled sardines are similar.

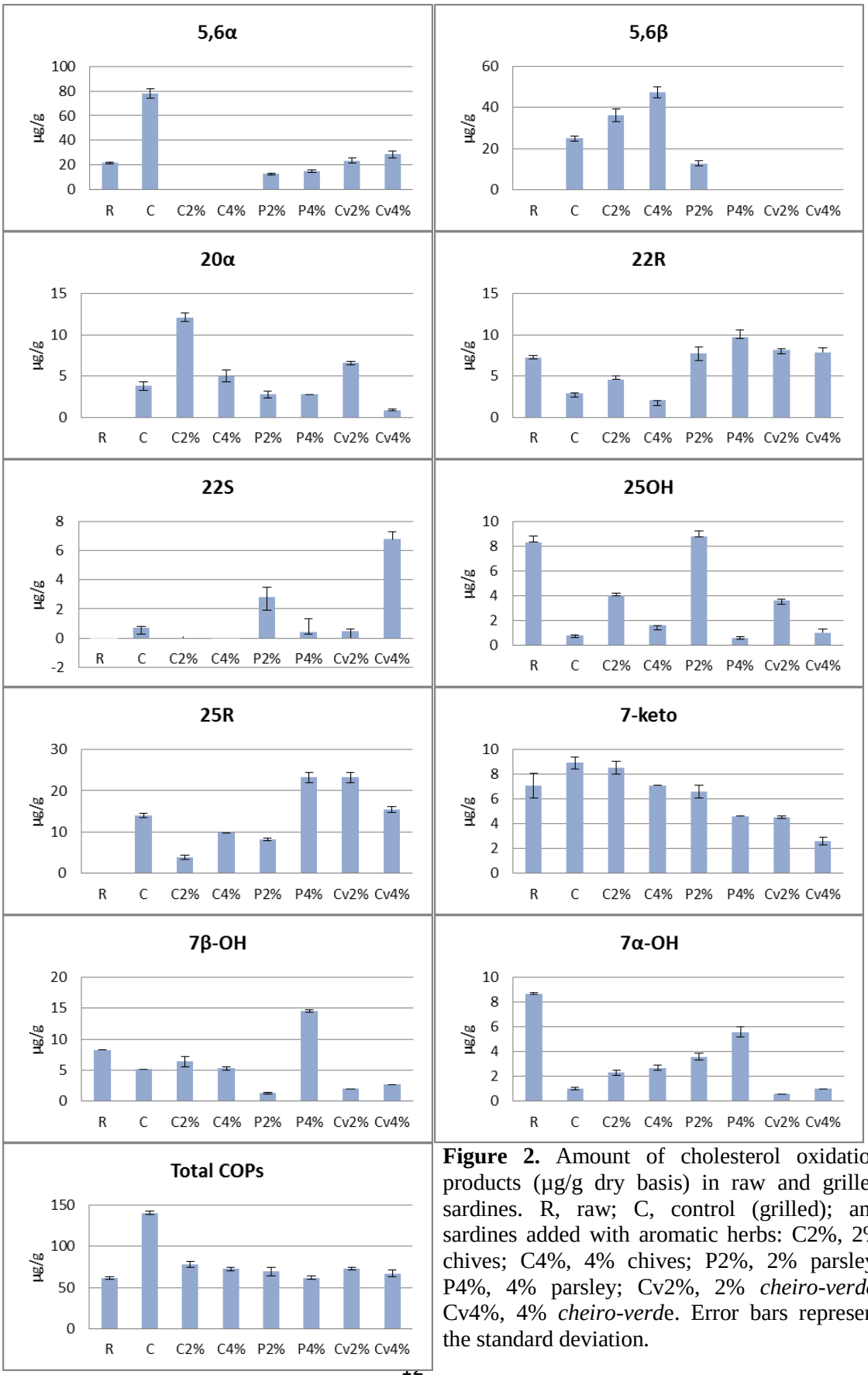

Figure 2. Amount of cholesterol oxidation products (µg/g dry basis) in raw and grilled sardines. R, raw; C, control (grilled); and sardines added with aromatic herbs: C2%, 2% chives; C4%, 4% chives; P2%, 2% parsley; P4%, 4% parsley; Cv2%, 2% *cheiro-verde*; Cv4%, 4% *cheiro-verd*e. Error bars represent the standard deviation.

The COPs found in grilled sardines were: 5,6α-EP, 5,6β-EP, 20α-OH, 22R-OH, 22S-OH, 25-OH, 25R-OH, 7-keto, 7β-OH and 7α-OH. The most affected oxides by the heat treatment were: 5,6α-EP, 5,6β-EP, 25R-OH and 7-keto (**Figure 3).** As was expected, the major cholesterol oxidation products found in fish were the 7-hydroxycholesterols, which are the main secondary oxidation products that are generated from the corresponding 7-hydroperoxy cholesterols (DANTAS et al., 2015).

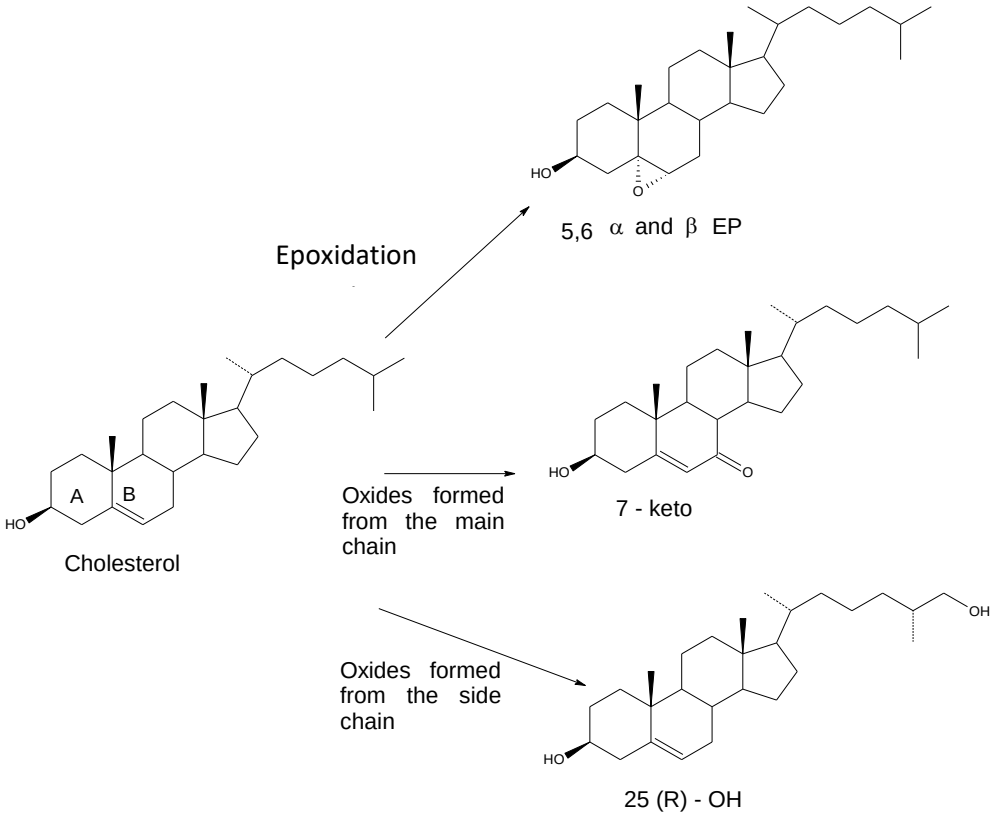

Figure 3. Chemical structures of cholesterol and cholesterol oxides more affected after grilling in control sardines.

This can be explained due to thermo-oxidation process, which the predominant compounds are those derived from the oxidation of C-7 carbon, although the epoxidation route includes the formation of 5,6 epoxides. The 7α- and 7β-hydroperoxides are formed simultaneously, but 7β-hydroperoxide is predominant because it is thermodynamically more stable. Then they are reduced to their corresponding alcohols (7α- and 7β-hydroxycholesterol). Moreover, the presence of unsaturated fatty acids (PUFA) in fish and seafood may accelerate the oxidation of cholesterol during high temperatures processing, forming radicals such as hydroperoxides (BARNABA et al., 2016).

Epoxides are originated by heat and oxygen, which dehydrates the molecule and facilitates epoxidation (CHIEN et al., 1998). This fact was observed in the present study, since, after the grilling, the control samples presented an increase in the levels of 5,6α-EP, 5,6β-EP and 7-keto. Moreover, the high levels of epoxides could be explained by the epoxidation of cholesterol molecule due to heating after the grilling process. In addition, the formation of 25R-OH was observed as the main oxide originating from the

side chain found in grilled sardine fillets. Oxides originated from the oxidation of lateral chain, which are common in fish, could be explained by where these products had been originated. This oxidation at position C 25 probably occurs due to a superposition of the bilayers of cholesterol making this position more reactive and more exposed to the attack of reagents than the other atoms (KORAHANI, BASCOUL e DE PAULET, 1982).

Considering the amount of PUFAs found in the fresh sardines and the temperature of grilling, the increase in oxide formation after thermal treatment can be understood. These can also be better explained due to a loss of antioxidant enzyme activity, disruption of cell membranes, which bring polyunsaturated fatty acids into contact with pro-oxidants, and thermal decomposition of hydroperoxides to pro-oxidant species that are responsible for the increase in cholesterol oxidation rate (HUR et al., 2007). Several studies have reported significant increases in the COP contents during thermal processing in fish (SALDANHA, BENASSI e BRAGAGNOLO, 2008; ANSORENA et al., 2013; DEREWIAKA e MOLIŃSKA, 2015; FREITAS et al., 2015;; TARVAINEN et al., 2016; XU et al., 2016; BARRIUSO et al., 2017; FERREIRA et al., 2017; LEAL-ESTRADA et al., 2017).

In sardines added with herbs, the total COPs ranged from 61.5 to 78 (µg/g dry basis) **(Figure 2**). It can be stated that the grilled sardine fillets added with 4% parsley showed the best effective protective, minimizing cholesterol oxidation products formation, presenting very similar levels of the total COPs to raw sardines; followed by sardines with 4% *cheiro-verde* and 2% parsley (66.9 and 69.2 µg/g), respectively. In contrast, 2% chives showed the highest level of COPs (78 µg/g), consequently the lowest protective effect (44%) among the sardine added with herbs (**Figure 2**).

The amount of COPs formed in fish or fish products depends on a number of parameters, such as preparation methods, antioxidant adding and thermal treatments. Comparing the COPs formation between control treatment and sardines with the addition of herbs, the grilled control sample generated a significantly higher amount of total COPs than the samples added with herbs. According to the degree of the cholesterol oxides formation in control samples, it can be concluded that the temperature employed is an important factor in the production of these compounds. However, as observed in this study the addition of aromatic herbs proved to be effective inhibiting high levels of COPs formation in grilled sardines ranging from 44 to 56% as shown on **Figure 4**.

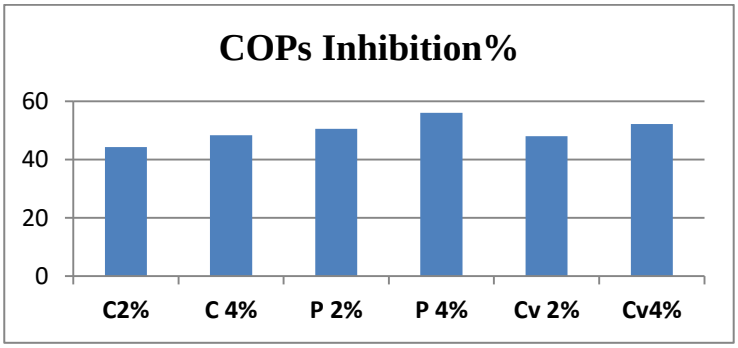

Figure 4. Percentage of COPs inhibition after grilling in sardines added with aromatic herbs: C2%, 2% chives; C4%, 4% chives; P2%, 2% parsley; P4%, 4% parsley; Cv2%, 2% *cheiro-verde* and Cv4%, 4% *cheiro-verde*.

As seen on the results, the aromatic herbs retarded the thermal oxidation of cholesterol, where 4% parsley presented the strongest effect among the studied herbs. This fact may be due the phenolic compounds present in these culinary herbs (**Table 2**), and in addition to the highest phenolic compound presented in parsley, and the greatest activity proprieties (**Table 1**). The antioxidant properties depend on several structural features of the molecule of polyphenols in its base structure and are primarily attributed to the high reactivity of hydroxyl substituents. Parsley also presented the highest result of ORAC assay. The advantage of the ORAC assay is that it combines both the inhibition time and inhibition degree of the radical generation, as it takes the oxidation reaction to completion (PRIOR et al., 2003).

The great results of the effects of the bioactive compounds found in parsley and chives in this study are in accordance with previous researches, which reported the their antioxidants activities *in vitro* (ZHANG et al.,2006; WONG e KITTS 2006; PARVU et al.,2010; FARZAEI et al.,2013; VLASE et al.,2013; PARVU et al., 2014; TANG et al., 2015), moreover, in different food model systems (JIA et al.,2012; PEREIRA e TAVANO 2014; SĘCZYK et al., 2015; SĘCZYK et al., 2016).

Other studies have evaluated the addition of natural antioxidants and their potential in the inhibition of lipid oxidation and COPs formation in thermally processed fish. TARVAINEN et al. (2016) evaluated the effect of three different extracts: rosemary leaf extract, oregano leaf extract, and a mixture of extracts of 7 herbs (turmeric, oregano, hops, cloves, sage, ajowan, and licorice, against oxidation of cholesterol in Atlantic salmon fillets during thermal preparation (180 °C for 20 minutes). The total COPs found in the control samples was 14 μg/g lipids. In contrast, lower levels were observed after processing in all samples containing the natural extracts (<1 μg/g lipid), confirming the potential of these natural antioxidants in retarding the thermo-oxidation of cholesterol.

Similarly, the effect of eleven antioxidants including nine phenolic compounds (rutin, quercetin, hesperidin, hesperetin, naringin, naringenin, chlorogenic acid, caffeic acid, ferulic acid), vitamin E (α-tocopherol), and butylated hydroxytoluene (BHT) were selected to investigate their inhibitory effects on thermal oxidation of cholesterol in lard (XU et al., 2016). Their results indicated that the unoxidized cholesterol decreased with heating time whilst cholesterol oxidation products (COPs) increased with heating time. The major COPs produced were 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, and 7-ketocholesterol. Therefore, according to the results caffeic acid, quercetin, and chlorogenic acid displayed the strongest potential to prevent thermal oxidation of cholesterol after 0.5 h of heating.

VAISALI et al. (2016) reported that phenolic compounds (quercetin, rutin and caffeic acid) were also very effective in preventing the oxidation of sardine oil. The mechanism of antioxidant action of phenolic compounds varies considerable from one compound to another. Different phenolics have shown varying efficiency in retarding lipid oxidation in seafood. Fish muscle is a complex system which offers a suitable environment for the lipids to undergo oxidation rapidly (MASQSOOD et al., 2014). As observed in the presented study and in the reported literature, phenolic compounds have the ability to delay the lipid oxidation process through the decay of hydroperoxides (MARINOVA, et al., 2006). As well as the presence of the phenolics like ferulic acid, caffeic acid, have shown preventive effects on lipid oxidation of fish in other study (MAQSOOD e BENJAKUL, 2010).

FERREIRA et al., 2017 also observed the effects of fresh aromatic herbs added as natural antioxidants to air-fried fillet sardines. Their results showed significantly increasing of total COPs from 61.2 (raw) to 283 μg/g ($p< 0.05$) in control air-fried

sardines. However, the sardines added with herbs proved to be effective reducing the levels of COPs in most samples. The best protective effect against lipid oxidation was observed in air-fried sardines with addition of 4% parsley (76.6%), followed by the treatment with 4% *cheiro-verde*.

Due to safety concerns and limitation on the use of synthetic antioxidants, the natural antioxidants are under intensive investigation to be used as safe alternatives to synthetic compounds (SHAHIDI e AMBIGAIPALAN, 2015, DE OLIVEIRA et al., 2018). Thus, the use of these selected plants in this study is being evaluated as potentially effective additives to prevent lipid oxidation in fish and fish products.

3.6 Principal Component Analysis (PCA) of Raw and Grilled Sardines

PCA provides information about the influence of the samples or process variables (**Figure 5b**) on the response variables (**Figure 5a**). The first two components account for 83.5% of variance explained. From **Figure 5a**, it is possible to see a strong correlation among cholesterol, PUFA, EPA, DHA, n-3 and the lower values of total COPs. On the other hand, the control sample presented higher values of total COPs and lower essential FA values. Thus, after grilling, it was observed higher increase in COPs and simultaneous decrease in the PUFA contents, probably as a consequence of the heating applied during the experiment combined with the appreciable amount of cholesterol and PUFAs in sardines. Similar results were found in previous studies with fish (SALDANHA e BRAGAGNOLO, 2007; SALDANHA et al., 2008).

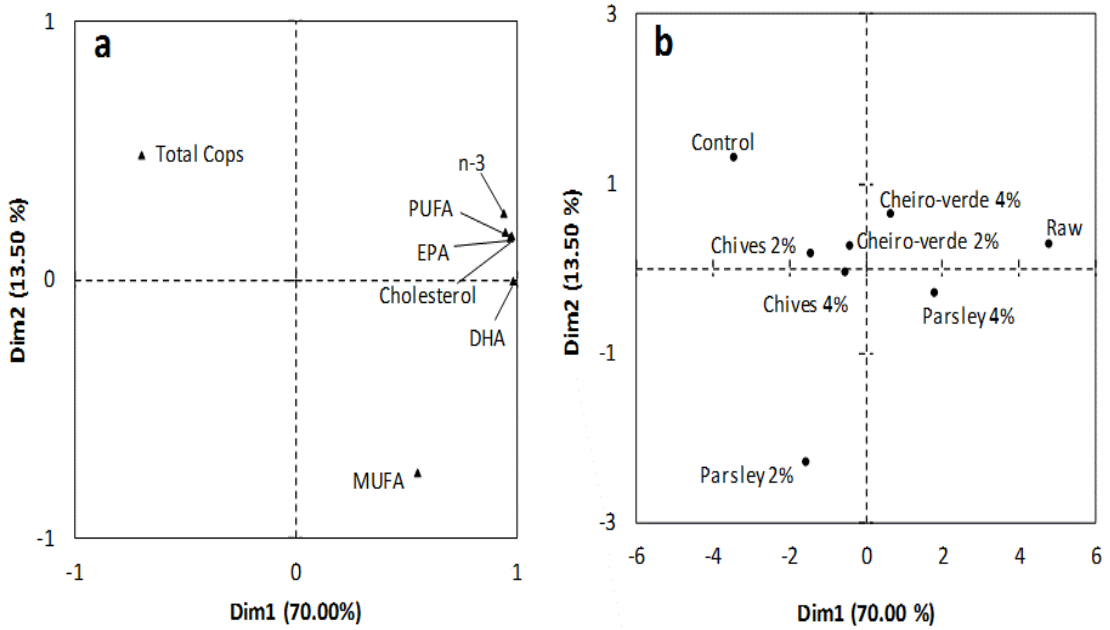

Figure 5. Principal components analysis (PCA) plots as a function of cholesterol oxides, cholesterol and fatty acids. (a) PCA loading plot for response variables (b) score plot for treatments of raw and grille sardines without herbs (control) and with addition of chives, parsley and *cheiro-verde* at two levels (2 and 4%).

In the current study, the PCA plot clearly showed the cholesterol, PUFAs, EPA, DHA and n-3 values in sardines decreased after grilling with concomitant increase of the total COPs content in control samples. However, the use of the aromatic herbs as

natural antioxidants proved to be effective in most samples, preserving PUFAs levels and inhibiting the high levels of COPs. The antioxidant effects of various plants, extracts, herbs, spices, rich in polyphenols, or isolated phenolic compounds have been evaluated to prevent lipid oxidation in fish and fish products (FIGUEIRÊDO et al., 2015; VAISALI et al., 2016; TARVAINEN et al, 2016; XU et al., 2016).

The cut of point to the HPCP suggested four groups by observing the inertia gain in **Figure. 6**. The raw and control samples formed two groups (Cluster 4 and 1, respectively) with one member in each group, as both treatments presented different results. In addition, the treatment of sardines with 2% chives, 4% chives, 2% *cheiro-verde*, 4% *cheiro-verde* and 4% parsley formed Cluster 3; while 2% parsley formed the Cluster 2 (**Figure 6**).

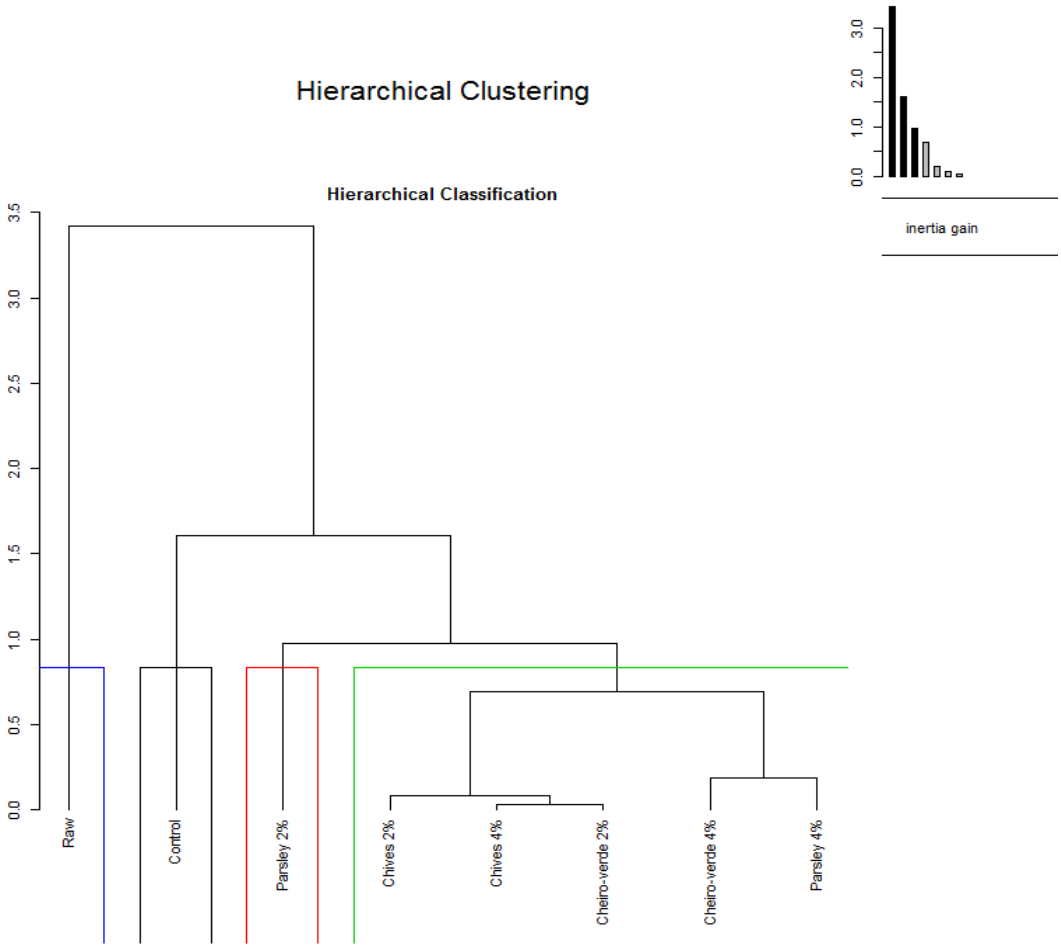

Figure 6. Hierarchical clustering on principal components of cholesterol oxides, cholesterol and fatty acids. The cut-off point of clusters was based on the inertia gain criterion.

The PCA analysis indicates that the addition of herbs protected the sardines during grilling, especially, 4% parsley and 4% *cheiro-verde.* Moreover, plant polyphenolic compounds could be potential additives for preventing lipid deterioration.

4 CONCLUSIONS

The data obtained in this study showed that the grilling in sardine samples presented changes of fatty acids contents mainly EPA and DHA and negative impact with cholesterol oxides formation concomitant decreases of total cholesterol. However, the addition of aromatic herbs proved to be effective against lipid oxidation, especially in sardines added with 4% parsley and 4 % *cheiro-verde*. Concluding, the presence of phenolic compounds, mainly (protocatechuic acid, apiin, *p*-coumaric acid, luteolin, apigenin, cathechin, ferulic acid, rutin, epicatechin and, hesperetin), also the stronger antioxidant properties observed in parsley and *cheiro-verde* corroborated to the efficacy inhibiting the lipid oxidation in grilled sardines. Thus, with the purpose of minimizing the oxidative processes that occur during the thermal preparation of fish, it is recommended to use the fresh herbs tested in this study.

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CONCLUSÕES GERAIS

Tendo em vista os efeitos benéficos à saúde do pescado, aliados às transformações químicas que oorrem durante o processamento térmico, este estudo avaliou duas técnicas de preparo térmico em sardinhas, *air-fryer e grill elétrico*, em combinação com a utilização de fontes alternativas de antioxidantes naturais, como a salsa, cebolinha e *cheiro-verde* visando minimizar a oxidação lipídica durante o processamento térmico, inibindo a formação de produtos de oxidação do colesterol (POCs) nas sardinhas. Os dados obtidos neste estudo mostraram que a caracterização e quantificação dos componentes químicos presentes na salsa, cebolinha e *cheiro-verde* revelaram um alto conteúdo de compostos bioativos devido à composição dos fitosteróis, perfil de ácidos graxos, compostos fenólicos e propriedades antioxidantes. A salsa apresentou melhores efeitos que a cebolinha e o *cheiro-verde* quanto ao potencial antioxidante. Os compostos bioativos presentes nos extratos de ervas aromáticas avaliados apresentaram também ação antioxidante no ensaio *in vivo*, utilizando leveduras. Dentre as três amostras avaliadas, a salsa apresentou o maior efeito redutor do estresse oxidativo causado pelo peróxido de hidrogênio nas células de *S. cerevisiae*. No estudo de sardinhas preparadas termicamente pela panela elétrica (*air-fryer*), os resultados mostraram um grande impacto na qualidade lipídica das sardinhas processadas termicamente, com diminuição significativa nos teores de ácidos graxos poli-insaturados (AGPIs) e formação de altos níveis de produtos de óxidos de colesterol (POCs) no tratamento controle. Por outro lado, o uso de ervas aromáticas, salsa (*Petroselinum crispum*), cebolinha (*Allium schoenoprasum* L) e sua mistura (*cheiro-verde*) mostraram-se eficazes para a maioria das amostras. A adição de 4% de cheiro-verde às amostras de sardinhas apresentou o melhor efeito protetor na oxidação lipídica. Nos resultados de sardinhas processadas no grill elétrico, as amostras de sardinhas apresentaram alterações nos teores de ácidos graxos principalmente EPA e DHA e impacto negativo na formação de óxidos de colesterol, concomitante decréscimo do colesterol total. No entanto, a adição de ervas aromáticas mostrou-se eficaz contra a oxidação lipídica, principalmente nas sardinhas adicionadas com 4% de salsa e 4% de cheiro-verde. Comprovando, que a presença de compostos fenólicos, principalmente (ácido protocatecuico, apiína, ácido *p*-cumárico, luteolina, apigenina, catequina, ácido ferúlico, rutina, epicatequina e hesperetina) presentes nas ervas aromáticas, apresentaram efeitos como antioxidantes naturais inibindo a oxidação lipídica em sardinhas grelhadas. Assim, o efeito protetor dos extratos *in vitro* converge com os resultados obtidos nos testes *in vivo* e com as aplicações das ervas nas sardinhas processadas termicamente, por *air-fryer* e *grill* elétrico. Comprovando que, embora a salsa tenha apresentado os melhores resultados antioxidantes nas análises *in vitro* e *in vivo*, o uso da mistura (*cheiro-verde*) também demonstrou um efeito sinérgico entre a salsa e cebolinha, quando usadas em conjunto, apresentando também bons resultados nas atividades antioxidantes. Sendo que nas aplicações das ervas culinárias nas sardinhas processadas termicamente, os resultados gerais mostraram o melhor efeito nas amostras de sardinhas adicionadas com 4% de salsa e 4% de *cheiro-verde*. Assim, conclui-se que as ervas culinárias avaliadas neste estudo podem ser consideradas como fontes de antioxidantes naturais alternativas, a serem utilizadas na inibição de oxidação lipídica, corroborando para indicar seu uso no combate aos radicais formados no processamento de alimentos.