UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Engenharia de Alimentos

THAIS CRISTINA LIMA DE SOUZA

BIOACTIVE COMPOUNDS IN NEGLECTED AND UNDERUTILIZED PLANTS

COMPOSTOS BIOATIVOS DE PLANTAS ALIMENTÍCIAS NÃO CONVENCIONAIS (PANC)

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COMPOSTOS BIOATIVOS DE PLANTAS ALIMENTÍCIAS NÃO CONVENCIONAIS (PANC)

Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Ph.D. grade, in Food Science.

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Orientadora: Profª. Drª. Helena Teixeira Godoy

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“Quanto mais aumenta nosso conhecimento, mais evidente fica nossa ignorância”

(John F. Kennedy)

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RESUMO

Plantas alimentícias não convencionais (PANC) podem ser classificadas como espécies alimentícias que não recebem a devida atenção por parte da comunidade técnico-científica e da sociedade como um todo, limitando seu consumo por apenas uma parcela da população em determinadas localidades ou regiões do país. Embora não haja um número exato de PANC que ocorrem no Brasil, cerca de 3.000 espécies já foram catalogadas como tais. Dentre elas, temos as folhas de alfavaca (*Ocimum gratissimum* Mill.), beldroega (*Portulaca oleracea* L.), coentro-bravo (*Eryngium foetidum* L.), ora-pro-nóbis (*Pereskia aculeata* Mill.), taioba (*Xanthosoma sagittifolium*), vinagreira (*Hibiscus sabdariffa* L.) e vinagreira roxa (*Hibiscus acetosella*). A literatura aponta que, em geral, as PANC possuem boas características nutricionais quando comparadas com plantas convencionais, além de apresentarem grande quantidade de compostos bioativos. Os compostos bioativos têm sido vastamente estudados devido à sua capacidade de promover benefícios à saúde humana, tais como a redução da incidência de algumas doenças degenerativas. Dessa forma, o levantamento de dados a respeito desses compostos funcionais e suas capacidades biológicas em PANC, podem gerar importantes informações para ampliar a ingestão das mesmas e aplicação destas em diversos setores industriais caso demonstrem expressivo potencial bioativo. Em face disto, este trabalho objetivou otimizar a extração de compostos fenólicos totais (CFT) em plantas classificadas como PANC. Os extratos das diferentes matrizes vegetais, alfavaca, coentro-bravo, beldroega, ora-pro-nóbis, taioba, vinagreira e vinagreira roxa, obtidos a partir da otimização foram avaliados frente a capacidade antioxidante *in vitro*, contra espécies reativas de oxigênio e de inibição da reação de nitrosação e oxidação frente à ação de angiotensina II (Ang II); bem como, avaliados em relação a sua atividade antiproliferativa e antimicroabiana *in vitro*. As plantas também foram analisadas para caracterização de seus compostos voláteis por microextração em fase sólida no modo headspace (HS-SPME) combinado à cromatografia gasosa (GC-FID/MS). O método otimizado demonstrou-se rápido e eficiente para o *screening* dos CFT das plantas, e proporcionou um aumentando entre 2% a 31% dos CFT em comparação com condições não otimizadas. Os resultados apontaram que os extratos foram capazes de inibir à oxidação contra o ácido hipoloroso, peróxido de hidrogênio e radical peroxila, mas não à oxidação por parte do ânion superóxido. No que diz respeito à inibição da reação de nitrosação, os extratos de *O. gratissimum* e *E. foetidum* destacaram entre as amostra, com uma inibição de 78% e 63%, respectivamente, dos compostos nitrosos totais aparentes. Os extratos de *O. gratissimum* também se destacaram na diminuição dos níveis de superóxido intracelular de células do músculo liso vascular. No tocante a avaliação da atividade antiproliferativa, todas as amostras atuaram a reduzir a proliferação das células tumorais testadas, sendo o extrato de *P. oleracea* o que apresentou, no geral, os menores valores de GI_{50}. Apenas os extratos de *O. gratissimum* demonstraram efetiva inibição antimicrobiana para o crescimento da bactéria *Salmonella choleraesuis*, com concentração inibitória mínima igual a 1.0 mg/mL. Os resultados da análise de voláteis evidenciaram a presença de um total de 81 compostos voláteis entre as sete plantas testadas, subdivididos entre as classes do álcool, aldeído, éster, éter, terpenos e cetona. Com base nos dados obtidos, as plantas estudadas apresentam interessantes capacidades bioativas, podendo contribuir para uma dieta mais saudável, além de apresentarem atraentes características para a utilização em diversos setores produtivos, tais como o alimentício, farmacêuticos, nutracêuticos e de cosméticos.

ABSTRACT

Neglected and underutilized species (NUS) receive this classification mostly because of the little attention paid by the technical-scientific community and society as a whole, which reflect on its quite local consumption by small communities or regions of the country. Although the exact number of NUS that occur in Brazil is not known, at least 3,000 species have already been categorized as such. Tree basil (*Ocimum gratissimum* Mill.), purslane (*Portulaca oleracea* L.), culantro (*Eryngium foetidum* L.), Barbados gooseberry (*Pereskia aculeata* Mill.), tannia (*Xanthosoma sagittifolium*), roselle (*Hibiscus sabdariffa* L.) and false roselle (*Hibiscus acetosella*) are examples of NUS. Studies report that NUS, generally, present good nutritional value when compared to conventional plants. Moreover, they comprise significant levels of bioactive compounds, which have been extensively investigated due to their association of lowering the risk of several chronic diseases. Data collection of the functional compounds and biological capabilities over NUS can generate important information to boost their intake and their application in several industrial sectors. Given such background, this study aimed to optimize the extraction of total phenolic compounds in NUS. The phenolic extracts obtained from leaves of tree basil, purslane, culantro, Barbados gooseberry, tannia, roselle and false roselle by the optimized protocol, were evaluated for their antioxidant capacity *in vitro*, against reactive oxygen species, inhibition of N-nitrosation and oxidative stress induced by angiotensin II (Ang II), as well as, their antiproliferative activity and antimicrobial effects. Their volatile compounds were characterized by headspace solid phase microextraction (HS-SPME) combined with gas chromatograph mass spectrometry and flame ionization detector (GC-FID/MS). The optimized method was time-efficient and useful for screening the preliminary TPC of the NUS, increasing TPC up to 2%-31%, when compared to non-optimized conditions. The results indicated that the extracts were able to scavenge induced oxidation by the hypochlorous acid, hydrogen peroxide and peroxyl radical, but not the oxidation induced by the peroxyl radical. Regarding the inhibition of the nitrosation reaction, *O. gratissimum* and *E. foetidum* stood out among the samples with 78% and 63% inhibition effect on apparent total nitroso compounds, respectively. The extracts of *O. gratissimum* also attenuated intracellular superoxide levels in a vascular smooth muscle cell line. All the samples presented antiproliferative activity toward, at least one, a tumor cell. However, *P. oleracea* presented the lowest GI_{50} values, overall. Only *O. gratissimum* extracts exhibited effective inhibition growth towards *Salmonella choleraesuis*, presenting 1.0 mg/mL as the minimum inhibitory concentration. Volatiles characterization demonstrated the presence of a total of 80 volatile compounds presented by the seven plants tested, comprising alcohols, aldehydes, esters, ethers, terpenes and ketones compounds. The finds indicate that the plants have interesting bioactivities and can contribute to a healthier diet. In addition, these plants present attractive characteristics to be exploited in various industrial sectors, such as food, pharmaceuticals, nutraceuticals, and cosmetics.

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CHAPTER II - SELECTED UNDERUTILIZED EDIBLE GREEN LEAVES: ATIOXIDANT, ANTIMICROBIAL, ANTIPROLIFERATIVE AND EFFECT ON ENDOGENOUS N-NITROSATION CAPACITY

INTRODUÇÃO GERAL

Nas últimas décadas, grande atenção tem sido dada aos compostos bioativos, devido à sua capacidade de promover benefícios à saúde humana, tais como a redução da incidência de algumas doenças degenerativas, como câncer e aterosclerose (D'Archivio, et al., 2008), redução dos fatores de risco de doenças cardiovasculares e hipertensão (Wildman & Kelley, 2016), bem como a redução significativa de doenças hematológicas, distúrbios neurológicos e neuropsiquiátricos (Giovannucci, 2002). Essas características benéficas estão relacionadas aos seus efeitos antioxidante, antiproliferativo, antimutagênico, anti-inflamatório, antimicrobiano, anticâncer, entre outros (Ham, et al., 2009; Šeruga, et al., 2011).

A investigação de compostos com capacidade antioxidante apresenta extrema relevância uma vez que, em sistemas biológicos, antioxidantes de origem externa são necessários quando ocorre o estresse oxidativo, isto é, quando a quantidade de radicais livres formados está acima da capacidade que o nosso sistema de defesa possui em combatê-los (Finkel & Holbrook, 2000; Subhasree, et al., 2009). O processo de oxidação também afeta a qualidade dos alimentos e, por esse motivo, antioxidantes sintéticos, como butil hidroxianisol (BHA) e o butil hidroxitolueno (BHT) são amplamente utilizados na indústria de alimentos como potenciais inibidores da oxidação (Antolovich, et al., 2002; Jiang & Xiong, 2016). Contudo, a utilização destes antioxidantes sintéticos está ligada a possíveis efeitos carcinogênicos e, portanto, existe um grande interesse na busca por fontes naturais ricas em compostos antioxidantes (Embuscado, 2015; Yehye, et al., 2015). Neste contexto, também podemos destacar o crescente interesse no uso de compostos voláteis para o desenvolvimento de alimentos funcionais, visando benefícios para a saúde, bem como para redução do emprego de

aditivos sintéticos em formulações alimentícias. Mais recentemente, além de suas propriedades aromatizantes, os compostos voláteis têm sido apontados como potenciais substâncias para promoção da saúde humana, com atuação antioxidante, anti-inflamatória, anticâncer e no tratamento da obesidade e complicações associadas (Ayseli & İpek Ayseli, 2016).

Investigações demonstram que vegetais de folhas verdes contêm importantes componentes bioativos, tais como β-caroteno, ácido ascórbico, riboflavina, ácido fólico e sais minerais, além de apresentarem grande quantidade de polifenóis, como ácidos fenólicos, flavonoides, e compostos voláteis (Kobori & Amaya, 2009; Kim, et al., 2013).

Algumas variedades de plantas no Brasil, classificadas como plantas não convencionais (PANC), fazem parte da dieta de populações rurais e urbanas, mas não recebem devida atenção por parte da comunidade técnico-científica e da sociedade como um todo (Rocha, et al., 2008; Padulosi, et al., 2013). De maneira geral, plantas dessa categoria não são produzidas comercialmente, resultado da globalização e do crescente uso de alimentos industrializados, que vem provocando mudanças no comportamento alimentar dos brasileiros, levando a perda de espaço e mercado destas plantas para espécies mais convencionais e mais facilmente encontradas nos mercados, feiras, etc. (Souza, et al., 2009; Brasil, 2010; EPAMIG, 2011).

Dentre as PANC, encontram-se as folhas de alfavaca (*Ocimum gratissimum*), coentro-bravo (*Eryngium foetidum*), beldroega (*Portulaca oleracea*), ora-pro-nóbis (*Pereskia aculeata*), taioba (*Xanthosoma sagittifolium*), vinagreira (*Hibiscus sabdariffa*) e vinagreira roxa (*Hibiscus acetosella*), que são empregadas para alimentação humana em diversas regiões do Brasil, onde inclusive fazem parte de pratos típicos destas regiões, como é o caso do ora-pro-nóbis (frango com ora-pro-nóbis, em Minas Gerais)

coentro-bravo (tacacá, no Pará) e vinagreira (arroz de cuxá, no Maranhão); são também utilizadas na medicina popular e como plantas ornamentais (Kinupp & Lorenzi, 2015). Embora seu consumo seja difundido em algumas localidades do país, não são encontrados trabalhos, até o momento, que contenham informações sobre a atividade antiproliferativa e capacidade antioxidante, contra espécies reativas de oxigênio, inibição da reação de nitrosação e oxidação pela ação da angiotensina II (Ang II) em células do músculo liso vascular; e poucos trabalhos relatam a composição volátil de tais plantas. Por essas razões, entende-se que a obtenção destas informações podem contribuir para o aumento do consumo destas plantas sub-utilizadas, tanto para alimentação direta, como para aplicação destas em diversos setores industriais. Promovendo assim, efeitos significativamente positivos do ponto de vista econômico, técnico-científico, sociocultural e de segurança alimentar. Com base nisso, o objetivo deste trabalho consistiu em otimizar a extração de compostos fenólicos totais e avaliar a capacidade antioxidante contra espécies reativas de oxigênio, contra oxidação induzida pela angiotensina II e inibição da reação de nitrosação por extratos de folhas de alfavaca, coentro-bravo, beldroega, ora-pro-nóbis, taioba, vinagreira e vinagreira roxa. Além disso, os extratos também foram avaliados frente suas atividades antimicrobianas e antiproliferativas contra células tumorais. Adicionalmente, realizou-se a caracterização dos compostos voláteis presentes nas folhas das setes PANC acima citadas, no qual foi aplicado um Plackett-Burman para seleção das variáveis mais importantes durante a microextração no headspace dos compostos voláteis (HS-SPME).

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Chapter I

SELECTED UNDERUTILIZED AND NEGLECTED PLANTS: PHENOLIC EXTRACTION OPTIMIZATION AND ANTIOXIDANT CAPACITY

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ABSTRACT

This study reports a sequential optimization strategy for extraction of total phenolic compounds (TFC) from five neglected and underutilized plants: *Pereskia aculeata*, *Eryngium foetidum*, *Hibiscus acetosella*, *Portulaca oleracea*, and *Xanthosoma sagittifolium*. To do so, a Placket-Burman (PB) design was performed to screening five variables for the solid-liquid extraction protocol, named: sequential extraction steps, extraction time (min.), temperature (°C), solid-to-liquid ratio (m/v, mg/mL) and methanol concentration (%). Based on PB results, time and methanol concentration were further optimized by Central Composite Rotational Design (CCRD) and the, validated in function of time, resulting in the final optimal conditions of two sequential extraction steps, at 42 ºC, 10 minutes each step, using 85% methanol in water (v/v) as the solvent, and a solid-to-liquid ratio of 1:75 (m/v, g/mL). Under optimized conditions, TPC extraction was increased by 31% for *X. sagittifolium*, 23% for *H. acetosella*, 15% for *E. foetidum*, 5% for *P. aculeata* and 2% for *P. oleracea*, when compared to PB_{12} results. The extracts were also evaluated for their antioxidant capacity *in vitro*, and results demonstrate that their antioxidant capacity is comparable to common vegetables, herbs and spices, which are widely known as good sources of antioxidant compounds. The sequential strategy enabled to obtain a single and time-efficient extraction protocol for all the samples and demonstrated the potential of the underutilized plants as sources of phenolic compounds with great antioxidant capacity.

Keywords: non-conventional, traditional, polyphenols, screening desing, response surface methodology, vegetables

1. INTRODUCTION

Neglected and underutilized species (NUS), also referred by minor, orphan, underexploited, alternative, forgotten, lost and traditional, are so called, because they receive little attention or are entirely ignored by researchers, plant breeders, policymakers and society as whole (Padulosi, et al., 2013; Chivenge, et al., 2015). They are rather locally than globally abundant species, and their current use is limited in relation to its economic potential (Gruère, et al., 2006). Many of these plants are considered invasive weeds and consequently they are removed from agricultural areas without any further use (Kinupp & Lorenzi, 2015). This practice, besides promotes food waste, result on sub-utilization of these plants, that could promoting food supplementation, nutritional diversification and income for poor and marginalized people (Padulosi, Thompson, & Rudebjer, 2013; FAO, 2017)

Brazil is a rich source of plant biodiversity comprising 15 to 20% of all plant species of the world (Barbieri, et al., 2014; Kinupp & Lorenzi, 2015), which includes wild and unexplored kinds, such as purslane (*Portulaca oleracea* L.), culantro (*Eryngium foetidum* L.), Barbados gooseberry (*Pereskia aculeata* M.), (*Xanthosoma sagittifolium* L.) and false roselle (*Hibiscus acetosella* W.). These NUS are consumed as vegetables and are used in folk medicine in some regions of Brazil.

The medicinal properties attributed to these NUS may be related to the presence of phenolic compounds, which are secondary plant metabolites, and thus, ubiquitous in all plant organisms and their derived foods (Dai & Mumper, 2010). The consumption of phenolic compounds, also called polyphenols, is associated to reduced risk of developing non-communicable diseases (Liu, 2013). Beside their health benefits, polyphenols have many industrial applications, as natural colorants and food preservatives, nutraceuticals, pharmaceuticals and cosmetics or even in the production

of paints (Ignat, et al., 2011; Arruda, et al., 2017). The extraction of polyphenols from rich natural sources has become a growing research field, especially because foodstuffs containing artificially produced additives are concerned about the possibility of adverse effects on human health (Pokorný, 2007; Díaz Reinoso, et al., 2012). In this context, NUS offer an unknown potential for use as natural and economic sources of polyphenols, once they are frequently described to concentrate high amount of these compounds (Baldermann, et al., 2016).

Extraction is the first step to recovery and purify phenolic compounds for their qualitative and quantitative characterization for industrial application (Azmir, et al., 2013). The efficiency of polyphenols extraction is strongly influenced by many factor, such as the type and concentration of solvent, extraction time, temperature, pH, solid-liquid ratio, number of extraction and the matrices particle size (Naczk & Shahidi, 2004; Xu, et al., 2017).

Considering the vast diversity of phenolic compounds and their food matrices, there is no single method that is suitable for phenolic extraction of all natural sources (Robbins, 2003; Ajila, et al., 2010; Dai & Mumper, 2010). For this reason, experimental design has been frequently applied to the optimization of phenolic extraction. This chemometric tool enables to determine the effects of multiple variables on the extraction process, as well as the interaction effects between the variables with a small number of experiments (Ferreira, et al., 2007; Hibbert, 2012).

In this context, Plackett–Burman (PB) designs are of great value in screening the most influence variables of complex process. Although PB designs do not allow the construction of predictive mathematical models, the number of experiments compared to a full factorial design is drastically small (Dejaegher & Vander Heyden, 2011; Rodrigues & Iemma, 2014; Borges, et al., 2016). Once the significant variables and

their most suitable domain are determined in the PB design, the optimal conditions are attained by using more complex experimental designs, such as Central Composite Rotational designs (CCRD) (Tarley, et al., 2009). CCRD results enable the development of models and response surface to predict the optimal conditions for depend variables (Dejaegher & Vander Heyden, 2011).

Few works regarding phenolic extraction optimization of *P. oleracea* are found in literature and, to the best of our knowledge, there are no reports of phenolic extraction optimization for leaves of *E. foetidum*, *P. aculeata*, *X. sagittifolium* and *H. acetosella*. The knowledge about the health promoting compounds of NUS may promote their cultivation and commercialization, and also increase their consumption at local and international level. An optimized protocol for phenolic extraction is of great important for preliminary evaluation of NUS potential industrial application as food ingredients, dietary supplements, nutraceuticals, pharmaceutical, and cosmetic products.

In light of this, the aim of this study was to optimize the extraction of total phenolic content (TPC) to obtain a simplified extraction protocol for *P. aculeata*, *E. foetidum*, *H. acetosella*, *P. oleracea*, and *X. sagittifolium*. For this purpose, a sequential extraction process was performed, comprising a screening PB design and optimization by CCRD for maximize the response. The optimal conditions were used to evaluate the antioxidant capacity by a combination of antioxidant assays.

2. MATERIAL AND METHODS

2.1 Plant material

Leaves of *Pereskia aculeata*, *Hibiscus acetosella*, *Portulaca oleracea* and *Xanthosoma sagittifolium* were harvest at Agronomic Institute of Campinas (IAC) (Campinas, Brazil) in January 2016. Leaves of *Eryngium foetidum* were purchased on a

local market in Belém, Brazil in February 2016. The leaves were manually separated from the twigs, washed in tap water and gently dried with absorbent paper. The material was frozen at –80 ºC for 24h then, freeze-dried (Terroni, LS 3000) and grounded by analytical mill (Quimis, Q-298A21).

The moisture percentage of each fresh sample was measured by oven-drying method at 105 ºC (AOAC, 2005) in triplicate. The samples exhibited the following moisture contents: *P. aculeata*, 88.92 ± 0.09 g/100 g; *E. foetidum*, 89.00 ± 0.24 g/100 g; *H. acetosella*, 82.04 ± 0.39 g/100 g; *P. oleracea*, 92.39 ± 0.60 g/100 g; and *X. sagittifolium*, 87.05 ± 0.99 g/100 g.

2.2 Chemicals

Unless stated otherwise, chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol, chloride acid, ferric chloride and sodium carbonate of analytical grade were purchase from LabSynth (São Paulo, SP, Brasil). Folin-Ciocaulteau was purchase from Dinâmica (Diadema, SP, Brazil). Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MS, USA).

2.3 Phenolic extraction procedure

The extraction of total phenolic content (TPC) from the plants materials was carried out using an orbital shaking water bath (Nova Técnica, banho Dubnoff). The freeze-dried samples were weighed, ca. 100 mg, and transferred into Falcon tubes, then, 7.5 mL of aqueous methanolic solution (85%, v/v) were added to the tube. The material was homogenized by vortex for 10 seconds and keep at 42 ºC for 10 minutes under orbital motion (200 RPM). The supernatant was centrifuged at 2182 g, filtered through a qualitative filter paper, and placed in a volumetric flask. The solid residue was re-

extracted once more following the same procedure described above. The supernatants were pooled together and adjusted to a known volume. The extracts were stored (maximum for one day) at –80 ºC prior to analysis.

2.4. Total phenolic compounds optimization

To optimize the extraction of total phenolic compounds from *E. foetidum*, *P. aculeata*, *H. acetosella*, *P. oleracea* and *X. sagittifolium*, a sequential strategy was adopted. Initially, a Plackett-Burman (PB_{12}) screening design was performed in order to select the main variables to increase total phenolic content (TPC). Base on the PB_{12} results, a central composite rotational design (CCRD) was applied for the selected significant variables.

2.4.1 Screening design – Plackett-Burman

Plackett-Burman design was performed in order to investigate the influence of the number of extraction steps (X_1), extraction time (minutes) (X_2), temperature (ºC) (X_3), solid-to-liquid ratio (m/v, mg/mL) (X_4), and methanol concentration (%) (X_5) on total phenolic content. Sixteen independent trials were performed for each plant material, including 12 trials at the upper (+1) and lower (- 1) levels, and 4 trials at the central point (0). The central points were included on the design matrix to evaluate the repeatability of the extraction procedure. Table 1 presents the design matrix and the coded and real values for each variable appraised.

The effect of each variable was analyzed by Protimiza Experiment Design Software. The confidence level adopted for variables significance were 10% ($p<0.1$). Such significance level was used to minimize the probability of exclude an important variable for the DCCR design, as recommended by Rodrigues and Iemma (2014). The

test of curvature was performed in order to evaluate the central conditions of the design matrix.

2.4.2 Central composite rotational design (CCRD)

The significant variables from PB results were further investigated in a CCRD. Methanol concentration (X_1, %) and time (X_2, minutes) were optimized for maximize TPC extractions at four levels of combinations (-α, +1, 0, -1, - α), and 3 replicates on central point, totalizing 11 independent experiments. The independent variables, their nomenclatures in dimensional and dimensionless terms, and their variation ranges are presented in Table 2.

In order to predict TPC, the following second-order polynomial equation (Eq. 1) was fit to the experimental data by multiple linear regression analysis:

$$Y = \beta_0 + \sum_j \beta_j X_j + \sum_{i<j} \beta_{ij} X_i X_j + \sum_j \beta_{jj} X_j^2 + \varepsilon \qquad (1)$$

where y is the predicted response (TPC as mg GAE/g d.w.), β_0 is global mean, β_j is the linear coefficient, ε is the error of the model, X_i and X_j are the coded values of the independent variables.

The good fit of the model and the significance of its coefficients were evaluated by analysis of variance (ANOVA), by Protimiza Experiment Design Software.

Table 1. Plackett-Burman design with coded and real values for the total phenolic content (TPC) from leaves of *P. aculeata*, *E. foetidum*, *H.acetosella*, *P. oleracea* and *X. sagittifolium*.

Run	Variables					TPC (mg GAE/g d.w.)[f]				
	X_1[a]	X_2[b]	X_3[c]	X_4[d]	X_5[e]	*Pereskia aculeata*	*Eryngium foetidum*	*Hibiscus acetosella*	*Portulaca oleracea*	*Xanthosoma sagittifolium*
1	1 (3)	-1 (10)	1 (60)	-1 (1:50)	-1 (0)	8.46	14.72	36.20	15.51	10.77
2	1 (3)	1 (60)	-1 (25)	1 (1:100)	-1 (0)	13.29	17.94	23.41	9.02	13.30
3	-1 (1)	1 (60)	1 (60)	-1 (1:50)	1 (100)	37.88	17.58	42.60	22.50	16.79
4	1 (3)	-1 (10)	1 (60)	1 (1:100)	-1 (0)	17.38	12.37	33.92	15.80	13.08
5	1 (3)	1 (60)	-1 (25)	1 (1:100)	1 (100)	34.1	20.41	57.46	23.08	16.00
6	1 (3)	1 (60)	1 (60)	-1 (1:50)	1 (100)	51.55	20.49	53.73	30.25	16.75
7	-1(1)	1 (60)	1 (60)	1 (1:100)	-1 (0)	5.31	11.94	30.92	13.31	10.58
8	-1 (1)	-1 (10)	1 (60)	1 (1:100)	1 (100)	28.98	17.89	35.00	22.50	19.22
9	-1 (1)	-1 (10)	-1 (25)	1 (1:100)	1 (100)	19.35	13.50	44.45	15.26	14.00
10	1 (3)	-1 (10)	-1 (25)	-1 (1:50)	1 (100)	24.48	18.12	48.98	20.05	16.38
11	-1 (1)	1 (60)	-1 (25)	-1 (1:50)	-1 (0)	6.22	12.34	16.30	5.60	11.46
12	-1 (1)	-1 (10)	-1 (25)	-1 (1:50)	-1 (0)	22.50	11.67	24.39	12.33	13.31
13	0 (2)	0 (35)	0 (42)	0 (1:75)	0 (50)	41.45	16.91	52.88	27.07	13.02
14	0 (2)	0 (35)	0 (42)	0 (1:75)	0 (50)	37.06	18.83	55.62	26.49	16.04
15	0 (2)	0 (35)	0 (42)	0 (1:75)	0 (50)	36.41	17.92	54.68	25.87	13.55
16	0 (2)	0 (35)	0 (42)	0 (1:75)	0 (50)	41.24	17.21	50.33	26.84	15.19

[a] Extraction steps (arbitrary unit); [b] Time (minutes); [c] Temperature (ºC); [d] Solid-to-liquid ratio (m/v, mg/mL); [e] methanol concentration (%); [f] mean values (n = 3).
TPC, total phenolic compounds; mg GAE/g d.w., mg gallic acid equivalents per gram of sample dry weight.

Table 2. Dimensional and dimensionless levels of the independent variables for central composite rotational design (CCRD).

Variables	Levels				
	-1.41	-1	0	1	1.41
Time (minutes)	30	47.45	90	132. 55	150
Methanol concentration (%)	0.0	14.54	50	85.46	100

2.5 Total phenolic compounds (TPC)

The Folin–Ciocalteu spectrophotometric method (Singleton & Rossi, 1965) with some modifications suggested by Singleton, et al. (1999) was used to quantify total phenolic content. Briefly, 25 µL of phenolic extracts/standard solution were mixed with 125 µL of Folin–Ciocalteu reagent (1:10, v/v, aq.) and 100 µL of sodium carbonate (7.5%, w/v, aq.), the last one added after 5 minutes of reaction. The result solution was allowed to react for 2h, and then, the absorbance was recorded at 750 nm (FLUOstar Omega, BMG Labtech). Each extractive solution (methanol 0%, 14.54%, 50%, 85.46% and 100%) used in the experimental design was adopted as blank control for their corresponding trial.

TPC was quantified, in triplicate, on the basis of a standard curve of gallic acid prepared in different extractive solutions, as aforementioned. The results were expressed as mg gallic acid equivalents per gram of sample dry weight (mg GAE/g d.w.). The linear fit of each calibration curve was evaluated by analysis of variance (ANOVA), at 95% confidence level.

2.6 Antioxidant Capacity

Antioxidant capacities were determined by FRAP, DPPH and ABTS assays using a microplate reader (FLUOstar Omega, BMG Labtech). Appropriate blanks were run in each performed assay and the antioxidant capacity was based on standard

calibration curves, prepared with different concentrations of Trolox in hydromethanolic solutions (85% MeOH, v/v). All the experiments were carried out in triplicate and results were expressed as μM Trolox equivalents per gram of dry weight (μM TE/g d.w.).

2.6.1 FRAP antioxidant assay

FRAP (Ferric reducing antioxidant power) was performed according to the methods of Benzie and Strain (1999) adapted for microplate. Briefly, 9 μL of phenolic extract/standard solution were mixed with 27 μL of distilled water and 20 μL of FRAP reagent. The mixture was incubated at 37 ºC for 15 minutes, then, absorbance was recorded at 593 nm. FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ, 10 mM in 40 mM HCl) and $FeCl_3 \cdot 6H_2O$ (20 mM) in the ratio of 10:1:1, respectively.

2.6.2 DPPH radical scavenging assay

Free radical scavenging activities to DPPH (α, α-Diphenyl-β-picrylhydrazyl) were determined as described by Brand-Williams, et al. (1995) with some modifications proposed by Mensor, et al. (2001) and adapted for microplate. Briefly, 180 μL of phenolic extract and 70 μL of DPPH solution (0.3 mM) were mixed together. The absorbance of the resulting solution was recorded at 518 nm at 0, 1 and every 10 minutes until reaction reached a plateau. The final absorbance, corresponding to the plateau, was used to calculate the results.

2.6.3 ABTS radical scavenging assay

ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay was performed following the procedure described by Re, et al. (1999) adapted for microplate. Briefly, ABTS radical was prepared by mixing ABTS stock solution (14 mM) with potassium persulphate (4.9 mM). The radical was kept in the dark at 18 ºC for 12-16h. ABTS solution was diluted in ethanol 50% (v/v) to an absorbance of 0.70 ± 0.02 at 734 nm. Three microliters (3 µL) of phenolic extract were mixed with 297 µL of diluted ABTS solution. The resulting solution was incubated at 30 ºC and absorbance was recorded at 1 minute of incubation and up to 30 minutes of incubation.

3. RESULTS AND DISCUSSION

3.1 Screening of variables

Total phenolic content variation resulting from PB_{12} assays (Table 1) ranged from 5.31 to 51.55 mg GAE/g d.w. for *P. aculeata*; 11.67 to 20.49 mg GAE/g d.w. for *E. foetidum*; 16.30 to 57.46 mg GAE/g d.w. for *H. acetosella*; 5.60 to 30.25 mg GAE/g d.w. for *P. oleracea*; and 10.58 to 19.22 mg GAE/g d.w. for *X. sagittifolium*.

The effects of the independent variables on TPC are presented in Table 3. The effect of each variable varied from -5,43 to 20,54% for *P. aculeata*; -0,15 to 4,50% for *E. foetidum*; 0,25 to 19,51% for *H. acetosella*; -1,21 to 10,34% for *P. oleracea*; and -0,31 to 4,44% for *X. sagittifolium*.

Table 3. Effects of the variables on total phenolic content (TPC), estimated from Plackett-Burman results.

Variables	*Pereskia aculeata*		*Eryngium foetidum*		*Hibiscus acetosella*		*Portulaca oleracea*		*Xanthosoma sagittifolium*	
	Effect (%)	p-value	Effect (%)	p-value	Effect (%)	p-value	Effect (%)	p-value	Effect (%)	p-value
Mean	22.47	0.000005	15.7	0.000000	37.28	0.000000	17.10	0.000000	14.30	0.000000
Curvature	33.15	0.006643	3.94	0.050958	32.19	0.000921	18.93	0.000080	0.30	0.883560
Numbers of extractions	4.85	0.331293	3.19*	0.005370	10.01*	0.014736	3.70*	0.026216	0.15	0.881872
Time (minutes)	4.55	0.360778	2.07*	0.042131	0.25	0.942193	0.39	0.789301	-0.31	0.762500
Temperature (ºC)	4.92	0.324683	0.17	0.853041	2.89	0.406643	5.75*	0.002563	0.46	0.659757
Solid-to-liquid ratio (m/v, g/mL)	-5.43	0.279859	-0.15	0.870486	0.49	0.885299	-1.21	0.406360	0.12	0.909241
Methanol concentration (%)	20.54*	0.001855	4.50*	0.000610	19.52*	0.000238	10.34*	0.000040	4.44*	0.001677

*Variable with significant effect ($p < 0.1$)

Except for *X. sagittifolium*, curvature was statistic significant ($p < 0.1$) for all the samples. This result indicates that there is a maximum extraction of TPC in the central region of the experimental domain. When curvature is not considered during the evaluation of the variables main effects, is high the probability of making an incorrect decision, as to underestimate the significance of a variable (Rodrigues & Iemma, 2014).

Methanol concentration was the main variable, within the ranged studied, to improve TPC for all the samples. This was expected, once solvent composition is one of the major factors influencing the efficiency of phenolic compounds extraction from plant material (Liyana-Pathirana & Shahidi, 2005; Do, et al., 2014; Xu, et al., 2017). The positive effect of this variable suggests that an increase in the percentage of methanol would increase total phenolic extraction from the plants. Methanol concentration was further evaluated on the CCRD, in order to optimize its value to maximize TPC.

Regarding on the number of extraction steps, increasing the steps from 1 to 3 produced a statistically significant positive effect for TPC extraction from *E. foetidum*, *P. oleracea* and *H. acetosella* leaves. According to Galili and Hovav (2014), multiple extraction steps enhance extraction efficiency, and then, total phenolic content. Although extraction efficiency increases dramatically with the first few multiple extractions, the effect quickly diminishes as this variable is still raised (Kolthoff, et al., 1969). This may reflect the low increase in phenolic content (<4%) (Table 1), when 3 steps rather than 2 were applied, for the aforementioned plants. In light of this, this variable was fixed in 2 steps, to minimize solvent consumption, lengthening on the extraction procedure, and phenolic compounds dilution.

Time and temperature were only significant for *E. foetidum* and *P. oleracea* samples, respectively. The increment on time and temperature, normally, promotes phenolic solubility

(Vajić, et al., 2015). As the temperature is increased, the average kinetic energy of the organic solvent also increases, allowing the solvent to diffuse more effectively across the sample matrix, and then, improving the extraction of phenolic compounds (Selvamuthukumaran & Shi, 2017). However, extended extraction times and high temperatures are linked to polyphenols degradation by undesirable reactions, such as enzymatic and oxidative reactions (Khoddami, et al., 2013). The positive effect for temperature promoted a small increase (<4%) (Table 1) on TPC for *P. oleracea*, and only for this sample. Based on this and keeping in mind that some phenolic compounds are thermosensitive (Silva, Rogez, & Larondelle, 2007), the temperature was set at 42 ºC to perform the CCRD, as a compromise between good solubility and prevention of phenolic compounds thermal degradation.

Although time had only significant effect on TPC for *E. foetidum*, this variable was further investigated in the CCRD. The main concern on include time for the CCRD lay down on the fact that the range selected for PB_{12} might not be adequate to leading to a high phenolic extraction. Then, for the CCRD, the range of this variable was extended from 60 to 150 minutes.

Solid-to-liquid ratio had not influence on TPC within the experimental domain. In fact, some works reports that an increase in sample-to-solvent ratio results in a decrease on TPC, mostly because of solvent saturation (Silva, et al., 2007; Bhuyan, et al., 2015; Chotphruethipong, et al., 2017). However, *P. aculeata* leaves produces large amounts of mucilage, a carbohydrate having high water absorption capacity (Martin, et al., 2017); *X. sagittifolium* and *P. oleracea* extracts also exhibited a slightly viscous texture, as also observed for Lim and Quah (2007) for antioxidant extraction from *P. oleracea* cultivars. The viscosity difficults their solubilization when a solid-to-liquid ratio < 1:50 (m/v, mg/mL) is applied, especially using extraction solvent

with low methanol concentration. For this reason and because curvature shows itself significant, the solid-to-ratio was set as 1:75 (m/v, mg/mL) for CCRD trials.

Then, base on these PB_{12} results, time (minutes) and methanol concentration (%) were investigated as independent variables by a central composite rotational design. Remain variables were fixed as following: 2 extraction steps extraction, temperature at 42 ºC and solid-to-liquid ratio as 1:75 (m/v, mg/mL).

3.2 Central composite rotational design (CCRD)

CCRD was performed to establish the optimal conditions of time and methanol concentration to maximize TCP extraction from *P. aculeata*. Since all the samples presented similar responses within PB_{12} experimental domain, CCRD were performed only for *P. aculeata*, because the main variable to affect TPC, i.e. % MeOH, was more crucial for this food matrix, on the matter to avoid mucilage co-extraction.

Total phenolic content for *P. aculeata* (Table 4) varied from 9.92 to 54.76 mg GAE/g d.w. in DCCR trials. The second-order equation for TPC, including only significant terms, is expressed by Equation (1):

$$TPC\ (mg\ GAE/\ g\ d.w.) = 40.70 + 15.42X_2 - 6.93X_2^2 \qquad \text{Eq.(1)}$$

Were X_2 is the coded value for methanol concentration (%).

Analysis of variance (ANOVA) demonstrated the high significant level ($p < 0.05$) of the quadratic model and a non-significant value for lack of fit, revealing the good fit of the model to the experimental data. Moreover, the model presented high value of determination coefficient ($R^2 = 0.9056$), indicating a good correlation between the experimental and predicted values.

The variation on TPC was mostly because methanol concentration, once time-related terms were not statistically significant ($p > 0.05$), within the range of the experimental design. The model suggests that enhancing methanol concentration from 0 to 85%, results in a significantly increase on TPC ($p < 0.05$) (linear effect). However, a further enhancing on methanol concentration, beyond 85%, promotes a significant decrease in TPC (quadratic effect), as can be seen in Figure 1, that presents the TPC versus methanol concentration. Aqueous methanol is usually described as one of the best solvents for phenolic extraction, likely because its polarity covers the varied chemical structures presented by the phenolic compounds, especially for medium to polar phenolic compounds, such as phenolic acids and flavonoids (Babbar, et al., 2014).

Table 4. Central composite rotational design for extraction of total phenolic content (TPC) from *Pereskia aculeata* leaves.

Run	X_1[a]	X_2[b]	Experimental TPC (mg GAE/g d.w.)	Predicted TPC (mg GAE/g d.w.)	Relative Error (%)
1	-1 (47)	-1 (15)	14.57	18.35	-25.96
2	1 (133)	-1 (15)	9.92	18.35	-84.98
3	-1 (47)	1 (85)	54.76	49.19	10.17
4	1 (133)	1 (85)	50.53	49.19	2.66
5	-1.41 (30)	0 (50)	41.47	40.70	1.86
6	1.41 (150)	0 (50)	43.75	40.70	6.96
7	0 (90)	-1.41 (0)	13.10	5.18	60.45
8	0 (90)	1.41 (100)	43.21	48.66	-12.62
9	0 (90)	0 (50)	41.35	40.70	1.58
10	0 (90)	0 (50)	40.81	40.70	0.28
11	0 (90)	0 (50)	40.06	40.70	-1.59

[a]Extraction time (minutes); [b] Methanol concentration (%).

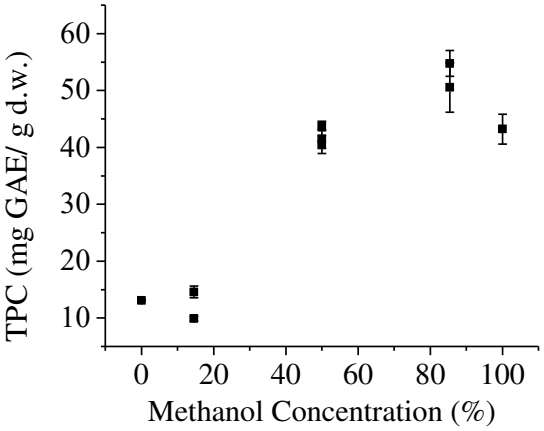

Figure 1. Curve of total phenolic compounds (TPC) (mg GAE/g d.w.) vs. methanol concentration (%) for *P. aculeata* leaves extracts. Values expressed as mean ± standard deviation (n = 3). Vertical bars denote standard error.

3.3 Kinetics of optimized conditions

Optimal conditions, named: 2 extraction steps, temperature at 42 ºC, solid-to-liquid ratio 1:75 (m/v, g/mL) and 85% hydromethanolic solution as the solvent extractor, were used to verify the extraction of TPC as function of time. Figure 2 presents the variation on TPC during 60 minutes of the extraction process. The TPC under optimized conditions is presented in Table 5.

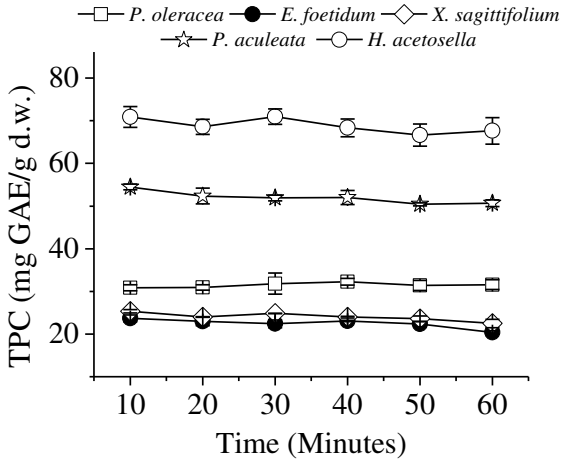

Figure 2: Total phenolic compounds, as a function of time, for leaves of *P. aculeata*, *E. foetidum*, *H. acetosella*, *P. oleracea* and *X. sagittifolium* (mean ± SD, n = 3). Vertical bars denote standard error.

Total phenolic compounds of *P. oleracea* and *H. acetosella* had not significant difference when the extraction time was in the range between 10 to 60 minutes. This behavior is predicted by the Fick's second law, which states that at some moment an equilibrium between the solute concentration in the solid matrix and bulk solution will be achieved and no more analyte will be extracted, even increasing the time of extraction (Silva, Rogez, & Larondelle, 2007).

Conversely, *E. foetidum* had a significant decrease on TPC when 60 minutes was applied for extraction. *X. sagittifolium* and *P. aculeata* had no significant difference between TPC extracted from 10 to 40 minutes. However, when extraction time exceeds 50 minutes, a decrease on TPC was also observed. The decrease in TPC for *E. foetidum*, *X. sagittifolium* and *P. aculeata* is likely related to phenolic degradation due the extended time.

Comparing the greatest TPC value from PB_{12} (Table 1) and the value from the optimized conditions (Figure 2 and Table 5), one can observe an increase from 51.55 to 54.48 mg GAE/g d.w. for *P. aculeata* (5%), from 20.49 to 23.67 mg GAE/g d.w. for *E. foetidum* (15%), 57.46 to 70.86 mg GAE/g d.w. for *H. acetosella* (23%), 30.25 to 30.87 mg GAE/g d.w. for *P. oleracea* (2%) and 19.22 to 25.33 mg GAE/g d.w. for *X. sagittifolium* (31%).

3.4 Antioxidant capacity

The antioxidant capacity exerted by NUS extracts, along with TPC obtained at the optimized conditions, are shown in Table 5. Total antioxidant capacity ranged from 50.63 ± 1.01 to 109.52 ± 10.86 µmol TE/g d.w. for FRAP, 102.96 ± 0.84 to 157.74 ± 2.15 µmol TE/g d.w. for DPPH, and 44.02 ± 2.48 to 200.08 ± 11.52 µmol TE/g d.w. for ABTS.

H. acetosella had the greatest antioxidant capacity measured by FRAP (109.52 ± 10.86 µmol TE/g d.w.) and $DPPH^{\bullet}$ (170.54 ± 1.74 µmol TE/g d.w.), and the second greater antioxidant

capacity for $ABTS^{\bullet+}$ (196.96 ± 11.40 µmol TE/g d.w.), only behind *P. aculeata* $ABTS^{\bullet+}$ scavenging capacity (200.08 ± 11.52 µmol TE/g d.w.). Conversely, *E. foetidum* presented the lowest $DPPH^{\bullet}$ (102.96 ± 0.84 µmol TE/g d.w.) and $ABTS^{\bullet+}$ (44.02 ± 2.48 µmol TE/g d.w.) scavenging capacity. These results appoint for a correlation between antioxidant capacity and total phenolic content, once *H. acetosella* and *E. foetidum* presented the greatest and lowest TPC, respectively.

Actually, a high positive and significant correlation among total phenolic compounds and ABTS ($r = 0.93$, $p < 0.05$) and DPPH ($r = 0.53$, $p < 0.05$) was observed. TPC had no significant correlation with FRAP. Antioxidant capacity assays measure the combined effect of many antioxidants that may be present in the extract, not only phenolic compounds. This could explain the lack of significant correlation between FRAP and TPC. The positive and significant correlation between TPC and both ABTS and DPPH antioxidant capacity highly indicates that phenolic compounds are responsible for the antioxidant capacity presented by the leaves.

Significant variations ($p < 0.05$) were found among the antioxidant capacity presented by some of the leaves. As aforementioned, this difference might be related to the TPC presented by each leave. However, one can note that some leaves presented higher antioxidant capacity, even when compared to others leaves with lower TPC, such as *P. oleracea* and *X. sagittifolium*, which presented higher DPPH scavenging capacity than *P. aculeata.* This is likely related to the phenolic composition of each sample and the fact that antioxidant capacity of foodstuffs occurs by multiple reaction and different mechanisms of their antioxidant constituents (Tabart, et al., 2009) and each phenolic compound can contribute distinctly for the overall antioxidant capacity (Heim, et al., 2002), which can result in a significant variation amongst antioxidant assays.

Table 5. Total phenolic content (TPC) and antioxidant capacity and of *P. aculeata*, *E. foetidum*, *H. acetosella*, *P. oleracea* and *X. sagittifolium* extracts by FRAP, DPPH and ABTS scavenging assays.

Leaves	**TPC** (mg GAE/g d.w.)[1]	**Antioxidant capacity** (μmol TE/g d.w.)[1]		
		FRAP	DPPH	ABTS
Pereskia aculeata	54.48 ± 0.69^{b}	50.63 ± 1.01^{d}	111.05 ± 0.38^{d}	200.08 ± 11.52^{a}
Eryngium foetidum	23.67 ± 0.80^{d}	102.76 ± 2.16^{ab}	102.96 ± 0.84^{e}	44.02 ± 2.48^{c}
Hibiscus acetosella	70.86 ± 2.45^{a}	109.52 ± 10.86^{a}	170.54 ± 1.74^{a}	196.96 ± 11.40^{a}
Portulaca oleracea	30.87 ± 0.70^{c}	91.20 ± 3.05^{bc}	157.74 ± 2.15^{b}	112.23 ± 4.02^{b}
Xanthosoma sagittifolium	25.33 ± 0.38^{d}	101.33 ± 3.81^{abc}	118.58 ± 0.61^{c}	58.86 ± 2.62^{c}

Different letters in the same column denote significant difference ($p < 0.05$) between the samples for the same antioxidant assay, by Tukey test; [1]Values expressed as mean ± standard deviation ($n = 3$); GAE, gallic acid equivalent; TE, trolox equivalent; d.w., dry weight; TPC, total phenolic content; ABTS, 2,2'-azinobis-3-ethylbenzotiazoline-6-sulfonic acid; FRAP, ferric reducing antioxidant power; DPPH, 2,2-diphenyl-1-picryl-hydrazine.

ABTS and FRAP values for NUS extracts are comparable, and even higher, to common vegetables, as spinach, broccoli, green pepper, purple onion and asparagus (Ou, et al., 2002; Tiveron, et al., 2012). DPPH and ABTS values found in this study are much higher than those reported by Wojdyło, et al. (2007) and Assefa, et al. (2018) for selected herbs and spices, which included rosemary, sage, clove, oregano, cinnamon, and many others.

Generally, the antioxidant capacity could be arranged in the following sequence: *H. acetosella* > *P. aculeata* > *P. oleracea* > *X. sagittifolium* > *E. foetidum*. Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and in biological systems. Excessive formation of free radicals accelerates the oxidation of lipids in foods, decreasing food quality and consumer acceptance (Schaich, 2017). The selected underutilized plants demonstrate great *in vitro* antioxidant capacity, suggesting that they might be promising sources of antioxidant to be used as additives in food manufacturing.

4. CONCLUSION

The Plackett-Burman and central composite rotational design were successfully employed to optimize the phenolic compounds extraction from leaves of *P. aculeata*, *E. foetidum*, *H. acetosella*, *P. oleracea* and *X. sagittifolium*. Optimal conditions consisted on 2 extraction steps, temperature at 42 ºC, solid-to-liquid ratio 1:75 (m/v, g/mL), 85% hydromethanolic solution as the solvent extractor and extraction time of 10 minutes. The optimized method was successfully applied to extraction phenolic compounds for investigation of its antioxidan capacity. The method is time-efficient and can be useful for screening the preliminary phenolic content of neglected and underutilized plants and consequently, their potential for technical functionality in industrial applications.

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Chapter II

SELECTED UNDERUTILIZED EDIBLE GREEN LEAVES: ANTIOXIDANT, ANTIMICROBIAL, ANTIPROLIFERATIVE AND EFFECT ON ENDOGENOUS N-NITROSATION CAPACITY

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ABSTRACT

Neglected and underutilized species (NUS) has been appointed as central key to overcome the challenges relate to food security. They can be offer as alternative sources of health promoting compounds, but most of them have been insufficiently studied with regard to their nutritional, technological and bioactive characteristics. In light of this, the present study was conducted to quantify total phenolic content (TPC), vitamin C and evaluate the bioactive potential, including antioxidant, antiproliferative, antibacterial and inhibition N-nitrosation capacity of the leaves of seven neglected plants, named: culantro (*Eryngium. foetidum*), false roselle (*Hibiscus. acetosella*), roselle (*Hibiscus sabdariffa*), tree basil (*Ocimum gratissimum*), *Barbados gooseberry* (*Pereskia aculeata*), *purslane* (*Portulaca oleracea*) and tannia (*Xanthosoma sagittifolium*). Total phenolic content varied from 12.55 mg GAE/g d.w. (*E. foetidum*) to 93.88 mg GAE/g d.w. (*O. gratissimum*). Total vitamin C varied from 5.36 mg AA/100g (*P. aculeata*) to 84.77 mg AA/100g d.w (*X. sagittifolium*). NUS extracts showed high scavenging capacity against HOCl, H_2O_2 and ROO$^{\bullet}$ induced oxidation, but no antioxidant capacity toward $O_2^{\bullet-}$ oxidation. *P. oleracea* presented, in general, the lowest GI_{50} values when compared with the others extracts. Maximum inhibition effect on apparent total nitroso compounds (ATNC) was presented by *O. gratissimum* and *E. foetidum*, nearly 78% and 63%, respectively. These results highlight the potential of the studied NUS as new sources of health-promoting compounds.

Keywords: non-conventional, functional food, polyphenols, NOCs, bioactive.

1. INTRODUCTION

In face of environment changes and growth of world population, project to reach 9.6 billion people in 2050, global food security is one of the greatest challenges of our era (Breene, 2016; Padulosi, et al., 2018). According to FAO (2017), the current rate of agricultural production will not be enough to eradicate hunger by 2050, but expanding food production in a not sustainable way can endanger biodiversity and undermine the world's capacity to meet its food needs. In this scenario, neglected and underutilized species (NUS) represent a great potential to address food security and diversify diets with a more sustainable production system (Patel, 2015; Baldermann, et al., 2016; Patel, 2017).

Neglected and underutilized species (NUS) refer to a category of domesticated, semi-domesticated and wild plants that are traditional managed and consumed in some local communities, contributing to the health status and income of these local populations, as well as their traditions and identity (Padulosi, et al., 2013; Barbieri, et al., 2014; Chivenge, et al., 2015). However, the lack of recognition of these species as food source have been contributing to the fast disappearing of these plants along with a wealth of traditional knowledge about their uses and cultivation (Padulosi, et al., 2013; Bacchetta, et al., 2016).

Cultivation and commercialization of a limited number of species has disturbing effects on global diet. It is estimated that, among the 300,000 edible plants currently available, fewer than 200 are commercially important and only four crops (rice, wheat, maize, and potato) account for more than 60% of the calories consumed by humans worldwide. Such narrow diet diversity has alarming consequences on the nutritional status and can contributes to increase undernourished and malnourished people (Padulosi, et al., 2013). Unbalanced diet and malnutrition have been associated to a more vulnerability to oxidative stress and associated

complications, such as cancer, cardiovascular diseases, inflammatory bowel disease, chronic kidney, lung and liver disease, as well as impaired development and allergies, to name just a few (Benković, et al., 2014; le Coutre, 2014; Liu, et al., 2018).

NUS are often related to contain high concentrations of minerals, proteins, vitamins, and significant percentages of fiber (Aberoumand & Deokule, 2009; Pinela, et al., 2017). They are also reported to contain health-promoting compounds, such as polyphenols, ascorbic acid, terpenoids, polysaccharides, which might be related to their use in folk medicine for alleviating a wide spectrum of diseases (Bacchetta, et al., 2016). Despite their nutritional and folk medicinal importance, there is a lack of scientific evidence to such medicinal properties for most of them, and a great number of NUS have never even been evaluated for biological activities, such as antioxidant, antiproliferative and antibacterial. Information regarding on NUS potentially health-promoting properties could promote their consumption and commercialization, improving dietary diversification and overall human nutrition and health.

For this reason, the aim of this work was to evaluate the bioactive potential, including antioxidant, antiproliferative, antibacterial and inhibition of N-nitrosation capacity of the leaves of seven neglected plants, named: culantro (*Eryngium foetidum*), false roselle (*Hibiscus acetosella*), roselle (*Hibiscus sabdariffa*), tree basil (*Ocimum gratissimum*), Barbados gooseberry (*Pereskia aculeata*), purslane (*Portulaca oleracea*), and tannia (*Xanthosoma sagittifolium*).

2. MATERIAL AND METHODS

2.1 Chemicals

Unless stated otherwise, materials were purchased from Sigma-Aldrich (USA). Fluorescein sodium salt, Tris buffer, HCl, glacial acetic acid, methanol and dimethylsulfoxide

(DMSO) were purchased from Synth (Brazil). Folin Ciocalteu reagent was purchased from Vetec (Brazil). Sodium hypochlorite and hydrogen peroxide were purchased from Dynamics (Brazil). HPLC grade acetonitrile and sulfuric acid were purchased from J. T. Baker (USA). L-ascorbic acid 99% was purschased from Mallinckrodt (USA) and formic acid from Emsure (USA). Ultra-pure water was obtained from a Milli-Q system (Millipore, USA).

2.2 Plant material

Pereskia aculeata M., *Hibiscus acetosella*, *Portulaca oleracea* L. and *Xanthosoma sagittifolium* were harvest at Agronomic Institute of Campinas (Campinas, SP, Brazil) in January 2016. *P. acuelata*, *H. acetosella*, and *P. oleracea* were identified by Dr. Eliane Fabri, and *X. sagittifolium* were identified by Dr. Jose Carlos Feltran from the Agronomic Institute of Campinas. *Eryngium foetidum* L., *Ocimum gratissimum* L. and *Hibiscus sabdariffa* L. were purchased on a local market in Belém, PA, Brazil in February 2016. Voucher specimens (*E. foetidum* L. – IAN 196530, *H. sabdariffa* L. – IAN 196531, *O. gratissimum* L. – IAN 196532) were identified and deposited at the Herbarium of the Botanical laboratory, at Embrapa Eastern Amazon.

Fresh leaves were manually separated from branches, washed with tap water and gently dried with absorbent paper. Then, the leaves were frozen at – 80 ºC (overnight), freeze-dried (Terroni LS 3000, Brazil) and stored at –80 ºC until further analysis. Prior to analysis, the freeze-dried leaves were grounded by analytical mill (Quimis, Q-298A21).

2.3 Phenolic extraction

Grounded freeze-dried leaves were successively extracted with two portions of aqueous methanol (methanol/water; 85/15; v/v) at 1:75 ratio (sample/extract solution; m/v; g/mL). Each extraction stage consisted of homogenization by vortex for 10 seconds, followed by shaking for 10 minutes in a water bath at 42 °C at 120 rpm (Nova Técnica, Dubnoff orbital shaking). The resulting mixture was centrifuged at 2000 g for 10 min and filtered through qualitative filter paper. The supernatants were pulled together and made up to a known volume with an aqueous methanol solution (methanol/water; 85/15; v/v).

For antiproliferative and antimicrobial analysis, the extracts were concentrated under reduced pressure, in a rotary evaporator (Fisatom Rotavapor 0925149, Brazil), at 40 °C. Then, the concentrated extract was freeze-dried (Terroni LS 3000, Brazil) and stored at –80 °C until analysis.

2.4 Determination of total phenolic content

Total phenolic content (TPC) was estimated by colorimetric Folin-Ciocalteu method following the modifications suggested by Singleton, et al. (1999) and adapted for a microplate reader. Briefly, 25 µL of extract/standard solution were mixed with 125 µL of diluted Folin-Ciocalteu in water (1:10, v/v). After 5 minutes, 100 µL of sodium carbonate (7.5%, w/v) was added and the mixture was left to react for 2h in the dark at room temperature. The absorbance of the resulting solution was measured at 760 nm (FLUOstar Omega – BMG Labtech, Germany). Results were expressed as mg gallic acid equivalents per gram of sample dry weight (mg GAE/g d.w.).

2.5 Determination of vitamin C

2.5.1 Extraction of ascorbic acid (AA) and dehydroascorbic acid (DHAA)

The extraction of ascorbic acid (AA) and dehydroascorbic acid (DHAA) were conducted as described by Cunha-Santos, et al. (2018). Briefly, 250 mg of freeze-dried samples were weighed and mixed with 15 mL of 0.05 M sulfuric acid solution. The mixture was shaken for 2 minutes, in orbital shaking water bath (Nova Técnica, banho Dubnoff) at 250 rpm and 25 ± 2 ºC. The extracts were then centrifuged for 10 minutes at 1890 g and 4 ºC. The supernatant was collected and the solid residue was re-extracted with 5 mL of the sulfuric acid solution. The supernatants were pulled together, and filtered through polyvinylidene difluoride (PVDF) membranes (0.22 μm) before chromatographic injection. The reducing procedure was performed in independent triplicates.

DHAA was reduced to ascorbic acid: 1 mL of AA extracts was mixed with 0.7 mL of Tris buffer (pH 8.0) and 0.3 mL of DTT (20 mg/mL). The mixed solution was allowed to stand for 120 minutes at room temperature (~27 ºC). Sulfuric acid (0.4 M) was added to reduce the pH to 2. Extracts were filtered in polyvinylidene difluoride (PVDF) membranes (0.22 μm) and immediately analyzed by UPLC. The reducing procedure was performed in triplicate.

2.5.2 Quantification of ascorbic acid (AA) and dehydroascorbic acid (DHAA)

Quantification of AA and DHAA were conducted by UPLC (Ultra high liquid chromatography), using a Waters ACQUITY system equipped with a binary pump, autosampler, and diode array detector. Separation was performed using a Kinetex C18 column (100 mm × 2.1 mm, 1.7 μm) (Phenomenex) kept at 25 ºC. An isocratic mobile phase composed by 0.1% (v/v) formic acid was used. The flow rate was 0.25 mL/min. and detection was recorded at 245 nm.

AA was identified by co-chromatography, comparing retention time of pure standard and AA extracts, as well as, by comparisons of their absorption spectra on UV-vis. Quantification was procedure on the basis of a standard curve of AA standard prepared in 0.05 M sulfuric acid, at eight concentrations (10 to 150 µg/mL). DHAA content was indirectly determined (Equation 1) by subtracting the total AA concentration from the converted AA by DHAA reduction:

$$DHAA = Total\ AA - AA_{reduced} \qquad \text{Eq. (1)}$$

2.6 *In vitro* antioxidant capacity

All the antioxidant capacity assays were conducted in four independent runs, in six concentrations and each one in duplicate. The absorbance/fluorescence/luminescence was recorded by a microplate reader (BGM Latech, Fluostar Omega, Germany) equipped with a thermostat. Results were expressed as the concentration (mg of freeze-dried sample/mL) required to inhibit 50% of the reactive species, expressed as IC_{50}. IC_{50} was calculated using GraphPad Prism version 5 (San Diego, CA, USA). Gallic acid (0.1 to 1000µg/mL) was used as the standard positive control. A blank and a control solution were run with each assay.

2.6.1 Peroxyl radical scavenging assay

Peroxyl radical ($ROO^{\bullet}$) scavenging capacity was expressed as the Oxygen Radical Absorbance Capacity (ORAC) following the protocol of Dávalos, et al. (2004). AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) was used as $ROO^{\bullet}$ generator and fluorescein as the fluorescent probe. The mixture of 20 µL phenolic extract/standard solution, 120 µL fluorescein (70 nM), and 60 µL AAPH (12 nM) was pre-incubated at 37 ºC. Fluorescence decay, resulting from fluorescent probe damage by $ROO^{\bullet}$, was monitored every minute, on a total of 80 minutes,

by florescence record at 485 nm (excitation wavelength) and 520 nm (emission wavelength). ORAC results were expressed as the net area under the fluorescein kinetic decay curve, expressed as μM TE/g d.w.

2.6.2 Superoxide radical scavenging assay

Superoxide radical ($O_2^{\cdot -}$) was generated by a non-enzimatic system name NADH/PMS/O_2. Nitro blue tetrazolium (NBT) was used as the oxidizable probe. The assay was conducted according to Gomes, et al. (2007). For the analysis, 50 μL of extract at different concentrations, 50 μL of NADH (996 μM), 150 μL of NBT (86 μM) and 50 μL of PMS (16.3 μM) were placed into the microplate. The plate was incubated at 37 ºC for 2 min and the absorbance was recorded at 560 nm.

2.6.3 Hypochlorous acid scavenging activity

The hypochlorous acid scavenging activity (HOCl) was dertermined according to Gomes, et al. (2007). HOCl was generated by adjusting NaOCl solution (1%, w/v) to pH 6.2 with H_2SO_4 (10%, v/v). Dihydrorhodamine 123 (DHR) (2887 μM) in DMF was the probe used to undergo HOCl-induced oxidation. The concentration of HOCl was determined spectrophotometrically at 235 nm, using the molar coefficient of 100 $M^{-1}cm^{-1}$. Immediately prior to the analysis, a 30 μM DHR working solution in 100 mM phosphate buffer (pH 7.4) was prepared. The reaction media contained: 150 μL phosphate buffer (pH 7.4) (100 mM), 50 μL phenolic extract, 50 μL DHR (5 μM), and 50 μL HOCl (5 μM). The mixture was incubated at 37 ºC and the fluorescence was recorded at 528 nm (emission wavelength) and at 485 nm (wavelength excitation).

2.6.4 Hydrogen peroxide scavenging assay

Hydrogen peroxide (H_2O_2) scavenging capacity was performed by chemiluminescence methodology, as described by Gomes, et al. (2007). This method measures the effect of the extracts or/and standards upon the oxidizable probe, lucigenin. Briefly, 91.5 µL tris buffer (pH 7.4) was added to the plate, mixed with 50 µL phenolic extract, 100 µL lucigenin (0.8 mM), and 8.5 µL of H_2O_2. The plate was incubated at 37 ºC and the luminescence was recorded immediately and after 5 minutes of incubation.

2.7 Minimal inhibitory concentration (MIC)

The method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2008, CLSI, 2015a, CLSI, 2015b) was performed to evaluate the antimicrobial effects of the freeze-dried extracts, determining the minimal inhibitory concentration (MIC) using the microdilution in a 96-well plate test. The samples were tested for the following microorganisms: *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538), *Salmonella choleraesuis* (ATCC 10708), *Streptococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 13388), all of them cultivated in nutrient agar (NA), and *Candida albicans* (ATCC 10231), cultivated in potato dextrose agar (PDA). The inocula for the assays were prepared by diluting scraped cell mass into 0.85% NaCl (m/v) solution, adjusting to McFarland scale 0.5, and confirmed by spectrophotometric method at 625 nm. Cell suspensions were finally diluted to 10^6 UFC/mL in Müller–Hinton Broth (DIFCO®).

The samples were diluted in 40% alcohol to 8 mg/mL, and transferred to the first well, before serial dilutions were performed to obtain concentrations between 15.62 and 2000 µg/mL. Chlorhexidine 0.12% (0.5 mg/mL, Sigma-Aldrich) and nystatin (1 mg/mL, Sigma-Aldrich) were

used as the positive control for antibacterial and antifugal activity, respectively; 40% alcohol was used as the negative control. The bacterial inocula (colony-forming units (CFU)/mL) and fungal inocula (CFU/mL) were added to the wells (100 µL/well) and the plates were incubated at 37 ºC for 24 h. MIC was determined as the lowest concentration of the extract that inhibited visible microbial growth, which was confirmed with 0.01% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich) for bacteria and by the change in color of the RPMI 1640 for yeast.

2.8 *In vitro* antiproliferative activity

The antiproliferative activity was performed as described by Monks, et al. (1991) on nine human cell lines: U251 (glioma), UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian expressing phenotype of multiple drugs resistance), 786-O (kidney), OVCAR-03 (ovarian), HT-29 (colon adenocarcinoma), K562 (leukemia), NCI-H460 (non-small cell lung cancer), cell lung cancer (NCI-H460) and a non-tumor cell line HaCat (human keratinocyte), purchased from National Cancer Institute (Frederick, MD, USA). Stock cultures were grown in 5 mL of RPMI-1640 supplemented with 5% fetal bovine serum (RPMI/FBS 5% Gibco, USA) at 37 ºC in 5% CO_2 with a 1% penicillin:streptomycin mixture (Nutricell1, Brazil, 1000 U/mL).

Freeze-dried extracts (10 mg) were diluted in DMSO (100 µL) and then, successively diluted in RPMI/FBS 5% to a final concentration of 0.25, 2.5, 25 and 250 µg/mL. Doxorubicin was used as a positive control at 0.025, 0.25, 2.5 and 25 µg/mL. Cells in 96-well plates (100 µL cells/well) were exposed to the samples and control for 48 h at 37 ºC and 5% CO_2, in triplicate. Before (T_0 plate) and after (T_1 plate) sample treatment, cells were fixed with 50% trichloroacetic acid, and submitted to sulforhodamine B assay for cell proliferation quantitation at 540 nm. The

GI_{50} (concentration that inhibits 50% cell growth or cytostatic effect) were determined to a nonlinear regression, type sigmoidal, using Origin 8.0 software (OriginLab Corporation, USA).

2.9 *In vitro* effect on the formation of nitroso compounds (NOC)

The samples were digested by simulated static *in vitro* model, based on Kuhnle, et al. (2007). The simulated gastric model was applied for samples and control (absence of the samples), as following: Freeze dried sample (ca. 0.5 g) was mixed with 20 mL of simulated gastric juice, containing NaCl (34 mM), pepsin from porcine (3.2 mg/mL), myoglobin (0.4 mg/mL), haemoglobin (0.13 mg/mL), and bovine serum albumin (0.05 mg/mL). Then, pH was adjusted to 2.2 with HCl 1M. The nitrosation reaction was started by adding $NaNO_2$ (0.015 mg/mL). The mixture was incubated in the dark, at 37 °C for 30 minutes. NOC production was measured as apparent total nitroso compounds (ATNC).

The ATNC from the *in vitro* digestion model were analyzed by chemiluminescence (CLD 88 Exhalyzer, Eco Medics, Switzerland). The analyzer was connected to a purge vessel and an ice cold NaOH trap. Briefly, 50 µL of digested material was incubated with 500 µL of sulphanilamide (50 mg/mL in 1 M HCl) for 3 minutes, to remove unbound nitrite. Then, 200 µL of this solution was injected into a purge vessel containing iodine/iodide reagent and kept at 60 °C. The cleavage of nitrosated compounds releases NO into the gas phase that was dragged by a helium stream to a scrubbing bottle containing 1 M of ice cold sodium hydroxide (NaOH). The cold NaOH traps traces of acid and iodine, while NO vapor reaches the analyzer, emitting light that can be quantified.

Discrimination between contribution of nitroso compounds NOC classes to ATNC, N-nitrosos, nitrosothiols, and nitrosyl heme compounds, was accomplished by reaction with group-

specific reagents: mercury (II) stable nitroso compounds (nitrosothiols) and potassium ferricyanide stable nitroso compounds (nitrosyl heme), determined under exactly the same way as described above with an additional step after the sulphanilamide reaction step, in which there was a additional incubation with $HgCl_2$ (53 mmol/L, 100 mL for 2 min) and $K_3Fe(CN)_6$ (4 mM, 100 mL for 2 min), for determination of nitrosothiols and nitrosyl heme, respectively. Calibration curves were obtained injecting freshly prepared sodium nitrite solution. Analyses were carried out in triplicate. Results were expressed as micromoles of NO released. Nitroso compounds were expressed as μM sodium nitrite, NO (μM).

2.10 Statistical analysis

All analyses were run in triplicate unless stated otherwise. Data were expressed as mean values with standard deviations. Linear fit of the standard calibration curves was evaluated by one-way analysis of variance (ANOVA), at 95% confidence level. When applied, comparison of the results was also made by ANOVA, followed by Tukey's test, with significant level of 5%, using Statistica 7.0 software (StatSoft Inc., Tulsa).

3. RESULTS AND DISCUSSION

3.1 Total phenolic content (TPC) and total vitamin C

Table 1 presents the total phenolic content (TPC) and vitamin C of the samples. *O. gratissimum* (93.88 mg GAE/g d.w.) presented the highest TPC, followed by *H. acetosella* (57.55 mg GAE/g d.w.), *P. aculeata* (42.04 mg GAE/g d.w.), *H. sabdariffa* (34.15 mg GAE/g d.w.), *P. oleracea* (27.85 mg GAE/g d.w.), *X. sagittifolium* (22.34 mg GAE/g d.w.) and *E. foetidum* (12.55 mg GAE/g d.w.). When compared with other fruits and vegetables, *O.*

gratissimum, H. acetosella and *P. aculeata* have a similar TPC as berries, such as strawberry (41.35 mg of GAE/g d.w.), raspberry (35.49 mg of GAE/g d.w.) and blueberry (35.40 mg of GAE/g d.w.) (Wu, et al., 2004); and some herbs, such as oregano (44.08 mg of GAE/g), rosemary (40.87 mg of GAE/g d.w.) and sage (36.79 mg of GAE/g d.w.) (Assefa, et al., 2018), which are well-known good sources of phenolic compounds.

Phenolic compounds are effective antioxidant constituents of plant foods that have been pointed out as one of the main phytochemical compounds related to biological properties in fruits and plants (Sun et al. 2002). A portion size of only 10 g (fresh weight) of *O. gratissimum and H. acetosella* leaves could account for 40% and 27%, respectively, of the mean intake of phenolic compounds in Brazil, which is estimated at 377 mg polyphenols/day (Miranda, et al., 2016).

Vitamin C is one of the highest effective water-soluble antioxidants present in foods (Carr & Maggini, 2017; Cunha-Santos, et al., 2018) comprising L-ascorbic acid (AA) and its degradation product, the dehydroascorbic acid (DHAA). Both ascorbic acid and dehydroascorbic acid exhibit biological activity of vitamin C (Mazurek & Jamroz 2014), so it is important to measure both molecules in order to predict total vitamin C.

The content of ascorbic acid (AA) of the samples varied from 2.98 mg AA/100g d.w for *E. foetidum* to 50.61 mg AA/100g d.w for *H. acetosella.* Dehydroascorbic acid (DHAA) results varied from 4.53 mg AA/100g d.w. for *P. oleracea* to 63.99 mg AA/100g d.w for *X. sagittifolium.* Giving a total of vitamin C ranging from 5.36 mg AA/100g d.w for *P. aculeata* to 84.77 mg AA/100g d.w for *X. sagittifolium*.

Table 1. Total phenolic compounds, total vitamin C (ascorbic acid and dehydroascorbic acid), and *in vitro* scavenging capacity for peroxyl radical ($ROO^{\bullet}$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet-}$) of *E. foetidum, H. acetosella, H. sabdariffa, O. gratissimum, P. aculeata, P. oleracea* and *X. sagittifolium* extracts.

	TPC (mg GAE/g d.w.) [1]	**Vitamin C** (mg AA/100g d.w.)[2]		**IC_{50}** (µg/mL)[3]			**ORAC** (µmol TE/g d.w.)[4]
		AA	DHAA	HOCl	H_2O_2	$O_2^{\bullet-}$	$ROO^{\bullet}$
E. foetidum	12.55±1.06^{f}	2.98±0.22^{e}	10.45±0.41^{c}	13.96±1.12^{d}	n.a	n.a	572.90±9.02^{d}
H. acetosella	57.55±5.13^{b}	50.61±0.60^{a}	n.d	19.89±0.63^{b}	388.90±35.08^{a}	n.a	778.63±18.79^{c}
H. sabdariffa	34.15±1.13^{d}	50.32±0.32^{a}	30.95±0.35^{b}	13.92±0.66^{d}	423.00±45.82^{a}	n.a	732.99±63.80^{cd}
O. gratissimum	93.88±3.74^{a}	10.26±0.86^{d}	4.75±0.42^{d}	20.35±1.69^{b}	n.a	n.a	1944.82±124.06^{a}
P. aculeata	42.04±1.61^{c}	5.36±0.39^{e}	n.d	16.94±1.18^{c}	n.a	n.a	1327.68±126.01^{b}
P. oleracea	27.85±1.75^{de}	23.87±2.31^{b}	4.53±1.58^{d}	16.84±0.57^{c}	n.a	n.a	590.31±39.43^{cd}
X. sagittifolium	22.34±0.33^{e}	20.78±0.92^{c}	63.99±0.72^{a}	35.21±0.94^{a}	n.a	n.a	632.26±33.70^{cd}
Gallic acid	–	–	–	4.12±0.15^{e}	203.00±15.32^{b}	118.76±20.84	–

Values expressed as the mean ± standard deviation (n = 3); n.d., not detected; n.a., no activity at the highest concentration tested (4 mg/mL).
[1]expressed as mg of gallic acid equivalents per gram of sample dry weight; [2]expressed as mg of ascorbic acid equivalents per 100 gram of sample dry weight; [3]IC_{50}, Inhibitory concentration to decrease by half the oxidative effect of the reactive specie expressed as mg of freeze-dried sample/mL; [4]expressed as µM Trolox equivalents per gram of sample dry weight;
AA, ascorbic acid; DHAA, dehydroascorbic acid; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; $O_2^{\bullet-}$, superoxide radical; $ROO^{\bullet}$ peroxyl radical.
Different letters in the same column denote significant ($p \leq 0.05$), by Tukey test, difference between the samples.
(–) not determined.

According to Spinola et al. (2014), DHAA represents less than 10% of total vitamin C in fresh horticultural products. However, AA is rapidly oxidized to DHAA, especially during storage, freezing or freeze-drying processes, which may be linked to the high concentration of DHAA presented by *X. sagittifolium* and *H. sabdariffa* leaves. Although DHAA exhibits active form of vitamin C, it presents less extension of activity which is about 80% of the vitamin C activity presented by AA (Cunha-Santos, et al., 2018). Moreover, DHAA has no radical scavenging activity, which highlight the importance to consume vegetables as fresh as possible (Wawire, et al., 2011).

Total vitamin C content of *X. sagittifolium, H. sabdariffa* and *H. acetosella* were comparable of those reported for apples (2.7 mg/100g w.b.), peaches (5.8 mg/100g w.b.) and lettuce (10.6 mg/100g w.b.), but were much lower than other fruits and vegetables, such as spinach (25.7 mg/100g w.b.), oranges (57.9 mg/100g w.b.) and broccoli (95.9 mg/100g w.b.) (Phillips, et al., 2018).

3.2 Antioxidant capacity

The scavenging capacity against HOCl, H_2O_2, $O_2^{\bullet-}$ and ROO exerted by the samples extracts is summarized in Table 1. Plant extracts presented effective protection against HOCl and H_2O_2 in a concentration-dependent manner (Figure 1).

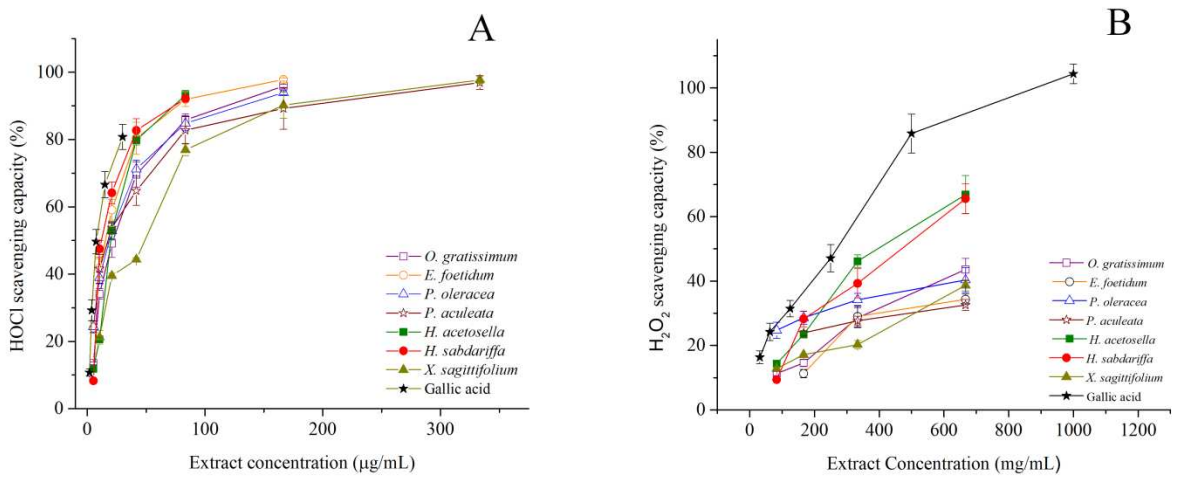

Figure 1. *In vitro* scavenging capacity for hypochlorous acid (HOCl) (A) and hydrogen peroxide (H_2O_2) (B) of *E. foetidum, H. acetosella, H. sabdariffa, O. gratissimum, P. aculeata, P. oleracea* and *X. sagittifolium* extracts. Values expressed as the mean ± standard deviation (n = 3). Vertical bars denote standard error.

The extracts promoted effective protection against HOCl, which presents a very fast reaction rate with various compounds in biological systems, such as sulfhydryl, polyunsaturated fatty acids, DNA pyridine nucleotides and amino acids. Moreover, HOCl is approximately 100–1000 times more toxic than $O_2^{\bullet-}$ and H_2O_2 (Conner & Grisham, 1996). *E. foetidum* and *H. sabdariffa* presented the lowest IC_{50} against HOCl (Table 1). Although *X. sagittifolium* presented the highest IC_{50}, i.e, the lowest scavenging capacity against HOCl, this extract was more efficient than 5-caffeoylquinic acid (56 µg/mL) and trolox (134 µg/mL) standards (Rodrigues, et al., 2013) for scavenging HOCl.

Except for *Hibiscus* species, the extracts were able to decrease the oxidizing effect of H_2O_2 by only 34–43%, at the highest concentration tested (4 mg/mL). It was already reported that the IC_{50} values needed for inhibiting the harmful effect of H_2O_2 are generally greater than those shown for other ROS (Pistón, et al., 2014; Berto, et al., 2015). *H. acetosella* and *H. sabdariffa* (Table 1) presented effective protection against H_2O_2, which was higher than those

reported for *Theobroma grandiflorum* (IC_{50} 700 μg/mL), *Spondias lutea* (IC_{50} 526 μg/mL) (Vissotto, et al., 2013) and quercetin standard (IC_{50} 509 μg/mL) (Berto, et al., 2015).

Although H_2O_2 alone is very little reactive, H_2O_2-induced oxidation is related to oxidative stress implications, once H_2O_2 may cross cell membranes and react with transition metals, producing hydroxyl radicals ($^{\bullet}OH$), which is considered one of the most reactive species in biological systems (Pistón, et al., 2004). Therefore, *H. sabdariffa* and *H. acetosella* phenolic extract may be significant for human health and may have potential to be used as pharmaceutical and antioxidants in food system.

$O_2^{\bullet-}$ is another important reactive specie to be considered in the implications of oxidative stress. This reactive specie is not considered as a potent pro-oxidant, but it is a key point in the oxidative stress as a primary generated ROS. $O_2^{\bullet-}$ may be dismutated by the superoxide dismutase (SOD) to H_2O_2 or by metal-catalyzed processes producing $^{\bullet}OH$. Plants/extracts which present high capacity to scavenge this reactive specie is highly desirable, once they can avoid the cascade of ROS formation (H_2O_2, OH, and $^1O_2$) and then inhibiting oxidative damage in lipids, proteins, and DNA (Gülçin, et al., 2006). None of the extracts tested in the present study could inhibited the formation of formazan induced by $O_2^{\bullet-}$ oxidation at the maximum concentration tested (4 mg/mL).

In general, the scavenging capacity towards $ROO^{\bullet}$ (as ORAC value) were high. The extracts activities were comparable to fruits and plants well known to possess high antioxidant capacity. Especially, for *O. gratissimum* and *P. aculeata*, which presented antioxidant capacity higher than reported for açaí (1014.67 μM TE/g d.w.) (Kang, et al., 2011), raspberry (568.8 μM TE/g d.w.) and strawberry (527.2 μM TE/g d.w.) (Wang & Lin, 2000).

The results of antioxidant capacity underline the interest for these underutilized plants as a new source of antioxidants that should be specifically studied for its potential effects on human health and its potential application in food and drug technologies. Correlation analysis demonstrated that the scavenging capacity of the extracts is more dependent on the phenolic compound contents than on the vitamin C contents, especially towards ROO•, which present significant positive correlation to TPC (r = 0.84) and non-significant correlation with vitamin C content (r = 0.43) at 95% of confidential level.

3.3 Minimal inhibitory concentration (MIC)

The results of MIC (Table 2) demonstrated that none of the phenolic extracts could inhibit growth of the gram-positive bacteria, named *P. aeruginosa*, *S. aures*, *and B. subtilis*. While the yeast (*C. albicans*) and the gram-negative bacteria, expect for *P. aeruginosa,* were sensitive to antimicrobial effects of some samples.

Gram-negatives bacteria present a peptidoglycan cell wall that is surrounded by an outer membrane. While, gram-positive bacteria lack this outer membrane, and then, are more sensitive to antimicrobial agents (Stover, et al., 2000; Silhavy, et al., 2010). Even though the outer membrane of gram-negative bacteria hinders the penetration of the antimicrobial compounds, the results demonstrated these bacteria were more sensible to the plants extracts tested.

In general, there was a correlation between total phenolic content and antimicrobial activities. Extracts of *X. sagittifolium* and *E. foetidum*, which presented the lowest TPC, could not inhibit any microbial growth, even at the maximum concentration tested (2 mg/mL). Among the extracts that exhibited antimicrobial activity, *Hibiscus sadbariffa* and *Ocimum campechianum* stand out as the extract with the highest inhibition spectrum and the extract

presenting the lowest MIC, respectively. Although *H. acetosella*, *P. oleracea*, *P. aculeata* and *H. acetosella* could inhibit bacterial and yeast growth, phytochemicals can only be classified as antimicrobials when producing a minimum inhibitory at extract concentrations from 100 to 1000 µg/mL (Simoes, et al., 2009). Considering this classification, only *O. gratissimum* presented effective inhibit growth of *S. choleraesuis*. In fact, *Ocimun* species are well recognized as antimicrobial agents (Politeo, et al., 2007; Pandey, et al., 2014; Baldim, et al., 2018; Piras, et al., 2018), although to date, there is no data reporting the antimicrobial activity by phenolic extract of *O. gratissimum* Mill, to the best of our knowledge.

Usually, phytochemicals present higher MIC (100–5000 µg/mL) when compared with antibiotics (0.031–512 µg/mL), for this reason, they are not suitable to be used in monotherapy (Barbieri, et al., 2014). However, there is a growing interest in developing products that combine chemicals and natural compounds traditionally used in medicine, as a co-administration of antimicrobial and an inhibitor that modulates or modifies the bacterial resistance mechanism, thus increasing the effectiveness of the antimicrobial product (Harvey, 2008; Abreu, et al., 2012). Therefore, *O. gratissimum* is a promising source to prospect research as a resistance-modify and preservative agent, with particular high antioxidant activity *in vitro.*

Table 2. Minimum inhibitory concentration (MIC) of *E. foetidum, H. acetosella, H. sabdariffa, O. gratissimum, P. aculeata, P. oleracea* and *X. sagittifolium* extracts.

Extracts	**Minimum inhibitory concentration (MIC) (mg/mL)[a]**						
	Candida albicans	*Escherichia coli* (–)	*Pseudomonas aeruginosa* (–)	*Salmonella choleraesuis* (–)	*Staphylococcus aureus* (+)	*Bacillus subtilis* (+)	*Streptococcus epidermides* (+)
E. foetidum	*	*	*	*	*	*	*
H. acetosella	*	2.0	*	*	*	*	*
H. sabdariffa	2.0	2.0	*	2.0	*	*	*
O. gratissimum	*	*	*	1.0	*	*	*
P. aculeata	*	*	*	2.0	*	*	*
P. oleracea	2.0	*	*	2.0	*	*	*
X. sagittifolium	*	*	*	*	*	*	*
Control[b]	0.002	0.004	0.062	0.004	0.008	0.004	0.008

*MIC > 2.0 mg/mL; [a]expressed mg of freeze-dried extract/mL; [b]chloramphenicol (0.5 mg/mL) for bacteria and nystatin (1.0 mg/mL) for yeast. (–), gram negative; (+) gram positive. Data calculated from a mean (n = 3)

3.4 Antiproliferative activity against human tumor cell lines

The antiproliferative activity (Table 3) was expressed as the growth inhibition 50 (GI_{50}), i.e., the concentration required to inhibit cell proliferation by 50%, in which a lower value indicates a higher activity. The antiprolifarative values of the extracts ranged from 3.6 µg/mL, for *P. oleracea* against ovarian (OVCAR-03) cell line, to 212.3 µg/mL, for *O. gratissimum* against ovarian (OVCAR-03) cell line.

All the samples, except for *H. sabdariffa*, exhibit growth arrest of leukemia (K562) cell line (Table 3). According to Fouche, et al. (2008), the antiproliferative activity of a sample may be classified as inactive (log GI_{50} > 1.50 µg/mL), weak activity (1.50 µg/mL ≥ mean log GI_{50} > 1.10 µg/mL), moderate activity (1.10 µg/mL > mean log GI_{50} > 0 µg/mL) and potent activity (mean log GI_{50} < 0 µg/mL). Considering this criteria, *H. acetosella* and *X. sagittifolium* extracts showed a selective, but weak activity for leukemia (K562) with log GI_{50} 1.4 µg/mL and 1.14 µg/mL, respectively. *P. oleracea* extract showed moderate antiproliferative effect against ovarian expressing phenotype of multiple drugs resistance (NCI-ADR/RES) (log GI_{50} 0.98), kidney (786-O) (log GI_{50} 0.93) and ovarian (OVCAR-03) (log GI_{50} 0.56) cells. *O. gratissimum* and *P. aculeata* extracts have the ability to reduce the proliferation of glioma (U251), kidney (786-O) and leukemia (K562), but with a weak activity (log GI_{50} > 1.17). *E. foetidum* extracts was the only one providing cytostatic activity against melanoma (UACC-62) line with log GI_{50} 1.48. None of the extracts could actively inhibit the proliferation of non-small cell lung cancer (NCI-H460), breast (MCF-7), and colon adenocarcinoma (HT-29) tumor related cells. The antiproliferative results also suggest no toxic effects exerted by the plants extracts on the proliferation of non-tumor cells of keratinocytes (HaCat). A successful anticancer molecule should kill or incapacitate cancer cells while having reduced toxicity toward normal tissues (Sharma et al. 2016).

Although all the plants extracts exhibit activity against some tumor cell lines, *P. oleracea* stood out among the samples presenting, in general, lower GI_{50} values. Bioassay-guided fractionation studies could be further conducted to evaluate individual fractions activity rather than the crude extracts of *P. oleracea*, in order to better explore and examine its *in vitro* potential as antiproliferative agent. Moreover, it is important taking into account that *in vivo* phenolic compounds undergo intense metabolization and may lose partially or entirely their bioactivities observed *in vitro*. Therefore, the proliferative effect of the extracts must be also investigated, accounting for their bioaccessibility and biodisponibility.

Table 3. *In vitro* antiproliferative activity (GI_{50}) of *E. foetidum, H. acetosella, H. sabdariffa, O. gratissimum, P. aculeata, P. oleracea* and *X. sagittifolium* extracts.

Extracts	The antiproliferative activity for different human cell lines GI_{50} (µg/mL)[a]									
	Glioma	Melanoma	Breast	Ovarian II	Kidney	Lung	Ovarian	Colon	Leukemia	Non-tumor
E. foetidum	51.9	30.3	42.2	81.9	70.8	*	42.7	*	11.1	40.0
H. acetosella	*	*	*	*	40.0	*	*	*	24.8	*
H. sabdariffa	*	*	*	*	*	*	*	*	55.4	*
O. gratissimum	39.1	189.4	51.4	63.6	16.2	*	212.3	200.5	14.7	90.6
P. aculeata	29.3	90.0	47.1	47.7	26.2	*	110.3	52.6	24.8	198.0
P. oleracea	82.9	*	37.07	9.5	8.6	*	3.6	*	27.9	36.5
X. sagittifolium	205.1	225.7	*	185.6	116.0	*	*	*	13.9	*
Doxorubicin	<0.025	0.19	<0.025	0.12	<0.025	0.032	0.29	0.14	0.095	0.098

[a] Human tumor cell lines: Glioma (U251); Melanoma (UACC-62); Breast (MCF-7); Ovarian II = Ovarian expressing phenotype of multiple drugs resistance (NCI-ADR/RES); Kidney (786-O); Non-small cell lung cancer (NCI-H460); Ovarian (OVCAR-03); Colon adenocarcinoma (HT-29); Leukemia (K562). Non-tumor cell line: Keratinocytes (HaCaT).

GI_{50}, concentration that inhibits 50% cell growth or cytostatic effect; *GI_{50} > 250 µg/mL. Data calculated from a mean (n = 3)

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3.5 Effect on the formation of nitroso compounds

According to latest report of Internal Agency for Research on Cancer (IARC), colorectal cancer is the third most commonly diagnosed cancer in the world, accounting for more than 1.8 million cases worldwide (IARC, 2018). Based on epidemiologic studies, the consumption of red and processed meat was associated as a risk factor to develop colorectal cancer (Bouvard, et al., 2015). Among other factors, this association has been linked to the endogenous formation of nitroso compounds (NOCs), that take places through nitrosation reaction when secondary amines react with nitrite-derived compounds, formed under the acidic conditions in the stomach (Bartsch, et al., 1988; Nadia M. Bastide, et al., 2015). On the other hand, studies have been demonstrated that diets rich in fruits and vegetables may reduce the risk for colorectal cancer development, mainly by providing vitamins and polyphenols capable of blocking nitrosation reaction and, therefore, decrease the formation of endogenous NOC (Lee, et al., 2006; Bastide, et al., 2017; da Silveira, et al., 2017).

The *in vitro* effect of endogenous formation of NOC by intervention of the seven selected leaves under gastric simulated digestion is shown in Figure 2. The effect responses on NOC formation, expressed on the bases of apparent total nitroso compounds (ATNC), nitrosothiols and nitrosylhaem formation varied from sample to sample. Contrary to expectations, some of the samples had no inhibition effect on ATNC and derivatives, but actually contributed to the formation of these compounds.

H. acetosella promoted a significant ($p < 0.05$) formation on nitrosothiols (0.10 NO µmol) and others NOC, once the concentration of ATNC also increased, but not because formation of nitrosyl heme, which was not detected on the digested sample. Similarly, *H. sabdariffa* and *X. sagittifolium* promoted significant increase ($p < 0.05$) on ATNC, but *H.*

sabdariffa intervention significant decreased ($p < 0.05$) nitrosothiols by 33.99%, while *X. sagittifolium* increased ($p < 0.05$) its concentration. Conversely, *P. aculeata* was effective on reducing ($p < 0.05$) ATNC formation by 54.38%, but increased nitrosothiols and nitrosyl heme concentration after simulated digestion. *P. oleracea* did not affect significantly the formation of ATNC, but significantly increased ($p < 0.05$) nitroshothiols level.

Hughes, et al. (2002) also reported no effect on levels of fecal ATNC with a supplemented vegetables diet. Although vegetables can be rich sources of phenolic compounds and vitamins, they can also provide substantial amount of nitrate, in levels that are influenced by the type of vegetables and conditions for its cultivation and storage (Gangolli, et al., 1994; Hughes, et al., 2002). Ingested nitrate present in foods is reduced to nitrite by the bacterial flora in the mouth and digestive tract. Later on, nitrite can reacts with amines, amides and other nitrosation precursors in the gastrointestinal tract to form nitroso compounds (Dellavalle, et al., 2013). Therefore, the leaves tested are likely to be sources of nitrated, which may be related to the increase on ATNC after digestion.

Maximum inhibition effect on ATNC was presented by *O. gratissimum* and *E. foetidum* intervention, nearly 78% and 63%, respectively. Nitrosothiols and nitrosyl heme were not detected after the *in vitro* digestion of *E. foetidum,* to which were attributed 100% inhibition of these compounds. *O. gratissimum* was effective in reducing the nitrosothiols and nitrosyl heme formation by approximated 64% and 60%, respectively. The active components of *O. gratissimum* exerting this effect are likely to be the ascorbic acid and polyphenols, which are well-known substances to suppress N-nitrosation. Phenolic compounds are specially found in high concentration on *O. gratissimum* leaves (Table 1). Phenolic compounds and ascorbic acid are capable of avoid the formation of nitrous acid, which is an oxidizing and nitrosating agent

that reacts with amines from food, generating potent carcinogenic compounds such as, nitrosoamines, nitrosamides, and nitrosoguanidines. During the inhibition process, phenolic compounds and the ascorbic acid not only avoid the formation of the nitrous acid, but also produces the nitric oxide (•NO) that is important for lowering the blood pressure and for the control of the platelet aggregation (Lee, et al., 2006; Takahama & Hirota, 2017).

Abraham & Khandelwal (2013) report that combination doses of ascorbic acid and some polyphenols are able to prevent nitrosation *in vitro*. According to the authors beneficial effects may occur when there is a complementary interaction between low doses of these compounds, rather than a high dose of a single one, highlight the importance of synergisms among bioactive compounds in foods. Therefore, the inhibition effect on ATNC formation by *E. foetidum* leaves may be associated to a synergetic action of polyphenols and ascorbic acid.

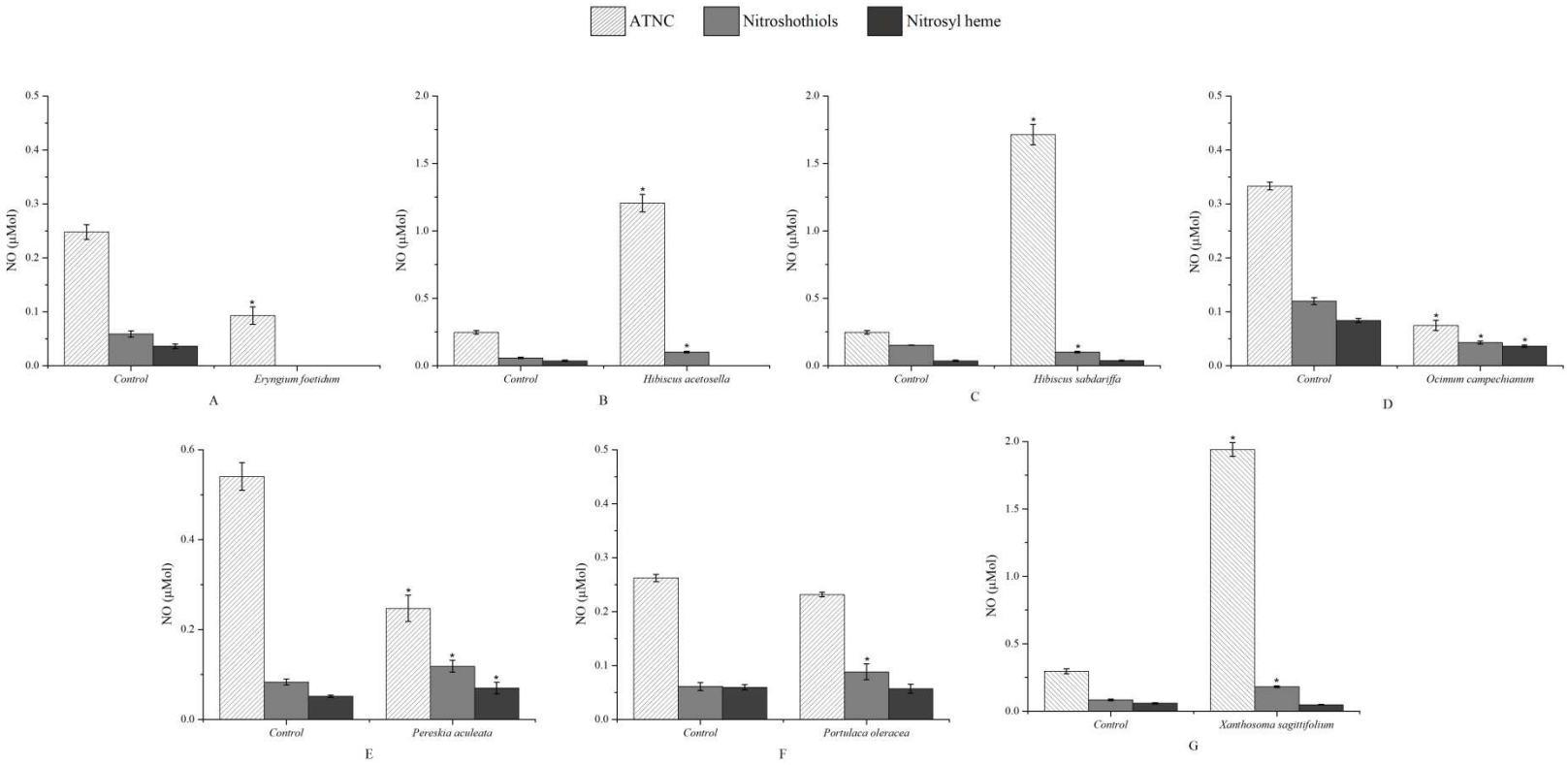

Figure 2. Concentrations of apparent total nitroso compounds (ATNC) and their derivatives (nitrosothiols and nitrosyl heme) after intervention with *E. foetidum* (A), *H. acetosella* (B), *H. sabdariffa* (C), *O. gratissimum* (D), *P. aculeata* (E), *P. oleracea* (F) and *X. sagittifolium* (G). Data shown as mean ± standard deviation (n = 3). Vertical bars denote standard error. * indicates significant difference ($p < 0.05$), by Tukey test, compared to the control.

4. CONCLUSION

Total phenolic compounds were found in high concentration for leaves of *O. gratissimum*, *H. acetosella and Pereskia aculeate,* while *X. sagittifolium, H. sabdariffa* and *H. acetosella* were the main source of total vitamin C. The results indicate the potential use of the selected NUS as sources of natural antioxidants, with great scavenging capacity towards oxygen reactive species, especially ROO•. Also, our work demonstrates the ability of *H. acetosella* and *H. sabdariffa* extracts to reduce H_2O_2-induced oxidation. Most of the leaves exhibit antimicrobial capacity at some extends, but only *O. gratissimum* presented effective inhibit antimicrobial growth of *Salmonella choleraesuis*. Overall, the leaves demonstrated activity against tumor cell lines, but in general *P. oleracea* stood out among the samples presenting the lowest GI_{50}. *O. gratissimum* and *E. foetidum* were capable of inhibiting nitrosation reaction after *in vitro* simulated gastric digestion, suggesting a possible protective effect of these plants on the nitroso compounds formation. The plants hereby studied have interesting bioactive capacities that should be further exploited by nutritionists, health specialists, as well as the food industry, and, which may promote the use of these neglected and underutilized plants.

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Chapter III

SELECTED EDIBLE GREEN LEAF ATTENUATES ANGIOTENSIN II-INDUCED OXIDATION

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ABSTRACT

Hypertension is a worldwide major public health problem and an important risk factor for other cardiovascular diseases (CVD), such as coronary artery disease, stroke, heart failure, atrial fibrillation, peripheral vascular disease, chronic kidney disease, and atherosclerosis. Angiotensin II (Ang II) is a vasoactive hormone that plays an important role in vascular remodeling, and consequently, hypertension and CVD. Consumption of fruits and vegetables is associated with decreased incidence of CVD, which has been mainly attributed to the polyphenol content of these foods. Thus, the objective of this study was to investigate crude and digest polyphenol extracts of *Eryngium foetidum*, *Hibiscus sabdariffa, Ocimum gratissimum, Pereskia aculeata*, *Portulaca oleracea* and *Xanthosoma sagittifolium* as attenuators of Ang II-induced oxidative stress in a vascular smooth muscle cell (VSMC). Pre-incubation with crude extracts of *H. sabdariffa, O. gratissimum, P. oleracea* and *X. sagittifolium* significantly decreased intracellular basal superoxide levels, as well as digest extracts of *E. foetidum, O. gratissimum* and *X. sagittifolium*. Extract of *O. gratissimum* stood out among the selected plants as the only one to attenuated superoxide levels in VSMC under Ang II-induced oxidative stress. These results suggest that *O. gratissimum* extract may have clinical potential for improved vascular function in hypertension.

Keywords: Antioxidant capacity, phenolic, reactive oxygen species, bioaccessibility, vascular dysfunction, antihypertensive effect.

1. INTRODUCTION

Cardiovascular diseases (CVD) have been the leading cause of mortality and morbidity in the world during the last 15 years, accounting for approximately one third of all deaths worldwide (WHO, 2018). Hypertension is the most common risk factor for cardiovascular diseases and its treatment dramatically contributes to decrease the incidence of mortality due to stroke and coronary heart disease (Lenfant, 2002; Wolf-Maier, et al., 2003).

Excessive and deregulated activity of the renin-angiotensin system (RAS) underlies the pathogenesis of many cases of hypertension (Putnam, et al., 2012). Angiotensin II (Ang II) is the main effector of the renin-angiotensin system, which consists of an enzymatic cascade beginning with the production of angiotensinogen (AGT), the precursor of angiotensin (Ang) peptides, that regulates cardiovascular function through Ang II binding to its AT1 and AT2 receptors on target tissues (Montezano, et al., 2014; Satou, et al., 2018). Ang II is a significant factor in blood pressure regulation and a key factor to cause vasoconstriction, salt retention, and inflammation (Li, et al., 2016). Ang II is also involved in the reactive oxygen species (ROS)-induced stress, another pathogenic factor that enhances pro-hypertensive factors, like reduce the bioavailability of important vasodilating agents (Vanhoutte, 2001; Aekthammarat, et al., 2019). Excessive production of ROS results in prolonged vascular injury and then, release of inflammatory cytokines that potentiate oxidative stress and perpetuate vascular inflammation, thereby resulting in hypertension and cardiovascular complications (Montezano, Nguyen Dinh Cat, Rios, & Touyz, 2014; Mahtta, et al., 2018)

Polyphenols are a wide variety of secondary metabolites that are present in all plant-based foods (Cheynier, et al., 2015), with numerous cardio-protective properties including anti-inflammatory, anti-aggregating and antihypertensive effects (Ludovici, et al., 2017).

Epidemiological and clinical studies suggests that polyphenol-rich foods can be important modulators of vascular risk and are associated to a reduction in mortality rates due to cardiovascular disease, mostly through the reduction of blood pressure, improvement of vascular function and reduction of platelet aggregation (Arts & Hollman, 2005; Chong, et al., 2010; Peterson, et al., 2012; Ludovici, et al., 2017; Al-Dashti, et al., 2018). Various chemical properties and biological effects of dietary polyphenols might be involved in protection against CVD (Stoclet, et al., 2004). An obvious hypothesis is that the antioxidant properties of polyphenols might protect blood vessels against the deleterious consequences of oxidant stress associated with many, if not all, cardiovascular risk factors (Stoclet, et al., 2004). The impact of the vasorelaxation and inhibiting oxidative stress properties of polyphenols is of public health importance and could help to inform policy on diet recommendations, to supply natural substances related to the treatment and prevention of blood pressure-related complications (Chong, et al., 2010; Zhu, et al., 2016; Aekthammarat, Pannangpetch, & Tangsucharit, 2019).

It is important to keep in mind that polyphenols biological action may change after human digestion process. In this way, determine polyphenols bioaccessibility, i.e., the potentially available fraction of polyphenols that is released from the food matrix during digestion process for further uptake and absorption, is of great importance to evaluate polyphenols health promote action in the body (Etcheverry, et al., 2012; De Santiago, et al., 2018). *In vitro* digestion studies have been developed to simulate the physical (agitation, temperature and pH) and chemical (enzymatic and salinity) conditions taking place in the human gastrointestinal tract (Lucas-González, et al., 2018). *In vivo* experiments in humans are definitely required in order to determine the bioaccessibility and bioavailability of bioactive compounds, as phenolic compounds (Gullon, et al., 2015). However, despite limitations that constitute a static model of

digestion, the evaluation of bioaccessibility by *in vitro* models can be well correlated with results from human studies and animal models (Bouayed, et al., 2011).

Given such a background, the aim of this study was to screening the antioxidant protective effects of phenolic-rich extracts, before and after gastrointestinal simulated digestion, on vascular smooth muscle cell (VSMC). Phenolic-rich extracts were obtained from six selected edible green leaves, named: culantro (*Eryngium foetidum*), roselle (*Hibiscus sabdariffa*), tree basil (*Ocimum gratissimum*), Barbados gooseberry (*Pereskia aculeata*), purslane (*Portulaca oleracea*) and tannia (*Xanthosoma sagittifolium*).

2. MATERIAL AND METHODS

2.1 Chemicals

Dulbecco's phosphate buffered saline (PBS), Ang II and dihydroethidium (DHE) were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen (Carlsbad, CA, U.S.A.). Penicillin-Streptomycin was from Life Technologies (Carlsbad, CA, U.S.A.). Methanol and ultrapure water were of HPLC grade purchased from Merck (Montreal, QC, Canada).

2.2 Sample preparation

Fresh leaves of *Pereskia aculeata* (Barbados gooseberry), *Portulaca oleracea* (purslane), identified by Dr. Eliane Fabri, and *Xanthosoma sagittifolium* (tannia), identified by Dr. Jose Carlos Feltran, were harvest at Agronomic Institute of Campinas (IAC) (Campinas, Brazil) in January 2018. Leaves of *Eryngium foetidum* (culantro), *Hibiscus sabdariffa* (roselle) and *Ocimum gratissimum* (tree basil) were purchased on a local market in Belém, PA, Brazil in

January 2018. Voucher specimens (IAN 196530, IAN 196531, and IAN 196532) were identified and deposited at the Herbarium of the Botanical laboratory, at Embrapa Eastern Amazon. The leaves were manually separated from the twigs, washed in tap water and gently dried with absorbent paper, then frozen at –80 ºC (overnight), freeze-dried (Terroni, LS 3000) and stored in airtight bags at –80 ºC. Prior to analysis, the freeze-dried material was ground to a powder using a domestic grinder (L'Equipe, Kitchen Resource Group, North Salt Lake, UT, USA).

Freeze-dried samples (ca. 200 mg) were mixed with 15 mL of aqueous methanol (85:15, methanol/water, v/v) and submitted to orbital motion at 200 rpm for 30 minutes at 42 ºC. The resulting mixture was centrifuged at 2000 g for 10 minutes and the supernatant was collected. The solid residue was re-extracted following the same procedure described above. The supernatants were pooled together and concentrated under reduced pressure (600 mmHg) in a rotary evaporator at 40ºC. The concentrated extracts were frozen at –80 ºC (overnight) and freeze-dried.

2.3 Simulation of *in vitro* digestion

The *in vitro* gastro-pancreatic digestion method was performed based on Flores, et al. (2014) with modifications. The composition of the simulated gastric, duodenal and bile juices are presented in Table 1. The freeze-dried plant material was weighted (ca. 2 g) and transferred to a jacket beaker equipped with a water bath (Lauda-Brinkmann, LaudaKönigshofenand, Germany) and Titrando (Metrohm, Herisau, Switzerland) for temperature and pH control, respectively. Then, 48 mL gastric juice was added, pH was adjusted to 2 and 0.25 g pepsin and 0.3 g mucin were added to the juice. This mixture was incubated for 2h under constant agitation at 37 ºC. After, 48 mL duodenal juice and 24 mL bile juice were added, pH adjusted to 7 and 0.9 g

pancreatin and 0.15 g lipase were incorporated to the digestion solution, which was incubated for 2h more at 37ºC under agitation. The reaction was ended by cooling the samples in ice bath. The resulting solution was centrifuged (2000 g for 15 minutes at 4 ºC) and the supernatant collected and freeze-dried. The freeze-dried material was submitted to extraction as previously described (section 2.2).

Table 1. Composition of the simulated gastrointestinal juices.

Gastric Solution	**Duodenal Solution**	**Bile Solution**
500 mL Deionized water	500 mL Deionized water	500 mL Deionized water
2.752 g NaCl	7.012 g NaCl	5.259 g NaCl
0.824 g KCl	0.564 g KCl	0.376 g KCl
0.266 g NaH_2PO_4	3.388 g $NaHCO_3$	5.785 g $NaHCO_3$
0.399 g $CaCl_2.2H_2O$	80.0 mg KH_2PO_4	0.25 g Urea
0.306 g NH_4Cl	50.0 mg $MgCl_2$	0.150 mL Concentrated HCl
0.085 g Urea	0.1 g Urea	
6.5 mL Concentrated HCl	0.180 mL Concentrated HCl	
Adjunts		
2.5 g Pepsin	9.0 g Pancreatin	30 g Bile salts
3.0 g Mucin	1.5 g Lipase	

2.4 Cell culture and viability

The A7r5 cells (ATCC CRL-1444, Manassas, VA, USA), a vascular smooth muscle cell line from rat aorta, were used in this study between passages 4 and 11. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Penicillin-Streptomycin and Gentamicin), and incubated in a humidified atmosphere with 5% CO_2 at 37 °C. Cells medium was replaced every other day and, when grown ~80% confluence, were sub-cultured using 0.25% trypsin-EDTA (Ethylenediamine tetraacetic acid) treatment.

Cell viability was determined by Alamar Blue fluorescent assay following the manufacturer protocol (Thermo Fisher Scientific, Burlington, ON, Canada). A7r5 cells were seeded in 96-well plates at a density of 1.8×10^4 cell/well for 24 h. Then, the cells were treated with 25 µg/mL of phenolic extract (crude and digest) for another 24 h in a fresh medium. After 24 h treatment, the medium was discarded and fresh medium with 10% Alamar Blue reagent was added and incubated, protected from light, for 4 h at 37 °C. The fluorescence intensity was recorded at emission wavelength of 590 nm and excitation wavelength of 570 nm. The viability of the treated cell was expressed as the percentage as compared to untreated cells.

2.5 Oxidative stress

Superoxide generation in VSMC was measured by dihydroethidium (DHE) staining method based on Peshavariya, et al. (2007). A7r5 cells were seeded in 48-well tissue culture plates at a density of 2×10^4 cell/well in DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum (FBS) and antibiotics (Penicillin-Streptomycin and Gentamicin). The plate was incubated overnight (24 h) in a humidified atmosphere with 5% CO_2 at 37 °C. The media was replaced with DMEM supplemented with 1% FBS and antibiotics prior to treatment. Then, cells were treated with crude and digest phenolic extracts for 30 minutes prior to addition of Ang II (angiotensin II). Following 1h incubation with Ang II, the medium was removed and fresh medium (DMEM with 1% FBS), contain 20 µM (final concentration) of DHE was placed. Once incubated in dark for 30 min, the cells were washed 3 times with PBS (phosphate-buffered saline) and the fluorescence was visualized under an Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group; Markham, ON, Canada). For each data point, 3 fields were

randomly chosen and images in each field were taken. The fluorescence was measured as mean fluorescence intensity per cell (MFI) by ImageJ software (http://imagej.net/Welcome).

2.6 Total phenolic content (TPC)

The Folin-Ciocalteau method was employed with modifications (Singleton, et al., 1999). Prior to analyze, the freeze-dried extracts were re-dissolved in aqueous methanol (85:15, methanol/water, v/v). Then, 25 µL of extract/standard solution were mixed with 125 µL Folin-Ciocalteu (10-fold diluted). After 5 minutes, 100 µL of sodium carbonate (7.5%, w/v) was added and the mixture was left to react for 2h in the dark at room temperature. Absorbance was measured at λ of 760 nm (FLUOstar Omega – BMG Labtech, Germany). The TPC was calculated from a standard curve prepared using gallic acid. Results are presented as mg gallic acid equivalents per gram of dry weight (mg GAE/g d.w.).

2.7 Statistical analysis

Analyses were run in triplicate and data were expressed as mean values with standard deviations. Comparative results was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test, at 5% significant level, using Statistical 7.0 (StatSoft, USA).

3. RESULTS

3.1 Total phenolic content of the extracts

The total phenolic content (TPC) of the crude extracts and subjected to *in vitro* simulated gastro-intestinal digestion (digest extracts) is presented in Figure 1. The TPC values of crude extracts ranged from 7.12 to 47.03 mg GAE/g d.w., while TPC values of digest extracts ranged

from 8.34 to 33.56 mg GAE/g d.w.; in both extracts (crude and digest) *Ocimum gratissimum* and *Xanthosoma sagittifolium* presented the highest and lowest TPC values, respectively. There was a significant ($p < 0.05$) decrease on TPC values after simulated digested, expect for *Xanthosoma sagittifolium* to which TPC did not significantly differ between the two extracts. The % relative recovery of TPC after grastro-intestinal digestion of *Eryngium foetidum*, *Hibiscus sabdariffa, Ocimum gratissimum, Pereskia aculeata*, *Portulaca oleracea* and *Xanthosoma sagittifolium* was 65.45%, 55.44%, 71.38%, 64.16%, 78.24% and 117.16%, respectively. The decrease on the absolute values of TPC of the digest extracts was different for each plant matrix.

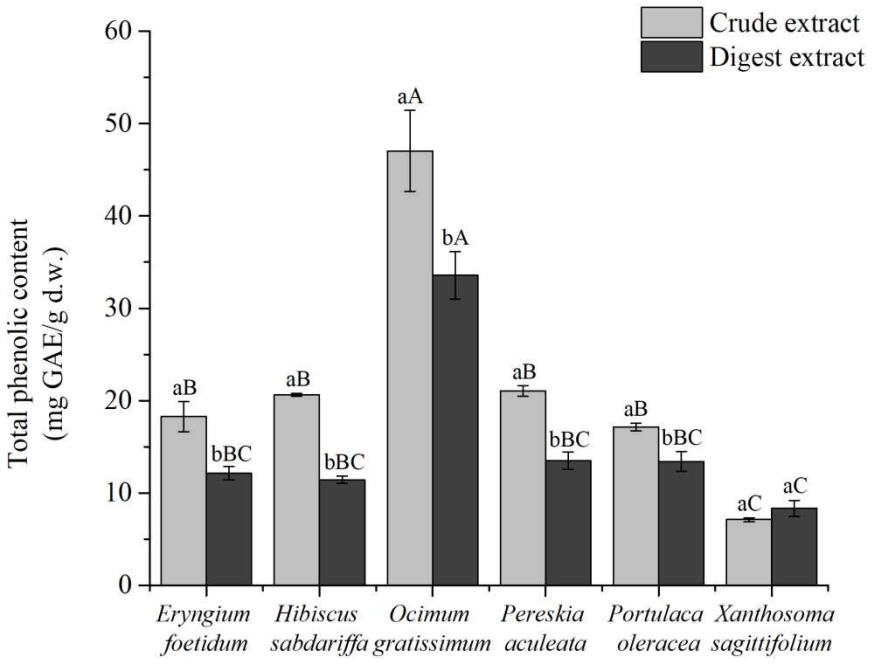

Figure 1. Total phenolic contente (TPC) of crude and digest extracts of *E. foetidum*, *H. sabdariffa, O. gratissimum, P. aculeata*, *P. oleracea* and *X. sagittifolium*. The data herein presented consist of average values ± standard deviation ($n = 3$) of triplicates. Different low case letters denote significant difference ($p < 0.05$), by Tukey test, within results of the same sample. Different upper case letters denote significant difference ($p \leq 0.05$), by Tukey test, within results of different samples.

3.2 Cell viability and oxidative stress

Alamar Blue fluorescent assay was used to assess the effect of crude and digest extracts on the viability of A7r5 cells in order to select the non-cytotoxic concentration to be use in our experiments. The results revealed that extracts exhibited cytotoxicity at concentrations > 25 µg/mL of phenolic extract (Anexo II). Thus, 25 µg/mL of phenolic extract was the selected concentration to evaluate the antioxidant activity on A7r5 cells.

The levels of oxidative stress marker were measured in order to assess effects of the extracts on levels of intracellular ROS on A7r5 cells. The ROS levels was measured by intracellular superoxide generation that engages a reaction with DHE to form ethidium, which then binds to nuclear DNA and releases nuclear fluorescence (Peshavariya, Dusting, & Selemidis, 2007). Then, samples that show high fluorescence emission are associated to a greater level of intracellular superoxide production. The influence of crude and digest extracts on superoxide levels is presented in Figure 2 and 3, respectively. We also determined the antioxidant activity of crude and digest extract on A7r5 cells ROS levels under Ang II-induced oxidative stress, as show in Figure 4 and 5, respectively.

Pre-incubation with crude extracts of *H. sabdariffa, O. gratissimum, P. oleracea* and *X. sagittifolium* significantly decreased ($p < 0.05$) the relative basal levels of ROS (Fig. 2), by 10.66%, 33.51%, 23.33% and 18.30%, respectively. Digest extracts of *E. foetidum, O. gratissimum* and *X. sagittifolium* significantly decreased ($p < 0.05$) the basal levels of ROS (Fig. 3) by 25.92%, 17.28% and 35.33%, respectively. Upon Ang II-induced oxidative stress, only crude extract of *O. gratissimum* was capable to significantly decrease ROS levels (Fig. 4) by 37.05%. None of the digest extracts, at tested concentration, had any effect on the ROS levels under Ang II-induced oxidative stress (Fig. 5).

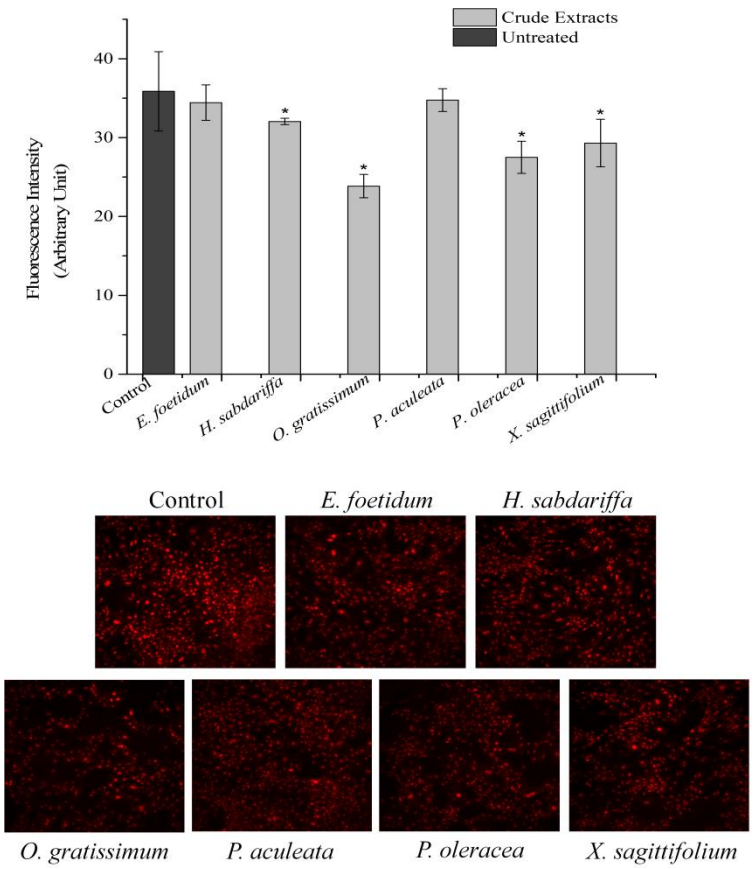

Figure 2. Antioxidant effect of untreated (control) and crude extracts on A7r5 cells. The cells were treated with extracts at 25 μg/mL for 30 min. Superoxide generation was expressed as mean fluorescence intensity (arbitrary unit). Mean ± SD of three independent experiments are shown. Asterisk indicates $p < 0.05$ compared to the untreated control by Tukey test.

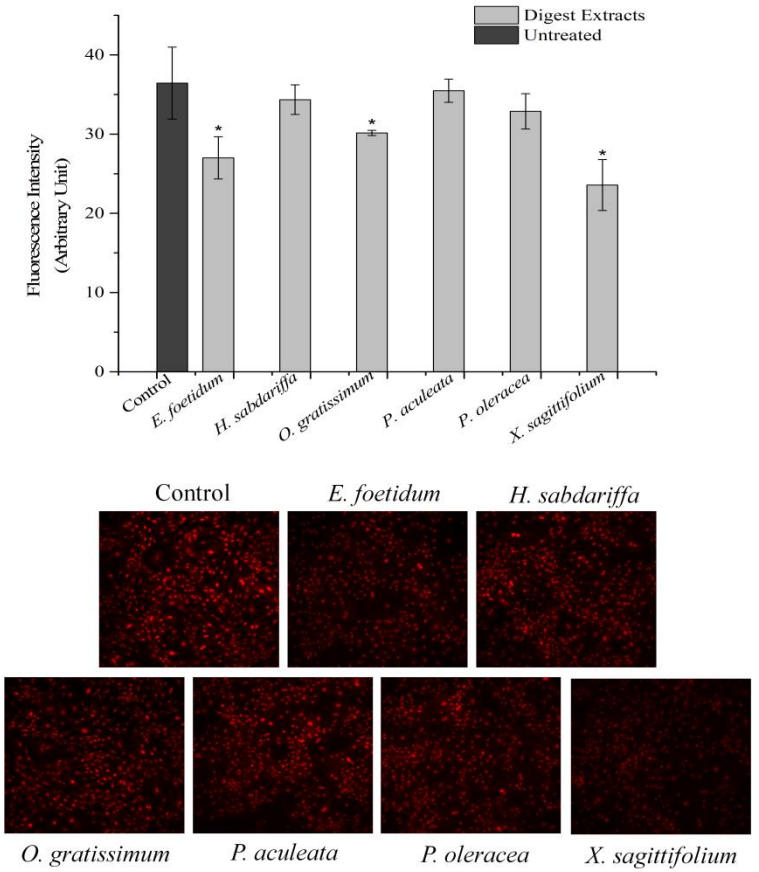

Figure 3. Antioxidant effect of untreated (control) and digest extracts on A7r5 cells. The cells were treated with extracts at 25 μg/mL for 30 min. Superoxide generation was expressed as mean fluorescence intensity (arbitrary unit). Mean ± SD of three independent experiments are shown. Asterisk indicates $p < 0.05$ compared to the untreated control by Tukey test.

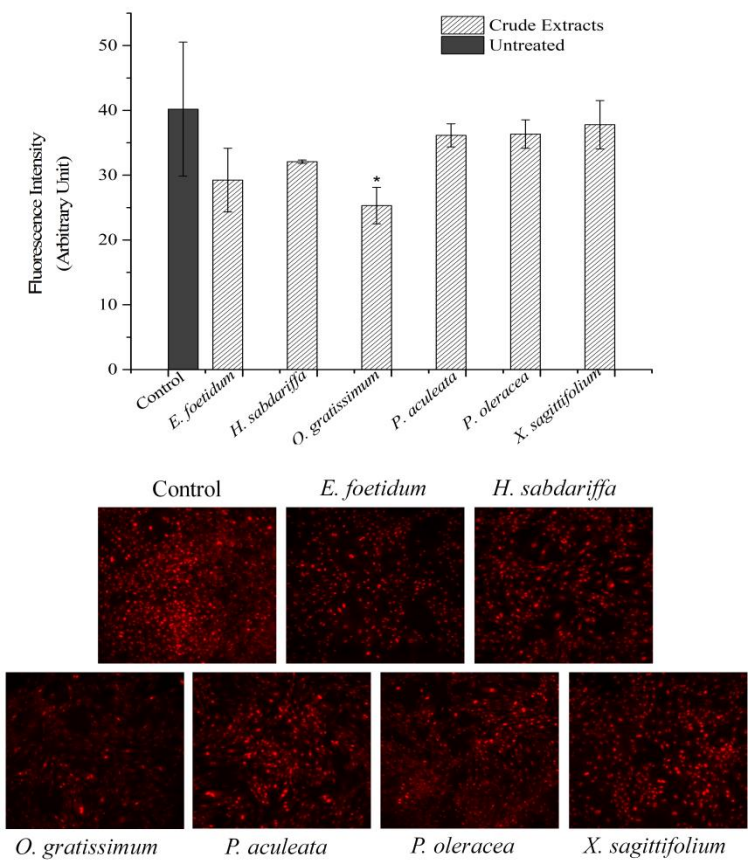

Figure 4. Antioxidant effect of untreated (control) and crude extracts on A7r5 cells under Ang II-induced oxidation. The cells were treated with extracts at 25 μg/mL for 30 min. Superoxide generation was expressed as mean fluorescence intensity (arbitrary unit). Mean ± SD of three independent experiments are shown. Asterisk indicates $p < 0.05$ compared to the untreated control by Tukey test.

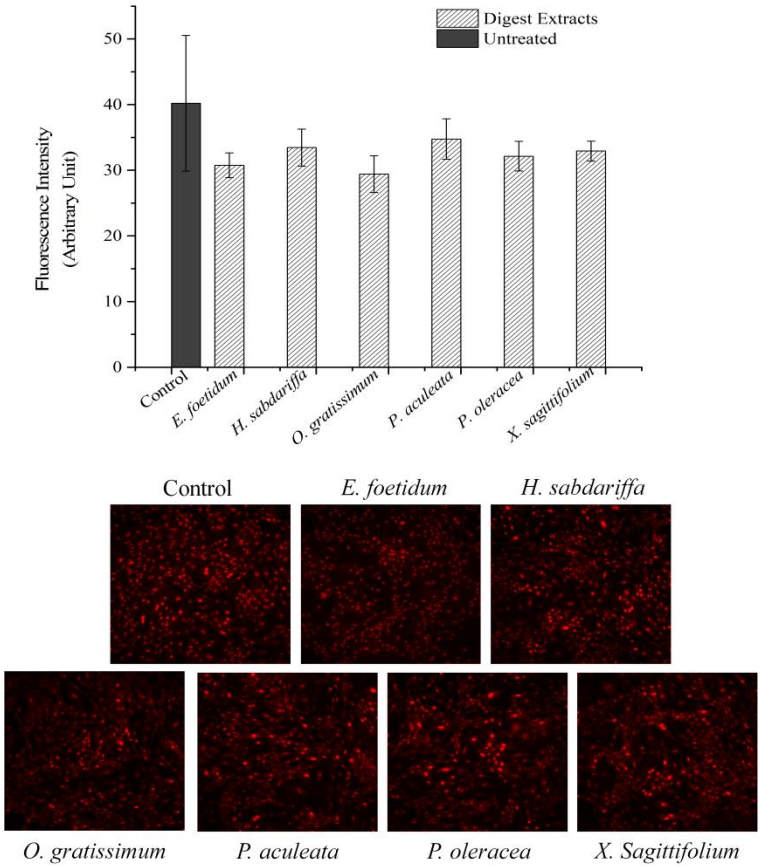

Figure 5. Antioxidant effect of untreated (control) and digest extracts on A7r5 cells under Ang II-induced oxidation. The cells were treated with extracts at 25 μg/mL for 30 min. Superoxide generation was expressed as mean fluorescence intensity (arbitrary unit). Mean ± SD of three independent experiments are shown. Asterisk indicates $p < 0.05$ compared to the untreated control by Tukey test.

4. DISCUSSION

Reactive oxygen species (ROS) are a family of highly reactive molecules, yielding superoxide anions and other species that plays prominent physiological processes, ranging from oxygen sensing and vasodilatation to smooth muscle cell proliferation and migration in angiogenesis (Peshavariya, Dusting, & Selemidis, 2007; Jamwal & Sharma, 2018). However, an imbalance between generation of reactive ROS and antioxidant defense systems can cause vascular dysfunction and inflammation, representing the primary cause of endothelial dysfunction, leading to hypertension and associated diseases (Incalza, et al., 2018; Jamwal & Sharma, 2018). Pharmacological treatment of hypertension have been improved greatly over the past decades and several drugs are now available (Tome-Carneiro & Visioli, 2016). However, these anti-hypertensive drugs usually require long-term and, often, life-long adherence, which is not only expensive but also contributes to numerous side-effects (Khanna, et al., 2008). Thus, there has been growing interest in developing safer and natural based compounds for the management of hypertension and its complications.

Numerous studies have shown that the intake of polyphenol-rich foods can be associated with improved vascular function and repair, as well as reduced blood pressure in both hypertensive and pre-hypertensive individuals (Taubert, et al., 2007; Grassi, et al., 2008; Chong, Macdonald, & Lovegrove, 2010; Heiss, et al., 2010; Peterson, et al., 2012). Several molecular mechanisms seem to contribute to the physiological effects of polyphenols on vascular function: (1) increasing the production of nitric oxide (NO), vital to maintain vascular homeostasis and acting as a signaling molecule that can mediate vascular smooth muscle relaxation; (2) exerting inhibitory effects on prooxidants (e.g., nicotinamide adenine dinucleotide [NADPH] oxidase), oxidants (e.g., superoxide and hydrogen peroxide) and angiotensin-converting enzyme (ACE);

and (3) inducing vasodilation via prostacylin (PGI_2) pathway (Holt, et al., 2012; Jimenez, et al., 2012; Al-Dashti, et al., 2018).

Our findings indicate for the first time, to the best of our knowledge, that polyphenol extracts of *E. foetidum, H. sabdariffa, O. gratissimum, P. oleracea* and *X. sagittifolium* leaves can decrease basal ROS production on A7r5 cells, a vascular smooth muscle cell line. The hypothesis is that the antioxidant capacity of theses extracts is related to the presence of the polyphenols compounds. *O. gratissimum* decreased ROS levels on A7r5 cells, even under Ang II-induced oxidative stress, likely because presented the highest amount of phenolic compounds. Although, *E. foetidum*, *H. sabdariffa*, *P. aculeata* and *P. oleracea* crude extracts presented no significant difference among each other, on the matter of TPC (Figure 1), only *H. sabdariffa* and *P. aculeata* were able to decrease basal ROS levels (Figure 2). *X. sagittifolium* crude extract, which presented the lowest TPC, also demonstrated antioxidant capacity. These results suggesting that not only total phenolic content, but also the composition of polyphenols may play an important role on the antioxidant capacity of the extracts at cellular level. Another indication of such theory is the lack of significant correlation between antioxidant activity of crude extracts and TPC: $r = 0.25$ ($p > 0.05$), for the assays not induced with Ang II, and $r = -0.40$ ($p > 0.05$) for the assays induced with Ang II.

In terms of biological relevance, it is important to keep in mind that before exerting any physiological effect, polyphenols must first survive the passage though the gastrointestinal tract. In general, polyphenols are highly sensitive to gastrointestinal pH variations and digestive enzymes, resulting in a considerable decrease of their amounts throughout the digestion process (Bermúdez-Soto, et al., 2007; Chiang, et al., 2014). All these limiting factors on the stability of polyphenols are likely to be associated to the decrease on total phenolic content of extracts from

digested leaves herein observed. However, even thought TPC decreased, overall ROS inhibition by digest extracts of *E. foetidum, O. gratissimum* and *X. sagittifolium* was higher than those presented by crude extracts, except for *P. oleracea* and *H. sabdariffa,* which did not show antioxidant activity after digestion process. This is another reason to point out the phenolic composition as possible key factor on the inhibition of intracellular superoxide levels.

Studies that simulated digestion have shown that the composition of bioactive compounds can be modified during gastrointestinal tract (Correa-Betanzo, et al., 2014; Boaventura, et al., 2015). In general, non-anthocyanin flavonoids and hydroxycinamic acids are reported to be slightly more stable under gastrointestinal environment when compared to hydroxibenzoic acids (Vallejo, et al., 2004; Tagliazucchi, et al., 2010). Interactions with the food matrix also strongly influence polyphenols stability (Karaś, et al., 2017), and then, might explain the variation, among the samples submitted to simulated digest, on TPC relative recovery (%).

O. gratissimum crude extracts were able to reduce superoxide levels on A7r5 under Ang II-induced oxidative stress, while digest extracts could not atenuate the Ang II-induced ROS generation. However, the tested concentration for all extracts (25 µg/mL) was lower compared to most concentrations reported in literature, ranging from 200 µg/mL to 2000 µg/mL (Kono, et al., 2013; Chan, et al., 2015; Feresin, et al., 2016). Also, the extracts were not purified or fractioned before the cellular assays, likely carrying inert and undesirable components and thus, diminishing the antioxidant activity of the extracts.

CONCLUSION

To the best of our knowledge, this is the first report on the effect of the crude and digest extracts of the selected plants leaves in ROS levels of VSMC. Our finds indicate that *E. foetidum, H. sabdariffa, O. gratissimum, P. oleracea* and *X. sagittifolium* extracts reduce basal

ROS levels in A7r5 cells, but only *O. gratissimum* attenuated superoxide levels in A7r5 cells under Ang II-induced oxidative stress. However, due to the use of whole extract of the plant rather than a purified component, the active components that are responsible for the decrease in intracellular superoxide levels are still unknown at this time. Overall correlation with antioxidant activity and TPC was observed, but samples with lower TPC, as *X. sagittifolium,* also presented antioxidant activity, suggesting that the polyphenolic profile presented by these plants may play important role on the polyphenolic profile presented these plants antioxidant activity.

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Chapter IV

VOLATILE COMPOUNDS OF SEVEN SELECTED UNDERUTILIZED PLANTS BY HEADSPACE SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY

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ABSTRACT

Volatiles were characterized by headspace solid phase micro extraction (HS-SPME) coupled with gas chromatography mass spectrometry (GC-MS) and flame ionization detector (GC-FID). The experimental parameters of HS-SPME were established based on Plackett-Burman screening design, in order to estimate the significant factors affecting volatile extraction efficiency in *Eryngium foetidum*, *Hibiscus acetosella, Hibiscus sabdariffa, Ocimum gratissimum, Pereskia aculeata, Portulaca oleracea* and *Xanthosoma sagittifolium* leaves. Assed variables and final concentration (values within parentheses) were: salt concentration (17.5%), temperature (65 °C), extraction time (40 minutes) and equilibrium time (12 minutes). In total, eighty one volatile compounds were identified, from each 46 were present in *O. gratissimum,* 24 in *E. foetidum,* 14 in *H. sabdariffa,* 11 in *H. acetosella,* 7 in *P. oleracea,* 5 in *P. aculeata* and 3 in *X. sagittifolium.* Terpenes constituted the most dominant volatile class in *O. gratissimum,* while aldehydes were mainly found in *E. foetidum, H. acetosella* and *H. sabdariffa* fresh leaves. *P. aculeata, P. oleracea*, and *X. sagittifolium* did not present a broad distribution of volatile compounds, but (E)-2-hexenal, a compound with strong antimicrobial activity, was the main volatile identified in these plants.

Keywords: aroma, functional foods, neglected plants, complex matrix, natural preservatives.

1. INTRODUCTION

Aroma and flavor are among the most important quality criteria of fresh and processed foods; they are invariably a key factor to influence selection, perception and acceptance of a given food product, according to consumer and marketing studies (Glanz, et al., 1998; Souza-Silva, et al., 2015). Flavor is usually the result of many volatile and non-volatile components interacting with the gustatory system to induce specific odor and taste sensations (Longo & Sanromán, 2006; Jeleń, et al., 2012). Flavor industry represents over a quarter of the world market for food additives and is expected to reach nearly USD 12.8 billion by 2023 (Longo & Sanromán, 2006; Strojnik, et al., 2019). Although most flavoring compounds are still produced by chemical synthesis, consumer's demands for functional foods and more natural products have been impose considerable changes, not only in the flavor industry but in the whole food chain (Younesi & Ayseli, 2015; Ayseli & İpek Ayseli, 2016).

Flavor precursor molecules, such as phenolic compounds, are well-known for their healthy-associated properties, and have been used as potential natural therapeutic agents and additives in food formulation (D'Archivio, et al., 2008). However, large amount of phenolic in food products may lead to consumer rejection due to astringent and bitter taste (Drewnowski & Gomez-Carneros, 2000). In this scenario, volatile compounds emerges with great potential to be used as natural preservative, mainly because of their antimicrobial properties, diminishing the use of synthetic additives and filling up the gap and demand for more natural ingredients for food formulations (Lanciotti, et al., 2003).

Volatiles compounds are typically mixtures of alcohols, aldehydes, carboxylic acids, furans, fatty acids, esters, ethers, hydrocarbons, ketones, lactones, pyrazines and terpenes, generally characterized by relatively high vapor pressures and low molecular size, not exceeding

300 Da (Longo & Sanromán, 2006; Jeleń, Majcher, & Dziadas, 2012; Wylock, et al., 2015). Qualitative and quantitative determination of volatile compounds can be quite challenge, especially in complex matrix such as food, in which volatiles are generally associated with others compounds, such as lipids, proteins and carbohydrates (Souza-Silva, Gionfriddo, & Pawliszyn, 2015). Several analytical techniques have been applied for the extraction of volatile compounds over the years: liquid-liquid extraction (LLE), simultaneous distillation liquid extraction (SDE), solid phase extraction (SPE), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) (Andujar-Ortiz, et al., 2009). HS-SPME (headspace solid phase microextraction), a SPME-based method, has several advantages over other sample preparation techniques, including its small sample size requirements, green analytical label, less labor-consuming and relatively high sensitivity (Burzynski-Chang, et al., 2018).

Headspace extraction involves two stages: (1) one between the sample matrix and the gaseous phase (headspace); (2) and the other between the headspace and the fiber coating (Balasubramanian & Panigrahi, 2011). HS-SPME can be affected by several parameters, including temperature, salting, fiber coating material, and organic solvent content in water (Pawliszyn, 2002). Proper optimizations of the parameters involved in the HS-SPME makes possible to estimate the significant factors to achieve adequate reproducibility and sensitivity with reduced number of experiments (Balasubramanian & Panigrahi, 2011; Abdulra'uf & Tan, 2015). Therefore, the aim of this study was to determine the profile of volatile compounds by HS-SPME combined with GC-MS and GC-FID of seven underutilized edibles plants, named *Eryngium foetidum*, *Hibiscus acetosella, Hibiscus sabdariffa, Ocimum gratissimum, Pereskia aculeata, Portulaca oleracea* and *Xanthosoma sagittifolium.* HS-SPME parameters conditions

were screened by Plackett-Burman design, in order to estimate the significant factors affecting extraction efficiency.

2. MATERIAL AND METHODS

2.1 Chemicals

2-methyl-4-pentanol and C6–C20 *n*-alkanes series were purchased from Sigma-Aldrich (St. Luis, USA). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MS, USA). Sodium chloride (NaCl) was purchased from Vetec (Xerém, RJ, Brazil).

2.2 Samples

Pereskia aculeata M., *Hibiscus acetosella*, *Portulaca oleracea* L. and *Xanthosoma sagittifolium* were harvest at Agronomic Institute of Campinas (IAC – Campinas, Brazil) in October 2017. The plants were botanically identified at the IAC – Campinas. *Eryngium foetidum L.*, *Ocimum gratissimum L.* and *Hibiscus sabdariffa* L. were purchased on a local market in Belém, Brazil in October 2017. Voucher specimens (*E. foetidum* L. – IAN 196530, *H. sabdariffa* L. – IAN 196531, *O. gratissimum* L. – IAN 196532) were identified and deposited at the Herbarium of the Botanical Laboratory, at Embrapa Eastern Amazon.

2.3 Solid phase micro-extraction (SPME) procedure

SPME conditions were based on PB-results, described as following: grounded plant material was weighted (ca. 1.50 g) and placed into 15-mL SPME flasks that already contained the appropriate amount of deionised water (5 g) and NaCl (according to the PB design). Right

after placing the sample into the SPME flask, 150 µL of internal standard (2-methyl-4-pentanol), at concentration of 5 mg/L, was immediately added to the flasks and sealed with screw tops fitted with PTFE/silicone septa (Supelco — Bellefonte, PA, USA). The flask content was vortexed (20 s) and incubated under continuous magnetic agitation at the temperature determined from the PB design. Finally, the fiber 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA) was exposed to the headspace and kept for the appropriate time. At the end of the extraction time, the fiber was immediately inserted into the gas chromatograph injection port for thermal desorption of the analytes. After each extraction and desorption procedure, the fiber was kept for two additional minutes within the injector, operating in purge mode, for recondition and to minimize carry-over effects. The same fiber was used for all the experiments.

2.4 Plackett-Burman screening design

Headspace solid-phase micro extraction (HS-SPME) conditions were investigated using the Plackett-Burman (PB) screening design, in order to determine the influence of NaCl concentration (X_1, %), temperature (X_2, °C), equilibration time (X_3, minutes), and extraction time (X_4, minutes) that could affect the extraction of volatile compounds from the plants matrix, in terms of the numbers of compounds for each class of volatiles and the amount extracted (total area). The amount of water was fixed in 5g. Only for the optimization analysis, the plant materials were mixed at equal parts.

Eleven independent trials were performed for PB design, including 8 trials at the upper (+1) and lower (-1) levels, and 3 trials at the central point (0). The central points were included to evaluate the repeatability of the extraction procedure. Table 1 presents the design matrix and the

coded and real values for each variable. The effect of each variable was analyzed by Protimiza Experiment Design Software. The confidence level adopted for variables significance were 10% ($p<0.1$). Such significance level was used to minimize the probability of underestimate an important variable for the final extraction protocol (Rodrigues & Iemma, 2014). Curvature test was also performed, in order to evaluate the central conditions of the design matrix. Tentative identification of the volatiles compounds was accomplished by spectrum comparison with those reported by National Institute of Standards and Technology (NIST) and linear retention indices (LRI) (as better described in 2.5 section). PB-results were expressed as arbitrary area.

2.5 Identification of volatiles compound by gas chromatography/mass spectrometry (GC–MS)

An Agilent 7890A GC system coupled to a quadrupole mass detector and equipped with an autosampler system was used for chromatographic analyses. Separation was carried out using a fused silica ZB-5HT capillary column (5% phenyl/95% dimethylpolysiloxane) (20 m×0.18 mm i.d.×0.18 μm of film thickness) from Phenomenex (USA). Helium was used as carrier gas at a constant flow rate of 1 mL/min. Injections were performed in the splitless mode at 260 °C and desorption of the volatiles from the fiber was carried out for 2 min. Initial oven temperature was set at 50 °C; then, it was increased at 4 °C/min. to 250 °C and held for 5 min., totalizing 55 minutes. Electronic ionization (EI) source was operated at 70 eV at temperature of 230 °C, the transfer line and quadrupole were set at 250 °C and 180 °C, respectively. Mass spectrometric detection was performed by a quadrupole in the range of mass/charge (m/z) of 35–400.

Identification of the volatile compounds was carried out by comparing their mass spectra with those from the database of the National Institute of Standards and Technology (NIST) MS

05, considering above 80% similarity, and comparing the linear retention indices (LRI) with those reported in the literature for similar chromatographic columns. LRI was determined on the basis of retention indices of a series of C6–C20 *n*-alkanes, which were extracted by HS-SPME following the same extraction conditions of the samples and calculated as recommended by Van Den Dool and Kratz (1963).

2.6 Quantification of volatiles compounds by gas chromatography (GC-FID)

Once the volatiles compounds were identified by GC–MS, their semi-quantification was estimated by gas chromatography coupled to a flame ionization detector (GC-FID). The samples were injected into a GC-FID (7890A Agilent Technologies gas) by applying the same sample preparation procedure, HS-SPME extraction, column and chromatographic conditions as used in the GC-MS. The GC-FID conditions were as following: injection of the volatiles compounds was carried out for 2 minutes in the splitless mode, at 250 °C; oven temperature program was from 40 °C to 240 °C at 3 °C/min and remained at this temperature for 4 min (with total run time of 55 minutes). The flow rate of the carrier gas (He), make-up gas (N_2), flame gases H_2, and synthetic air were 1 mL/min, 30 mL/min, 30 mL/min, and 300 mL/min, respectively. Concentration levels were calculated based on internal standard (IS) calibration using 2-methyl-4-pentanol and expressed in the same unit of the IS (µg/g).

2.7 Statistical analysis

The samples were analyzed in triplicate and the results presented as mean values ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Screening design

The factors and level of the HS-SPME variables were selected to cover the optimal range conditions that could influence the maximum number and quantity of the volatile compounds. Table 1 presents the sum of total area and the number of compounds comprising each volatile classes. The effects of the variables on these responses are presented in Table 2.

The total area variation within PB trials ranged from 1.10 to 48.75. Although a clear variation within the total area results could be noticed, the variables assed for this response had no significant effect ($p < 0.1$). This may be related to a contra balance observed within the trials, i.e., some conditions were favorable to a particular class and concomitantly bad for others, while other conditions favored that less intense classes, but disfavored the ones with greater intensity, making the sum of the areas equivalent for the different trials.

The numbers of extracted peaks by each class were evaluated during PB analysis, in other to avoid an experimental condition that would lead to a high concentration of only some compounds rather than a condition able to extract more compounds, even at lower levels, that could be important for the sample characterization. The numbers of extracted peaks varied from 34 to 48 compounds, comprising the classes of volatiles compounds of alcohols, aldehyde, ethers, esters, hydrocarbons and ketones.

Table 1. Plackett-Burman experimental design with coded and real values.

	Variables				Classes of the volatile compounds						
Run	X_1[a]	X_2[b]	X_3[c]	X_4[d]	Alcohols	Aldehyde	Ethers	Esters	Hydrocarbons	Ketone	Total area[e]
1	1 (30)	-1 (35)	-1 (4)	1 (60)	3	11	6	2	15	2	6.93
2	1 (30)	1 (65)	-1 (4)	-1 (20)	7	10	7	7	16	1	14.44
3	1 (30)	1 (65)	1 (20)	-1 (20)	2	13	7	4	17	2	11.45
4	-1 (5)	1 (65)	1 (20)	1 (60)	4	6	4	9	16	1	1.10
5	1 (30)	-1 (35)	1 (20)	1 (60)	5	9	6	1	20	1	7.90
6	-1 (5)	1 (65)	-1 (4)	1 (60)	9	11	8	7	19	2	48.75
7	-1(5)	-1 (35)	1 (20)	-1 (20)	1	10	3	1	21	0	3.16
8	-1 (5)	-1 (35)	-1 (4)	-1 (20)	5	7	3	2	17	0	5.07
9	0 (17.5)	0 (50)	0 (12)	0 (40)	5	12	3	4	18	4	12.13
10	0 (17.5)	0 (50)	0 (12)	0 (40)	5	12	3	4	18	4	15.01
11	0 (17.5)	0 (50)	0 (12)	0 (40)	5	12	3	4	18	4	14.72

[a] NaCl concentration (%); [b] extraction temperature (ºC); [c] equilibration time (minutes); [d] extraction time (minutes); [e] Peak area divided by 10^8 (arbitrary unit).

Table 2. Effects of each classes and total area on volatile compounds extraction, estimated from Plackett-Burman results.

Variable		Effects						
		Alcohols	Aldehydes	Ethers	Esters	Hydrocarbons	Ketones	Total area[a]
NaCl concentration (%)	*Effect (%)*	-0.50	2.25	2.00*	-1.25	-1.25	0.75*	-4.34
	p-value	0.7171	0.2011	0.0493	0.1782	0.4103	0.0990	0.6510
Temperature (ºC)	*Effect (%)*	2.00	0.75	2.00*	5.25*	-1.25	0.75*	13.17
	p-value	0.1856	0.6445	0.0493	0.0012	0.4103	0.0990	0.2044
Equilibration time (min.)	*Effect (%)*	-3.00*	-0.25	-1.00	-0.75	1.75	-0.25	-12.90
	p-value	0.0697	0.8765	0.2532	0.3907	0.2642	0.5301	0.2125
Extraction time (min.)	*Effect (%)*	1.50	-0.75	1.00	1.25	-0.25	0.75*	7.64
	p-value	0.3020	0.6445	0.2532	0.1782	0.8645	0.0990	0.4360
Mean	*Effect (%)*	4.50*	9.63*	5.50*	4.13*	17.63*	1.13*	12.35*
	p-value	0.0010	0.0001	0.0000	0.0001	0.0000	0.0018	0.0410
Curvature	*Effect (%)*	1.00	4.75	-5.00*	-0.25	0.75	5.75*	3.36
	p-value	0.7053	0.1656	0.0199	0.8765	0.7897	0.0005	0.8535

*Variable with significant effect ($p < 0.1$); [a]Total area of each PB-trial divided by 10^8

Temperature is often reported as one of the most positive and significant factor for the extraction of volatile compounds (Moreira, et al., 2019). Temperature influences the partition coefficient of the compounds between the sample and the headspace, and between the headspace and the fiber (Pellati, et al., 2005). Temperature increment improves the concentration of analytes into the headspace, and subsequently, onto the fiber coating. However, temperature can exert dual effect (kinetic and thermodynamic) on extraction efficiency (Es-haghi, et al., 2014). Rising the temperature can increase extraction yield, but also decreases analytes distribution constants and then, their adsorption onto the fiber (Matin, et al., 2014; Souza Silva, et al., 2017; Mehta, de Sousa Galvão, Soares, Nogueira, & Narain, 2018).

The temperature presented significant and positive effect on the numbers of ethers, ester and ketones compounds (Table 2). According to Souza, et al. (2017), increasing the temperature can significantly reduce the equilibration time required for an efficient extraction. In fact, we observe that for those classes in which the temperature was significant (ethers, ester and ketones), the equilibration time presented negative effect. This could be due to a favorable extraction of less volatile compounds that may compensate the decrease in adsorption, induced by this high temperature (Mehta, de Sousa Galvão, Soares, Nogueira, & Narain, 2018). For this reason, temperature was set as the maximum level from PB design (65 ºC) for volatile extraction of the samples.

Salt addition is frequently used to enhance volatile compounds from aqueous solutions due to the ‘salting out’ effect (Monteiro, et al., 2014). The specific effect depends on the compound characteristic and salt concentration (Purcaro, et al., 2018). PB results demonstrate that salt concentration (NaCl %) presented significant and positive effect on the number of peaks for ethers and ketones classes. According to Kudlejova, et al. (2012), salt addition increases the

ionic strength, which modifies the solubility of the analytes, increasing their partition coefficients and their concentration in the headspace before extraction. Once NaCl concentration presented both positive significant effects and negative effects (not significant, though) and curvature was also significant for some of the volatiles classes, salt addition of 17.5% (central point) was selected for the HS-SPME final protocol.

HS-SPME is an equilibrium extraction technique, and as such, optimization of extraction and equilibration time are critical factors to ensure method efficiency (Souza Silva, Saboia, Jorge, Hoffmann, dos Santos Isaias, Soares, & Zini, 2017). Multi-component systems, such as foods, present a wide range of volatile compounds that reach equilibrium at different times and competition occurs for the active sites on the fiber coating (Keszler & Héberger, 1999). The experiments demonstrated that equilibration time affected negatively the number of alcohols compounds, while extraction time had a positive effect on ketones compounds. In general, longer time favors filling up the sites of the fiber by the volatile molecules. However, prolonged time might also causes desorption of some analytes, when all sites have been already occupied (Zhang & Pawliszyn, 1993). Keeping this in mind, equilibrium and extraction times were set at the central point as a compromise to ensure enough time exposure, avoiding fiber saturation and initiation of desorption processes.

Base on these results, final conditions used for the HS-SPME protocol were as following: 17.5% of salt concentration (NaCl), extraction temperature at 65 °C, extraction time for 40 minutes and equilibrium time for 12 minutes.

3.2 Volatile compounds of the selected edible leaves

Volatile profile isolated by HS-SPME for the samples resulted in 80 tentatively identified volatile compounds from which 46 of them were identified for *O. gratissimum,* 25 for *E. foetidum,* 14 for *H. sabdariffa,* 12 for *H. acetosella,* 8 for *P. oleracea,* 5 for *P. aculeata* and 3 for *X. sagittifolium.* Components were categorized into alcohols, aldehydes, esters, ethers, terpenes and ketones for comparative convenience. The details of the tentatively identified mass peaks are shown in Table 3, with their average concentrations and linear retention indices.

Terpenes constitute the largest class of plant volatile compounds and play important roles for both human and plant species (Abbas, et al., 2017). They protect many plant species against pathogens, predators and competitors; and are extensively used as scent compounds in cosmetic products, and as potential antioxidant and anti-inflammatory pharmacological products (Yu & Utsumi, 2009; Brokl, et al., 2013; Hijaz, et al., 2016).

Terpenes constituted the most dominant class of volatile compounds in *O. gratissimum,* accounting for a total of 25 indentified compounds. Of all the terpene compounds, caryophyllene (52.88 µg/g), α-selinene (45.44 µg/g) and β-selinene (32.09 µg/g) were the main terpenes present in the samples studied. Three compounds, previously described as the major aroma-active components in *Ocimum,* were among the identified terpenes: α-pinene (spicy-herbal), eucalyptol (mint-caramel) and γ-terpinene (herbal-leafy) (Sonmezdag, et al., 2018). Among these compounds, eucalyptol is point out to possess the greatest role in the odor characterization of *Ocimum* species, due its generally higher concentration and odor activity (Sonmezdag, Amanpour, Kelebek, & Selli, 2018). Besides for their well-known pleasant aroma, terpenes are also associated to the taste and pharmacological activities of *Ocimum* species (Sonmezdag, Amanpour, Kelebek, & Selli, 2018).

Table 3. Volatile compounds identified in *E. foetidum, H. acetosella, H. sabdariffa, O. gratissimum, P. aculeata, P. oleracea* and *X. sagittifolium* leaves with their respective retention index.

			Concentration (µg/g)*						
Compound	LRI_{cal}	LRI_{tab}	*Eryngium foetidum*	*Hibiscus acetosella*	*Hibiscus sabdariffa*	*Ocimum gratissimum*	*Portulaca oleracea*	*Pereskia aculeata*	*Xanthosoma sagittifolium*
Alcohols									
(Z)-3-Hexen-1-ol	855	857	-	0.29	-	-	-	-	-
1-Hexanol	867	867	-	0.08	-	tr	-	0.14	-
1-Octen-3-ol	982	979	-	-	-	6.24	-	-	-
Hemellitol	995	996	0.74	-	-	-	-	-	-
3-Octanol	999	995	-	-	-	0.55	tr	-	-
Linalool	1105	1098	-	-	0.18	17.45	0.18	-	-
Camphol	1168	1167	-	-	-	tr	-	-	-
L-terpinen-4-ol	1180	1182	-	-	-	0.32	-	-	-
α-terpineol	1194	1191	-	-	-	2.51	-	-	-
2-Hydroxycineol	1228	1229	-	-	-	0.28	-	-	-
Espatulenol	1581	1578	-	-	-	1.20	-	-	-
Globulol	1588	1580	-	-	-	0.76	-	-	-
Rosifoliol	1606	1611	-	-	-	0.53	-	-	-
10-epi-γ-Eudesmol	1636	1624	-	-	-	tr	-	-	-
β-Eudesmol	1654	1650	-	-	-	1.87	-	-	-
Juniper camphor	1658	1688	-	-	-	3.18	-	-	-
Aldehydes									
Hexanal	801	798	0.13	0.54	tr	0.77	0.38	0.11	0.68
(E)-2-Hexenal	849	855	-	3.23	13.35	4.85	0.98	2.34	6.16
Sorbaldehyde	914	909	-	-	0.53	-	-	-	-
Benzaldehyde	964	961	0.06	0.02	-	-	-	-	-
(E) 4-oxo-2E-Hexenal	967	958	-	0.11	tr	-	-	-	-
(E,E)-2,4-Heptadienal	1002	1015	-	tr	-	-	-	-	-

Table 3 (*continued*)

Benzeneacetaldehyde	1048	1044	0.11	-	-	-	-	-	-
Nonanal	1106	1104	0.48	0.06	-	-	0.04	-	-
2,4-Dimethylbenzaldehyde	1188	1181	0.62	-	-	-	-	-	-
Decanal	1206	1207	1.83	-	-	-	-	-	-
2,4-Nonadien-1-al	1217	1220	0.17	-	-	-	-	-	-
Undecanal	1308	1310	0.42	-	-	-	-	-	-
Mesitaldehyde	1319	1323	2.02	-	-	-	-	-	-
Eugenol	1340	1356	-	-	-	640.38	-	-	-
Duraldehyde	1364	1364	1.28	-	tr	-	-	-	-
Methyl eugenol	1376	1362	-	-	-	86.62	-	-	-
Dodecanal	1411	1409	16.76	-	-	-	-	-	-
(E)-2-Dodecenal	1451	1462	837.21	-	-	-	-	-	-
2-Dodecenal	1467	1462	-	-	tr	-	-	-	-
Z-7-Tetradecenal	1601	1597	1.13	-	-	-	-	-	-
Tetradecanal	1614	1615	4.48	-	-	-	-	-	-
Pentadecanal-	1715	1713	-	-	-	-	-	-	0.74
Esters									
(4E)-4-Hexen-1-ol acetate	1010	1013	-	-	-	tr	-	-	-
Palmitic acid ethyl ester	1995	1992	-	-	-	0.45	-	-	-
Ethers									
Eucalyptol	1034	1033	-	-	-	84.48	-	-	-
Terpenes									
α-Thujene	925	923	0.03	-	-	tr	-	-	-
(R)-α-Pinene	931	935	1.86	-	-	1.92	-	-	-
Camphene	945	959	-	-	-	0.28	-	-	-
β-Thujene	972	920	0.07	-	-	-	-	-	-
β-Myrcene	991	992	-	-	-	0.09	-	-	-
β-Pinene	992	984	1.07	-	-	0.98	-	-	-
α-Phellandrene	1006	1004	-	-	-	0.42	-	-	-

Table 3 (*continued*)

α-Terpinene	1018	1018	-	-	-	0.26	-	-	-
o-Cymene	1024	1021	3.14	-	-	-	-	-	-
D-Limonene	1028	1035	0.58		tr	-	-	-	-
β-trans-Ocimene	1039	1044	-	-	-	tr	-	-	-
β-Ocimene	1049	1050	-	-	-	0.96	-	-	-
γ-Terpinene	1058	1064	3.64	-	-	0.38	-	-	-
Isoterpinolene	1089	1081	-	-	tr	-	-	-	-
Naphthalene	1184	1177	-	-	2.99	-	-	-	-
δ-Elemene	1338	1337	-	-	-	0.79	-	-	-
Dehydro-ar-ionene	1354	1354	-	-	tr	-	-	-	-
β-Damascenone	1386	1381	-	0.33	tr	-	-	-	-
β-Elemene	1391	1391	-	-	-	17.42	-	-	-
Caryophyllene	1425	1431	-	-	-	52.88	-	-	-
Aromandendrene	1444	1447	-	-	-	15.82	-	-	-
Humulene	1458	1455	-	-	-	13.67	-	-	-
4,11-selinadiene	1479	1485	-	-	-	9.15	-	-	-
D-Germacrene	1484	1480	-	-	-	tr	-	-	-
β-Selinene	1492	1487	-	-	-	32.09	-	-	-
α-Selinene	1502	1494	-	-	-	45.44	-	-	-
β-Bisabolene	1516	1509	1.09	-	-	-	-	-	-
γ-Cadinene	1517	1513	-	-	-	1.58	-	-	-
(-)-α-Panasinsen	1521	1527	-	-	-	0.86	-	-	-
δ-Cadinene	1526	1524	-	-	-	3.96	-	-	-
α-Cadinene	1540	1524	-	-	-	tr	-	-	-
Selina-3,7(11)-dien	1545	1542	-	-	-	2.12	-	-	-
α-Calacorene	1546	1546	-	-	-	tr	-	-	-
2-Phenyltridecane	1909	1916	-	-	-	-	-	tr	-
Ketones (5 compounds)			-	-	-	-	-	-	-
3-Hexen-2-one	803	834	-	-	-	-	tr	-	-

Table 3 (*continued*)

3,4-Hexanedione	834	816	-	tr	-	-	-	-	-
Isobutyl ethyl ketone	834	865	-	tr	-	-	-	-	-
Diisobutyl ketone	969	951	0.12	-	-	-	-	0.10	-
β-Ionone	1488	1500	-	-	tr	-	0.18	-	-

LRI_{Cal} = retention indexes calculated as recommended by Van Den Dool and Kratz (1963), using a 5% phenyl/95% dimethylpolysiloxane capillary column.
LRI_{Tab} = Van Der Dool retention indexes from the literature determined using a 5% phenyl/95% dimethylpolysiloxane capillary column.
*Data expressed as equivalent concentration of the internal standard (IS) (2-methyl-4-pentanol) area in µg/g.
tr = trace level (area < 0.06 µg/g, limit of quantification)
(–) = not detected.

Essential oils derived from *Ocimum* species have been reported to be active against several Gram-positive and Gram-negative bacteria, as well as against yeasts and fungi, mostly related to their terpenic constituents (Pandey, et al., 2014).. The anti-inflammatory, anti-bacterial, anti-mutagenic and antioxidant activities of eugenol has been reported several times (Murakami, et al., 2003; Kamatou, et al., 2012; da Silva, et al., 2018). Others important chemical groups found in *O. gratissimum* were aldehyde and alcohol, especially eugenol (640.38 µg/g) methyl eugenol (86.62 µg/g) and linalool (34.90 µg/g), which is also reported as one of the main constituents of *Ocimum* essential oil (Calín-Sánchez, et al., 2012; Oliveira, et al., 2013). Aldehydes and alcohols have been described as important contributores to the overall aroma of the *Ocimum* species (Lee, et al., 2005; Calín-Sánchez, et al., 2012). A total of 14 alcohols and 4 aldehydes were identified in *O. gratissimum* leaves.

Aldehydes were the overall second main class of volatile compounds identified in the samples, from which hexanal and (E)-2-hexenal were the only compounds identified in all the samples, except for the later that was not present in *E. foetidum* leaves. These aldehydes are often related to contribute to sweet, green, beany, floral and tea leaf-like odors (Chung, et al., 2005). Aldehydes comprised the most abundant volatile class of *E. foetidum* leaves, accounting for 14 identified compounds. *E. foetidum* has a pungently smelling and an intense coriander-like aroma, which make this plant to be commonly used interchangeably with *Coriandrum sativum* L. (coriander) for culinary purposes (Eyres, et al., 2005; Paul, et al., 2011). Beside its use for gastromic purposes, this plant is an important item in the perfumery and cosmetic industries, and its essential oil is of high economical value in international trade markets (Paul, Seaforth, & Tikasingh, 2011). (E)-2-dodecenal (1674.42 µg/g) was by far the most abundant compound present in the *E. foetidum* leaves, followed by dodecanal (33.51 µg/g) and tetradecanal (8.96

µg/g), as also reported by Eyres, Dufour, Hallifax, Sotheeswaran, and Marriott (2005) for the *E. foetidum* essential oil. These authors also reported that (E)-2-dodecenal is likely to be the most odour-active compound and the one responsible for the coriander-like, pungent and spicy aroma aforementioned. Eryngial (E-2-dodecenal) rich-extracts have been reported as potent bactericidal activity against *Salmonella choleraesuis* and remarkably anthelmintic (*in vitro*) against *Strongyloides stercoralis*, an infective larva that is responsible for the most severe parasitic disease in humans in the Caribbean region (Forber, et al., 2002; Kubo, et al., 2004; Paul, Seaforth, & Tikasingh, 2011).

Similarly to *E. foetidum*, *H. acetosella* and *H. sabdariffa* volatile profiles were mainly constituted by aldehydes, from which (E)-2-hexenal was the most abundant compound. *Hibicus* exotic aroma has been previously described as sweet and tart (Farag, et al., 2015). α-terpineol and linalool in *H. sabdariffa* are likely responsible for the floral notes exhibited by this plants; while fatty acids derivative volatiles, such as hexanal and 1-hexanol might be related to the acidic notes (Pino, et al., 2006).

(E)-2-Hexenal was also the most abundant volatile compound present in leaves of *P. aculeata* (2.34 µg/g), *P. oleracea* (0.98 µg/g) and *X. sagittifolium* (6.16 µg/g). Few reports are available regarding volatile and essential oils of *Pereskia* species. Souza, et al. (2016) reported 24 compounds present in the essential oil from *P. aculeata* leaves. Our data, however, demonstrated the presence of only 5 identified volatiles compounds. These variations may be attributed mainly to the method used to isolate the analytes. We performed HS-SPME in fresh leaves for volatile identification, while the aforementioned authors firstly dried the leaves to finally obtain the essential oil. Another explanation lay on environmental conditions, age of the plant and method of harvesting.

P. oleracea is the most worldwide spread plant with a long history of use for human food, animal feed and medicinal purposes (Teixeira, et al., 2010). *P. oleracea* presents a variable natural chemical constituents, including terpenoids and fatty acids (Iranshahy, et al., 2017). Until now, *P. oleracea* remains one of the major vegetable sources of omega-3 fatty acids, particularly α-linolenic acid (Petropoulos, et al., 2016). This relatively high amount of fatty acid might be related to presence of fatty acids derivative volatiles identified in *P. oleracea* leaves, such as linalool, hexanal, (E)-2-hexenal and nonanal. The same can be extended for *X. sagittifolium*, in which only three compounds were identified: hexanal, (E)-2-hexenal and pentadecanal. To the best of our knowledge, this is the first report of volatile by SPME for *P. aculeata*, *P. oleracea* and *X. sagittifolium*.

The presence of volatile compounds, especial hexanal and (E)-2-hexenal, that show strong antimicrobial properties against pathogen microorganisms at low concentrations may provide great opportunity for this plants to be used as natural preservatives and in formulation of functional foods. However, future researches should focus on addressing the mode-of-action f these compounds in foods, synergic actions, organoleptic impacts, acceptable flavor profile, process/storage stability and efficacy dosage (Ayseli & İpek Ayseli, 2016).

CONCLUSION

Plackett-Burman screening design allowed estimating the significant factors affecting efficiency on HS-SPME, as NaCl concentration (17.5%), temperature (65 °C), extraction time (40 minutes) and equilibrium time (12 minutes). A total of 80 volatile compounds were identified, comprising the following classes: terpenes (34 compounds), aldehydes (22 compounds), alcohols (16 compouds), ketones (5 compounds), esters (2 compounds) and ethers

(2 compounds). Terpenes constituted the most dominant class of volatile compounds in *O. gratissimum*, while aldehydes were the most abundant one in *E. foetidum, H. acetosella* and *H. sabdariffa* leaves. Only few volatiles compounds were identified for *P. aculeata*, *P. oleracea* and *X. sagittifolium.* The volatile compounds, especially present in *O. gratissimum* and *E. foetidum* may improve food quality and, to some extent, might be used as substitutes of synthetic additives in food formulations.

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DISCUSSÃO GERAL

Mundo afora, milhares de plantas foram negligenciadas e subutilizadas durante séculos, sendo consumidas apenas por comunidades indígenas, tradicionais ou em determinadas localidades de um país (Padulosi, et al., 2013). No Brasil, essas plantas são comumente classificadas como 'Plantas alimentícias não convencionais (PANC)', mas são ainda pouco conhecidas e utilizadas como fontes de alimentos (Kinupp & Lorenzi, 2014). As previsões futuras para as mudanças climáticas e o aumento da população mundial, saltando de 7,7 bilhões em 2019 para 9,8 bilhões em 2050 (UN, 2019), representam um grande desafio para a segurança alimentar. Especialistas acreditam que, em um futuro próximo, as PANC assumirão papel fundamental para garantia o acesso à comida e a uma dieta balanceada (FAO, 2017; Padulosi, et al., 2018). Isso porque, estas plantas são mais resistentes a climas adversos, sobrevivendo à secas ou inundações; são normalmente muito nutritivas, podendo nos oferecer uma dieta mais equilibrada e diversificada; além de apresentam potencial para favorecer a complementação de renda de pequenos agricultores (FAO, 2017).

Apesar de todo seu potencial nutricional, agronômico e cultural, algumas destas PANC possuem pouco ou nenhum relato acerca das suas características nutricionais e seu potencial benéfico para saúde humana. Tais informações podem contribuir para estimular o consumo destas plantas, e um possivel emprego destas em diversos setores industriais, devido suas características nutricionais, tecnológicas e seus princípios bioativos. Neste contexto, podemos citar as PANC coentro-bravo (*Eryngium foetidum*), vinagreira roxa (*Hibiscus acetosella*), vinagreira (*Hibiscus sabdariffa*), alfavaca (*Ocimum gratissimum*), ora-pro-nóbis (*Pereskia aculeata*), beldroega (*Portulaca oleracea*) e taioba (*Xanthosoma sagittifolium*), que possuem

consideráveis concentrações de compostos fenólicos totais (CFT) : *O. gratissimum* (93,88 mg GAE/g d.w.), *H. acetosella* (57,55 mg GAE/g d.w.), *P. aculeata* (42,04 mg GAE/g d.w.), *H.sabdariffa* (34,15 mg GAE/g d.w.), *P. oleracea* (27,85 mg GAE/g d.w.), *X. sagittifolium* (22,34 mg GAE/g d.w.) e *E. foetidum* (12,55 mg GAE/g d.w.). Quando comparados com fontes notáveis de fenólicos, *O. gratissimum, H. acetosella* e *P. aculeata* possuem concentrações similares ao morango (41,35 mg of GAE/g d.w.), framboesa (35,49 mg of GAE/g d.w.) e mirtilo (35,40 mg of GAE/g d.w.) (Wu, et al., 2004), ou ainda orégano (44,08 mg of GAE/g), alecrim (40,87 mg of GAE/g d.w.) e sálvia (36,79 mg of GAE/g d.w.) (Assefa, et al., 2018).

A extração dos compostos fenólicos de matrizes vegetais pode ser influenciada por vários fatores, dentre eles a concentração do solvente, o tempo e temperatura de extração, razão sólido-líquido e número de ciclos de extrações (Naczk & Shahidi, 2004; Xu, et al., 2017). A otimização sequencial realizada neste trabalho, composta por um Plackett-Burman (PB) e um delineamento composto central rotacional (DCCR), demonstrou que todas as variáveis acima mencionadas foram, de fato, significantes para a extração dos compostos fenólicos. Dentre elas, a concentração do solvente (metanol) foi a variável com maior efeito, especialmente para *P. aculeata, X. sagittifolium* e *P. oleracea*, cujo extrato exibiu textura viscosa, devido a presença de mucilagem, e por isso, requisitou maior concentração de solvente orgânico para extração dos compostos fenólicos totais.

Todos os extratos fenólicos das sete PANC estudadas apresentaram capacidade antioxidante contra oxidação do ácido hipocloroso (HOCl), sendo o *E. foetidum* o mais eficaz por apresentar o menor IC_{50} (13,96 µg/mL). Os extratos das espécies de *Hibiscus,* os extratos também foram capazes de diminuir o efeito oxidante do peróxido de hidrogênio (H_2O_2) em 34 a 43%, na máxima concentração de extrato testada (4 mg/mL). No entanto, nenhum dos extratos

testados pôde inibir a oxidação por parte do radical superoxido ($O_2^{\bullet-}$). No que diz respeito à atividade antimicrobiana, apenas os extratos de *H. sabdariffa*, *P. oleracea*, *P. aculeata* e *H. acetosella* foram capazes de inibir o crescimento de bactérias e leveduras na máxima concentração testada (2 mg/mL). Entretanto, segundo a classificação de Simoes, et al. (2009), apenas extratos com concentração entre 100 e 1000 µg/mL podem ser, de fato, considerados extratos antimicrobianos eficazes. Dessa forma, dentre as plantas estudadas, apenas os extratos de *O. gratissimum* (1 mg/mL) apresentaram inibição efetiva ao crescimento de bactérias do gênero *Salmonella choleraesuis*.

No geral, todos os extratos demonstraram atividade antiproliferativa contra as linhagens de células tumorais relacionadas ao glioma, leucemia, melanoma, câncer de mama, ovários, rins, pulmão e do cólon. Entretanto, os extratos de *P. oleracea* destacaram-se entre as amostras apresentando valores de GI_{50} bem menores, especialmente para as células relacionadas ao câncer nos ovários (GI_{50} 3,6), ovários exibindo o fenótipo de resistência a múltiplas drogas (GI_{50} 9,5) e ao câncer nos rins (GI_{50} 8,6). Em relação à inibição da reação de nitrosação, destaque pode ser dado aos extratos de *O. gratissimum* e *E. foetidum*, que apresentaram máxima inibição na formação de compostos nitrosos totais aparentes, com aproximadamente 78% e 63%, respectivamente.

Os extratos de *E. foetidum*, *H. sabdariffa*, *O. gratissimum*, *P. oleracea* e *X. sagittifolium* também demonstraram efeito na redução dos níveis basais de superóxido em células A7r5, uma linhagem de células do músculo liso vascular. No entanto, apenas os extratos de *O. gratissimum* foram capazes de atenuar os níveis de superóxido nestas células sob estresse oxidativo induzido pela ação da angiotensina II (Ang II). O que demonstra um possível potencial clínico, por parte

do extrato fenólico de *O. gratissimum*, para futuras investigações na prevenção de doenças relacionadas ao estresse oxidativo.

Além dos compostos fenólicos, os compostos voláteis também possuem grande potencial para utilização como conservantes naturais, principalmente pelas suas propriedades antimicrobianas (Lanciotti, et al., 2003). Assim como os compostos fenólicos, vários fatores podem interferir na extração dos voláteis, em especial no emprego da técnica por microextração em fase sólida no modo headspace (HS-SPME). Ao empregar um planejamento de 'screening desing' do tipo Plackett-Burman, observamos que a concentração de NaCl (17,5%), temperatura (65 °C), tempo de extração (40 minutos) e tempo de equilíbrio (12 minutos) afetaram positivamente, em geral, a extração de compostos voláteis por HS-SPME. Para as amostras, um total de 80 compostos foi identificado, compreendendo as seguintes classes: terpenos (34 compostos), aldeídos (22 compostos), alcoóis (16 compostos), cetonas (5 compostos), ésteres (2 compostos) e éter (1 composto). Os terpenos constituíram a classe dominante de compostos voláteis presentes nas folhas de *O. gratissimum*, dentre os quais, três compostos são relatados na literatura como primordiais para o aroma característico desta planta, sendo estes: α-pineno (aroma picante e herbal), eucaliptol (aroma de menta e caramelo) e γ-terpinene (aroma herbal e de folha). Segundo Sonmezdag, et al. (2018), os terpenos são os principais compostos responsáveis pelas ações farmacológicas descritas para as espécies *Ocimum*.

Os aldeídos foram os compostos mais abundantes nas folhas de *E. foetidum*, *H. acetosella* e *H. sabdariffa*. O (E)-2-dodecenal (1674,42 µg/g) foi, de longe, o composto mais abundante nas folhas de *Eryngium foetidum*. Segundo Forber, et al. (2002) e Paul, et al. (2011), extratos ricos em (E)-2-dodecenal são correntemente relatados como potentes anti-bactericidas. Apenas alguns compostos voláteis foram identificados nas folhas de *P. aculeata*, *P. oleracea* e *X. sagittifolium*.

No entanto, com exceção ao *E. foetidum,* todas as plantas apresentaram (E)-2-hexenal em seu perfil volátil. (E)-2-hexenal é descrito na literatura como composto que apresenta importante propriedade antimicrobiana contra micro-organismos patogênicos, mesmo em baixas concentrações (Ayseli & İpek Ayseli, 2016).

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CONCLUSÃO GERAL

As sete plantas alimentícias não-convencionais (PANC) estudadas, apresentaram concentração de fenólicos totais similares ao encontrado em frutas e ervas, que são amplamente apontadas como fontes ricas de fenólicos. Os resultados de atividade antioxidante contra espécies reativas de oxigênio (ROS) destacam as espécies de *Hibiscus* como capazes de reduzir, não só a oxidação por parte do $ROO^{\bullet}$ e HOCl, mas como as únicas plantas capazes de inibição a ação do H_2O_2. Extratos de *O. gratissimum* foram os únicos a serem classificados como eficazes antimicrobianos. No que diz respeito à atividade antiproliferativa, o extrato de *P. oleracea* destacou-se com baixos valores de GI_{50}. *O. gratissimum* e *E. foetidum* foram capazes de inibir a reação de nitrosação, sugerindo um possível efeito protetor destas plantas na formação de compostos nitrosos. *O. gratissimum* também foi capaz de atenuar os níveis de superóxido em células do músculo liso vascular sob stress oxidativo induzido pela ação da Ang II. Em relação aos voláteis, *O. gratissimum* e *E. foetidum* foram as amostras que apresentaram maiores número de compostos voláteis em sua composição. Com exceção do *E. foetidum* todas as amostras apresentaram em seu perfil o composto (E)-2-hexenal, que é comumente descrito com potente ação antimicrobiana, mesmo em baixas concentrações.

As plantas aqui estudadas demonstraram capacidades bioativas interessantes que devem ser, melhor e mais profundamente, exploradas como fontes de alimentos, bem como por diversos setores industriais, a fim de promover o uso dessas plantas negligenciadas e sub-utilizadas. No entanto, apesar dos resultados obtidos, deve-se manter em mente que muito ainda precisa ser pesquisado à respeito dessas plantas, em especial sobre suas características bioativas *in vivo*.

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ANEXO I

DECLARAÇÃO REFERENTE AO PATRIMÔNIO GENÉTICO

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso
Cadastro nº A9E8DBC

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A9E8DBC**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Pereskia aculeata
Portulaca oleracea
Xanthosoma sagittifolium
Hibiscus acetosella
Eryngium foetidum
Ocimum gratissimum
Hibiscus sabdariffa

Título da Atividade: **Plantas alimentícias não convencionais (PANC): compostos bioativos**

Equipe

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Parceiras Nacionais

06.517.387/0001-34 / **Universidade Federal do Piauí**

Parceiras no Exterior

University of Alberta

University of Reading

Data do Cadastro: **03/11/2018 11:23:00**

Situação do Cadastro: **Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **10:54** de **11/12/2018**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

ANEXO II

CITOTOXIDADE *IN VITRO* PARA CÉLULAS A7r5

Table AII. Cytotoxicity test for the crude and digest extracts, at 25 µg/mL, from *E. foetidum, H. sabdariffa, O. gratissimum, P. aculeata, P. oleracea* and *X. sagittifolium*

	Absorbance (nm)	
	Crude extract	**Digest extract**
Control	4053.29 ± 91.82	3912.65 ± 82.62
E. foetidum	4439.91 ± 474.67	4667.52 ± 124.17
H. sabdariffa	3889.46 ± 98.05	3868.96 ± 53.47
O. gratissimum	3789.43 ± 150.63	3882.89 ± 156.29
P. aculeata	3922.72 ± 213.32	3838.97 ± 95.54
P. oleracea	3937.41 ± 68.44	4006.52 ± 130.19
X. sagittifolium	3641.48 ± 142.00	4028.06 ± 220.39

None of the samples presented significant difference ($p > 0.05$) with the control, by Tukey test.