UNIVERDIDADE FEDERAL DO ESPÍRITO SANTO

CENTRO DE CIÊNCIAS HUMANAS E NATURAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL

MAINÃ MANTOVANELLI DA MOTA

CARACTERIZAÇÃO GENÉTICA, FITOQUÍMICA E DAS ATIVIDADES BIOLÓGICAS DE DIFERENTES POPULAÇÕES NATURAIS DE *Varronia curassavica* Jacq. E *Momordica charantia* L. NO ESPÍRITO SANTO

VITÓRIA - ES
2019

MAINÃ MANTOVANELLI DA MOTA

CARACTERIZAÇÃO GENÉTICA, FITOQUÍMICA E DAS ATIVIDADES BIOLÓGICAS DE DIFERENTES POPULAÇÕES NATURAIS DE *Varronia curassavica* Jacq. E *Momordica charantia* L. NO ESPÍRITO SANTO

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Centro de Ciências Humanas e Naturais da Universidade Federal do Espírito Santo como parte dos requisitos exigidos para a obtenção do título de Doutor em Biologia Vegetal.

Área de concentração: Fisiologia Vegetal.

Orientador(a): Prof.ª. Dr.ª Maria do Carmo Pimentel Batitucci

VITÓRIA - ES
2019

[PÁGINA DA FICHA CATALOGRÁFICA]

CARACTERIZAÇÃO GENÉTICA, FITOQUÍMICA E DAS ATIVIDADES BIOLÓGICAS DE DIFERENTES POPULAÇÕES NATURAIS DE *Varronia curassavica* Jacq E *Momordica charantia* L. NO ESPÍRITO SANTO

MAINÃ MANTOVANELLI DA MOTA

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Centro de Ciências Humanas e Naturais da Universidade Federal do Espírito Santo como parte dos requisitos exigidos para a obtenção do título de Doutor em Biologia Vegetal na área de concentração Fisiologia Vegetal.

Aprovada em _________ de ________ de 20___.

Comissão Examinadora:

Drª. Maria do Carmo Pimentel Batitucci - UFES
Orientadora e Presidente da Comissão

Drª. Silvia Tamie Matsumoto - UFES
Examinador Interno

Dr. José Aires Ventura - INCAPER
Examinador Interno

Dr. Ricardo Machado Kuster- UFES
Examinador Externo

Drª. Taís Cristina Bastos Soares - UFES
Examinador Externo

“A educação é a arma mais poderosa para mudar o mundo.”
Nelson Mandela

AGRADECIMENTOS

À Universidade Federal do Espírito Santo por ter possibilitado a realização do meu Doutorado e por todo conhecimento que adquiri ao longo desses dez anos de formação acadêmica.

Ao Programa de Pós-Graduação em Biologia Vegetal pela oportunidade de desenvolver minha pesquisa.

À Fundação de Amparo à Pesquisa e Inovação do Estado do Espírito Santo pela concessão da bolsa, pelo fomento financeiro e incentivo à pesquisa.

Aos professores Drª. Silvia Tamie Matsumoto, Dr. José Aires Ventura, Dr. Ricardo Machado Kuster e Drª. Taís Cristina Bastos Soares por gentilmente aceitarem compor a banca de avaliação e contribuir com o meu trabalho.

À Maria do Carmo, que foi mais do que uma orientadora, mas também uma amiga. Muito obrigada por você ter depositado sua confiança em mim, em um momento de tamanha incerteza, por sempre valorizar o meu trabalho, por seus ensinamentos e conselhos preciosos. Você é um exemplo como pessoa e profissional por sempre trilhar seus caminhos pautados na ética, justiça e dedicação.

Ao Jean, um grande amigo que a UFES me deu e o qual levarei comigo, e o que posso dizer dele? Só coisas boas, ele me tranquiliza, me aconselha, me incentiva, está sempre disposto a ajudar e ainda é capaz de tirar uma risada noss até nos piores momentos.

Á Anny, uma bela surpresa que esse período reservou pra mim, uma parceira de extração de DNA e preparo de géis, que aos poucos foi se transformando em uma grande amiga. Obrigada por todos os ensinamentos, discussões e questionamentos que levantava e que você mesmo respondia isso foi muito importante na construção do meu senso crítico enquanto pesquisadora. Muito obrigada por tudo!

À Suiany, Irany e Juliana, por todo apoio e ajuda nos experimentos e pela amizade que tornou mais leve o meu trabalho.

À toda equipe do Laboratório de Genética Vegetal e Toxicológica, Mirieli, Monique, Patrícia, Paula, Judá, Alex, Sávio, Larissa, Felipe, Vanessa, Lana, Ana Júlia, Renê, Sula, Yasmim e Maria Gabriela, pelo bom convívio, pelas boas discussões e pelos momentos de descontração que por muitas vezes se instalava em nosso laboratório.

À Juliana Justino e aos professores responsáveis pelo Núcleo de Genética Aplica à Conservação da Biodiversidade, que tornaram possível a realização das amplificações de DNA e as análises de géis. Ao Professor Alexandre, responsável pelo Laboratório Multiusuário de Análises Biomoleculares, que juntamente com as técnicas, Caroline e Natércia, possibilitaram as análises de HPLC.

À todos os meus amigos pelos estímulos e compreensão dos momentos de ausência.

Às minhas irmãs, Paula, Manuela e Bete, e ao meu pai, Adilson, que se orgulham de mim, me incentivam e torcem pela minha vitória. Obrigada por todo amor e apoio que sempre dedicaram a mim.

À minha mãe, Sandra, uma mulher forte, guerreira e batalhadora pela qual me espelhei para chegar até aqui. Ela sempre me dizia: “minha filha, o conhecimento é algo que você adquire e que ninguém jamais poderá tirar de você”. Muito obrigada mãe por nunca medir esforços em proporcionar uma educação de qualidade para mim e para minhas irmãs.

Ao meu amor, Claudio Júnior, por sempre apoiar e incentivar as minhas decisões, por ser meu companheiro em todas as horas, por aceitar coletar plantas comigo, por me fazer companhia aos finais de semana no laboratório, por compreender minha ausência em muitas ocasiões e por me acalmar dizendo: “fica tranquila, meu amor, vai dar tudo certo!”. Obrigada por sempre estar ao meu lado, foram muitos momentos vividos juntos, desde a graduação até hoje. Que felicidade poder compartilhar mais essa conquista com você!

RESUMO

As plantas medicinais têm sido usadas para tratamento, cura e prevenção de doenças por milhares de anos. *Varronia curassavica* Jacq. e *Momordica charantia* L. são espécies de plantas amplamente exploradas para fins terapêuticos.*Varronia curassavica* Jacq., popularmente conhecida como "erva-baleeira", pertence à família Cordiaceae e é tradicionalmente usada para tratar inflamações, além disso, é descrita por apresentar propriedades antibacterianas, antifúngicas, antialérgicas, antitumorais e antioxidantes.Já a *Momordica charantia* L. é uma espécie herbácea pertencente à família Cucurbitaceae comumente conhecida como melão-de-são-caetano e na medicina tradicional, é utilizada para o tratamento de diabetes, cólicas, câncer, entre outras desordens. As propriedades medicinais apresentadas pelas diferentes plantas medicinais estão relacionadas com o conteúdo dos metabólitos secundários presentes na planta. No entanto, fatores genéticos e ambientais, como a composição do solo, a temperatura, a precipitação pluviométrica e a incidência de radiação ultravioleta podem afetar as concentrações desses componentes químicos que refletem em suas atividades biológicas. Portanto, objetivou-se com este estudo avaliar a influência dos fatores ambientais e genéticos na produção de metabólitos secundários e nas atividades antioxidante, citotóxica e antiproliferativa de populações naturais de diferentes regiões do Espírito Santo Brasil das espécies *V. curassavica* Jacq. e *M. charantia* L.. Os resultados obtidos a partir das análises utilizando marcadores moleculares ISSR mostraram que tanto as populações de *V. curassavica* quanto as de *M. charantia* apresentaram baixa diferenciação genética entre as populações analisadas, provavelmente devido ao fato dessas espécies possuírem uma grande variedade de polinizadores e animais dispersores de sementes que facilitaram o fluxo gênico entre as populações. As análises fitoquímicas de *V. curassavica* revelaram uma diferença significativa entre as amostras testadas, o que refletiu na variabilidade em sua atividade antioxidante e antitumoral. Os resultados sugerem fortemente que os fatores ambientais são mais determinantes para a variação dos compostos fenólicos do que os fatores genéticos. As análises com *M. charantia* demostraram que não há uma grande variação entre as suas populações, aqui avaliadas, com relação ao seu conteúdo químico e de atividade biológica, sugerindo que para esta planta a localização geográfica não foi determinante para a variação quantitativa e qualitativa dos compostos fenólicos. O extrato de ambas as espécies apresentou maior citotoxicidade seletiva *in vitro* contra células tumorais, sarcoma 180, demonstrando que *V. curassavica* e *M. charantia* apresentam potencial terapêutico para o desenvolvimento de novos fármacos. Dessa forma, este trabalho é importante para auxiliar na elucidação das condições ótimas para o uso etnofarmacológico dessas plantas medicinais.

Palavras-chave: *Varronia curassavica*• *Momordica charantia*• ISSR• compostos fenólicos• DPPH• ABTS • FRAP • atividade antitumoral• MTT

ABSTRACT

The medicinal plants have been used for treatment, cure and prevention of diseases for several thousands of years. *Varronia curassavica* and *Momordica charantia* are widely exploited plant species for therapeutic purposes. *Varronia curassavica* Jacq. popularly known as “erva-baleeira”, belongs to the family *Cordiaceae* and is traditionally used to treat inflammation, in addition it is described by present antibacterial, antifungal, anti-allergic, antitumor, and antioxidant properties. Already the *Momordica charantia L.* is a species herbaceous belonging to the family Cucurbitaceae commonly known as bitter gourd or bitter melon and in tradicional medicine, it is used for the treatment of diabetes, colics, cancer, among other disorders. The medicinal properties shown by different medicinal plants are due to the secondary metabolites present in the plant. However, genetics and environmental factors, such as soil composition, temperature, rainfall and ultraviolet radiation incidence can affect the concentrations of these chemical components that reflect on their biological activities. Therefore, the aim of this study was to evaluated the environmental and genetic factors influence on the production of secondary metabolites and the antioxidant, cytotoxic and antiproliferative activity of populations from different regions of Espírito Santo/Brazil of *V. curassavica* and *M. charantia*. The results obtained from the analyzes using ISSR molecular markers showed that both V. curassavica and M. charantia species showed a significant similarity between the analyzed populations, probably due to the fact these species possess variety of pollinators insect and seed dispersal animals which facilitated the gene flow. The phytochemical analyzes of *V. curassavica* revealed a significant quantitative difference between the samples tested, which reflected in variability in their biological antioxidant and antitumoral activities. Results strongly suggest that these variations were caused by environmental rather than genetic factors. The analyzes with the species *M. charantia* showed there is not a very large variation among the populations related as to their chemical content and biological activity, suggesting that for this plant the geographic location is not determinant for the quantitative and qualitative variation of phenolic compounds. The extract of both species showed a higher selective cytotoxicity *in vitro* against sarcoma 180, demonstrating that *V. curassavica* and *M. charantia* presents therapeutic potential for the development of new drugs. This work are important to help in elucidation optimal conditions for ethnopharmacological use of these medicinal plants.

Keywords: *Varronia curassavica*• *Momordica charantia*• ISSR• phenolic compounds• DPPH• ABTS • FRAP • antitumoral activity• MTT

LISTA DE FIGURAS

SUMÁRIO

1 INTRODUÇÃO GERAL

A Organização Mundial da Saúde (OMS) caracteriza como medicinal, plantas que contêm propriedades ou compostos que possam ser utilizados para fins terapêuticos. O uso de plantas medicinais para cunho medicinal é uma das estratégias mais antigas empregada pela humanidade, e até hoje é utilizada como terapia alternativa, principalmente em países subdesenvolvidos, em que os tratamentos convencionais são de difícil acesso para uma grande parcela da população.

As espécies *Varronia curassavica* Jacq. e *Momordica charantia* L. são muito utilizadas na medicina tradicional. *Varronia curassavica* Jacq. é uma espécie arbustiva pertencente à família Cordiaceae e é comumente conhecida como erva-baleeira, possui diversas propriedades medicinais, mas a sua ação anti-inflamatória comprovada se destaca. *Momordica charantia* L. é uma planta trepadeira originária da Ásia e África que pertence à família Curcubitaceae e no Brasil ela é mais conhecida como melão-de-são-caetano e apresenta diversos usos terapêuticos: hipocligemiante, antioxidante, antitumoral, antiinflamatório, antimicrobiano, hepatoprotetivo e neuroprotetivo.

Essas propriedades biológicas e farmacológicas das plantas medicinais que permitem a sua utilização para a prevenção, tratamento e cura de doenças, estão relacionadas ao seu conteúdo de metabólitos secundários. Os metabólitos secundários são substâncias que geralmente não estão envolvidas em funções vitais das plantas, assim como os metabólitos primários, mas desempenham um importante papel na adaptação das plantas aos seus ambientes. A síntese destes metabólitos é um resultado da estrutura genética do indivíduo associada às condições ambientais, dessa forma, os fatores como a composição do solo, a temperatura, a precipitação pluviométrica e a incidência de radiação ultravioleta podem afetar a concentração desses componentes químicos, e consequentemente nas suas ações terapêuticas.

Nesse sentido, estudos que visam à compreensão da interação entre as características genéticas das plantas medicinais e as condições do ambiente em que essas plantas se desenvolvem, são de grande relevância, pois podem sugerir condições que resultem em produto final de qualidade, além de contribuir para a conservação e o manejo adquado dessas espécies de plantas. Dessa forma, realizou-se um estudo comparativo das características genéticas e químicas de diferentes populações, das espécies *V. curassavica* e *M. charantia*, coletadas em regiões distintas do estado do Espírito Santo.

2 REVISÃO BIBLIOGRÁFICA

Plantas Medicinais: Dos saberes populares aos científicos

A utilização de plantas com fins terapêuticos para o tratamento e prevenção de doenças é uma prática milenar que está associada aos saberes populares e é aplicada nas diversas culturas ao redor do mundo, há registro de uso das plantas medicinais, em algumas civilizações antigas, que datam mais de 3.000 anos antes de Cristo (LONG et al., 2003; FERNANDES, 2004; AZEVEDO, 2017). Nas Américas, registros arqueológicos datam que os povos ameríndios utilizavam as plantas como remédio ou alimento há mais de dez mil anos (SIMÕES et al., 2017).

As plantas medicinais e seus extratos faziam parte da composição da maioria dos medicamentos utilizados até o século XIX. Entretanto, a partir de meados do século XX com o advento da medicina moderna e o crescimento da indústria de medicamentos, esse quadro sofreu grandes alterações e devido aos interesses da indústria em aumentar seus lucros passaram a desqualificar o saber popular sobre as plantas e por isso foram substituídas pela terapia sintética e altamente industrializada (SCHENKEL et al., 2002; FERNANDES, 2004; AZEVEDO, 2017).

Apesar do grande e contínuo avanço da tecnologia e da ciência na área da medicina, os medicamentos industrializados não atenderam a todas as expectativas geradas em torno destes, além disso, os efeitos colaterais e tóxicos produzidos pelo seu uso e a dificuldade de acesso por parte da população levaram ao ressurgimento e à expansão das plantas medicinais como um importante recurso para o tratamento de doenças (CAROLLO, 2008; AZEVEDO, 2017).

A Organização Mundial de Saúde (OMS), a partir do reconhecimento da incapacidade da medicina tecnológica em atuar de maneira eficaz, atendendo, por meio de suas terapias específicas, a todas as camadas da população, vem estimulando o uso da medicina tradicional, sendo que, alguns países, inclusive o Brasil, já possuem políticas nacionais que regulamentam o uso de plantas medicinais (FERNANDES, 2004; CAROLLO, 2008).

O Brasil através do Ministério da Saúde, no ano de 2006, implementou a Política Nacional de Práticas Integrativas e Completamentares (PNPIC) ao Sistema Único de Saúde (SUS) (BRASIL, 2006a). Utilizando como suporte a PNPIC, também no ano de 2006, o Presidente da República aprovou a Política Nacional de Plantas Medicinais e Fitoterápicos (PNPMF), tendo por objetivo garantir o acesso seguro e o uso racional de plantas medicinais e fitoterápicos pela população brasileira (BRASIL, 2006b).

Porém, o aumento do consumo de ervas medicinais tem levado a uma exploração desenfreada dos ecossistemas para obtenção de princípios ativos que possam ser utilizados no tratamento e prevenção de doenças. Dessa forma, o mercado de plantas medicinais está crescendo em um ritmo dramático e às custas de populações naturais já declinantes de espécies de plantas, muitas das quais estão à beira da extinção (LEAMAN, 2001; CAROLLO 2008).

A conservação de plantas medicinais é um desafio, no entanto, é amplamente aceito que a preservação dessas espécies pode ser alcançada através de um balanceamento entre as estratégias de conservação *in situ* e *ex situ*. Assim, a domesticação e o cultivo aparecem como opções para obtenção da matéria prima de interesse farmacêutico e redução do extrativismo nas formações florestais. Além da manutenção de bancos de germoplasma para manutenção dos recursos genéticos (CAROLLO 2008; HAWKIN, 2008).

Portanto, estudos que visam a caracterização completa de espécies de plantas medicinais se tornam imprescindíveis neste cenário, pois torna possível o desenvolvimento e aplicação de novas tecnologias para o cultivo e comercialização dessas plantas, além de contribuir para o uso adequado desses recursos terapêuticos, tendo em vista que o uso indevido de plantas medicinais fora de seu contexto original e sem respaldo científico pode resultar em agravos à saúde e, eventualmente, levar a quadros fatais de intoxicação (LEAMAN, 2001; PIRES et al., 2016).

2.2 Metabólitos secundários: a interface química planta-ambiente

As plantas sintetizam uma quantidade imensurável de compostos orgânicos que são tradicionalmente divididos em metabólitos primários e secundários. Os metabólitos primários incluem todos os compostos que são considerados imprescindíveis à vida, portanto, são fundamentais para os processos de fotossíntese, respiração, crescimento e desenvolvimento da planta (VERPOORTE, 2000; TAIZ; ZEIGER, 2013; SIMÕES et al., 2017). Já os produtos do metabolismo secundário não estão envolvidos em processos cruciais para a manutenção da vida, entretanto exercem um importante papel na interação do organismo com o ambiente, por isso, esses compostos estão frequentemente envolvidos na proteção de plantas contra os estresses biótico e abiótico (CROZIER; JAGANATH; CLIFFORD, 2007; PAGARE et al., 2015).

Esses compostos apresentam baixo peso molecular e para classificá-los como metabólitos secundários uma série de características devem ser observadas: (1) sua distribuição taxonômica é restrita, sendo encontrados em apenas alguns grupos de organismos; (2) apresentam uma ampla variabilidade química estrutural e a sua composição química distinta pode ser utilizada na distinção de táxons (quimiotaxonomia); (3) os metabólitos secundários não são necessariamente sintetizados durante todo o período de desenvolvimento da planta, podendo ser produzidos apenas em fases muito específicas; (4) em plantas superiores, essas moléculas são frequentemente armazenadas nos vacúolos das células produtoras (VILADOMAT; BASTIDA, 2015; SIMÕES et al., 2017).

As estimativas apontam que existam mais de 200.000 metabólitos secundários conhecidos (KUTCHAN et al., 2015; YANG et al., 2015) e toda essa gama de substâncias é sintetizada a partir de precursores provenientes do metabolismo primário (Figura 1), sendo os mais importante a acetil coenzima A (acetil-CoA), ácido chiquímico, ácido mevalônico e o metileritritol fosfato. Estes são utilizados respectivamente nas vias do acetato, chiquimato, mevalonato e na via do metileritritol fosfato (DEWICK, 2009; SIMÕES et al., 2011; TAIZ, 2013).

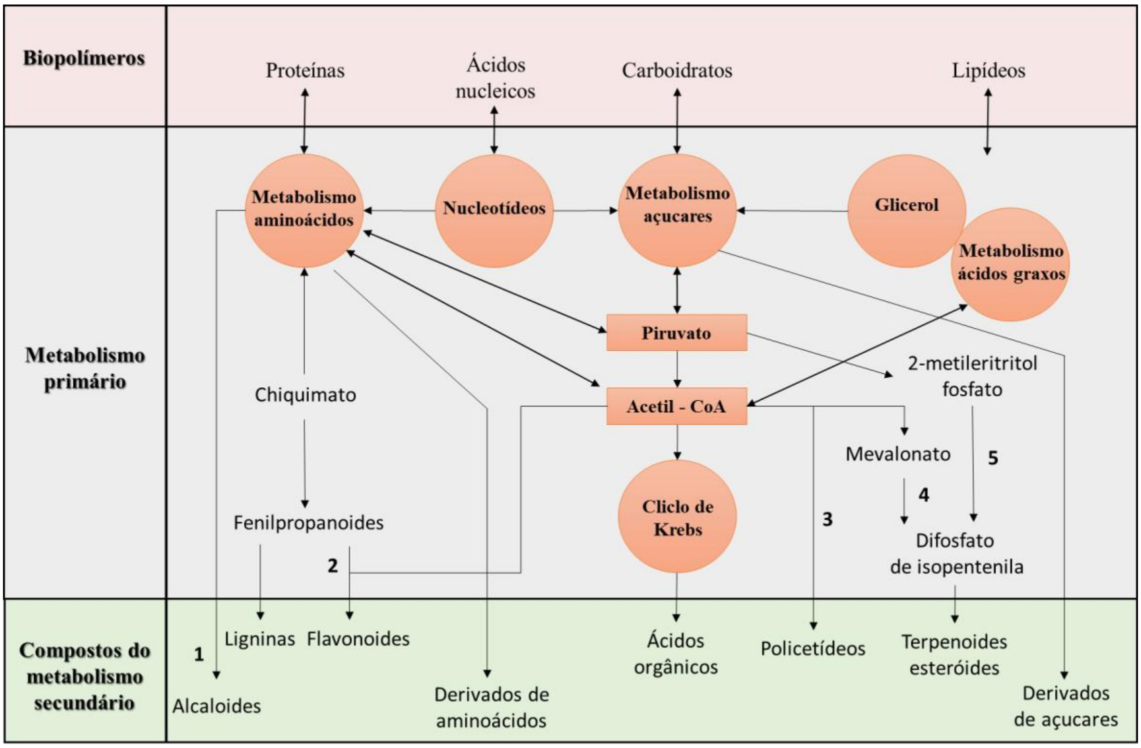


Figura 1 -Relação entre o metabolismo primário e secundário da planta. Fonte: Adaptada de Simões e colaboradores, 2011.

Acetil-CoA é formada pela descarboxilação oxidativa do piruvato pela via glicolítica ou pela β-oxidação dos ácidos graxos, esta pode seguir em três diferentes rotas metabólicas: 1) a do ácido tricarboxilíco, onde poderá ocorrer a síntese dos alcalóides pirrolidínicos, tropânicos, pirrolizidínicos, piperidínicos e quinolizidínicos; 2) a via do ácido mevalônico, que dará origem aos terpenóides, fenóis e os esteróis e por fim; 3) a via do acetato, na qual são formados os poliacetilenos (DEWICK, 2009; TAIZ, 2013).

O ácido chiquímico é produzido ao longo de uma série de quatro reações que começa com a condensação de dois intermediários do metabolismo dos carboidratos, o fosfenolpiruvato, proveniente da via glicolítica, e a eritrose 4-fosfato que é originada a partir da via das pentoses fosfato. Essa reação é catalisada pela enzima 3-desoxi-d-arabino-heptulosonato-7-fosfato (DAHP) sintase (Figura 2). Após a incorporação de uma molécula de fosfoenolpiruvato ao ácido chiquímico há a formação do ácido corísmico. Este atua como precursor de ácidos fenólicos simples e de aminoácidos aromáticos, triptofano, tirosina e fenilalanina, que são muito importantes para a síntese dos fenilpropanoides, derivados do ácido cinâmico, ligninas e alcaloides (TZIN et al., 2012; CORUZZI et al., 2015).

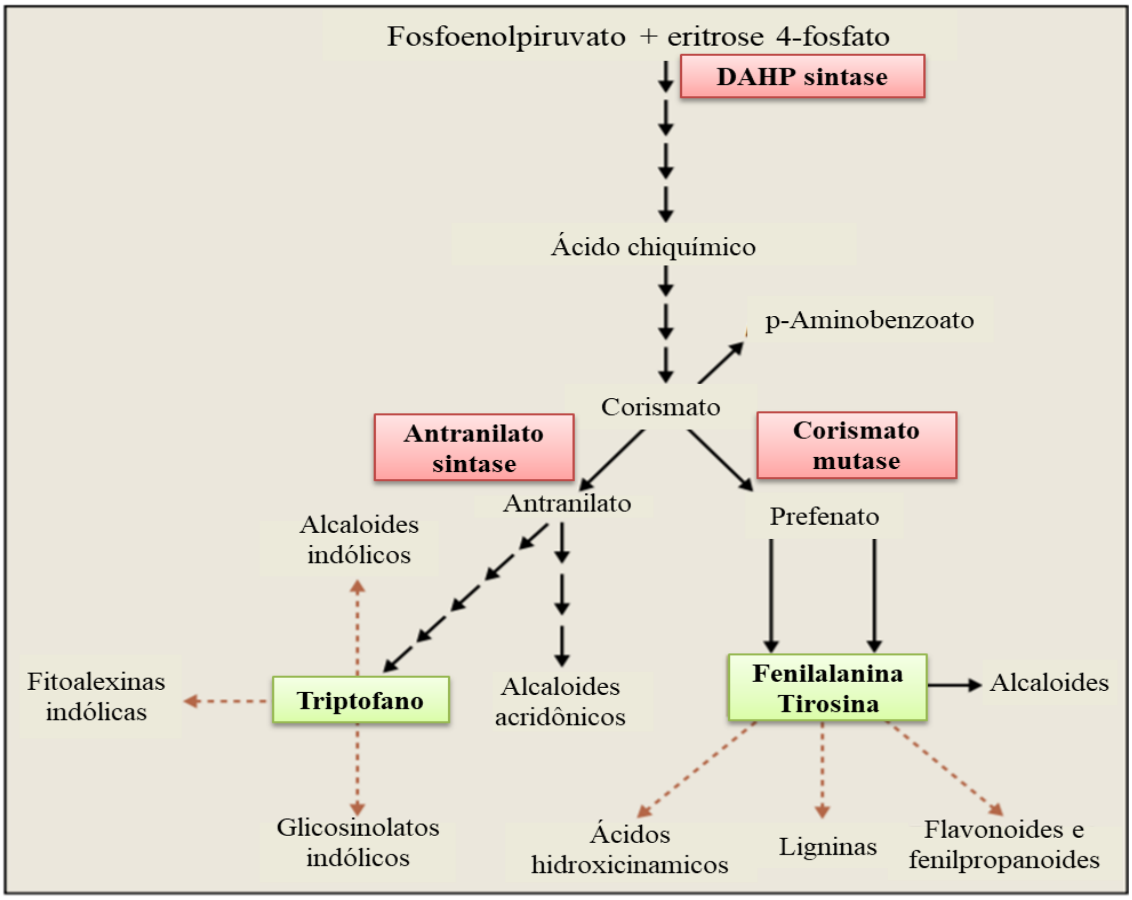


Figura 2 - Via do ácido chiquímico. Fonte: Adaptada de Coruzzi et al., 2015.

Para a síntese de todos os terpenos utiliza-se como matéria-prima os isoprenos difosfato de isopentenila (IPP) e o difosfato de dimetilalila (DMAPP), que são as unidades pentacarbonadas que se unem para formar moléculas de terpenos maiores, sendo os monoterpenos (C10), sesquiterpenos (C15), diterpenos (C20), triterpenos (C30) e os carotenoides (C40). Estes intermediários são sintetizados em plantas por duas rotas completamente diferentes que são espacialmente separadas: a via do mevalonato (MEV), localizada no citosol, e a via do metileritritol 4-fosfato (MEP) que ocorre nos plastídeos (Figura 3).

Na via do MEV há a condensação de três moléculas de acetil-CoA para formar o ácido mevalônico e em uma sequência de quatro reações é produzido o IPP ou o seu isômero DMAPP. Já na via do MEP, o IPP é formado por uma série de reações que se dá início a partir da combinação de dois intermediários da via glicolítica, o piruvato e o gliceraldeído 3-fosfato (DEWICK, 2009; SIMÕES et al., 2011; KUTCHAN et al., 2015).

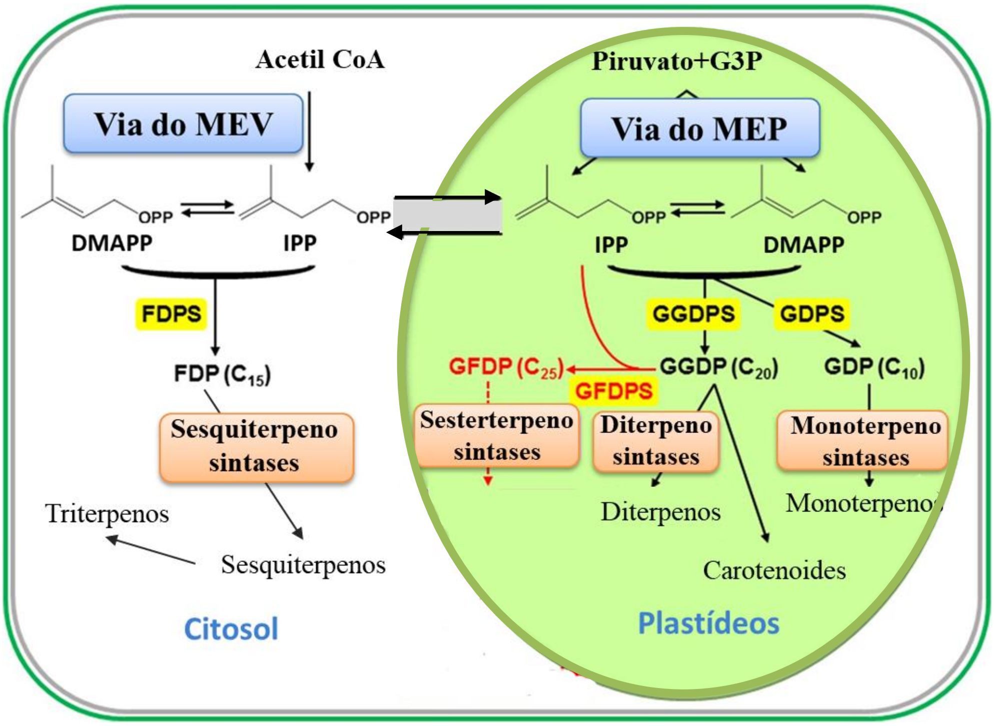


Figura 3 - Vias do metabolismo dos terpenoides. Fonte: Adaptada de Yan Liu et al. (2016).

A biossíntese dos metabolitos secundários representa a interação química entre a planta e o ambiente circundante, já que os metabolites secundários desempenham papéis biológicos como resposta adaptativa ao meio ambiente (VERMA; SHUKLA 2015; ZHI-LIN et al. 2007). Embora exista uma regulação genética, as alterações no conteúdo e nas proporções desses compostos em plantas podem sofrer variações que resultam da interação de processos bioquímicos, fisiológicos, ecológicos e evolutivos. Assim, dependendo do tipo e da intensidade do estímulo ambiental, a planta poderá sintetizar diferentes compostos em função do redirecionamento de vias metabólicas responsáveis pela produção de compostos bioativos. Dentre os estímulos ambientais associados a alterações na síntese de metabólitos secundários, destacam-se os fatores bióticos como a interação entre as plantas e outros organismos, a idade e o estádio de desenvolvimento da planta e o seu ciclo circadiano; e os fatores abióticos: temperatura, umidade, intensidade da luz, sazonalidade, disponibilidade de nutrientes, água e CO_2 (GOBBO-NETO; LOPES, 2007; BORGES et al., 2017).

Diversos estudos têm demonstrado que a síntese de metabólitos secundários ocorre mediante controle genético (DAVIES; SCHWINN, 2003; YANG et al., 2012; PATRA et al., 2013; LI et al., 2015). Estima-se que 15 a 25% dos genes identifcados no genoma de plantas contribuam para o metabolismo secundário, levando à síntese de compostos bioativos. Esses genes são regulados por diferentes classes de fatores de transcrição que afetam o fluxo metabólico, influenciando a expressão gênica dos genes que controlam a biosíntese e acúmulo dos metabólitos secundários (YANG et al., 2012; PATRA et al., 2013; VERMA; SHUKLA 2015).

Li et al. (2015) ao realizarem o sequenciamento de RNA da planta *Camellia sinensis,* identificaram 1719 genes que estariam relacionados com a regulação de vias de síntese de metabólitos secundários. Os genes que regulam a via biossintética da artemisina, uma importante lactona sesquiterpênica isolada da planta *Artemisia annua* e é bastante conhecida por sua ação antimalárica, foram identificados por Abdin e Alam (2015). Enquanto Yu et al. (2012) observaram que a superexpressão dos fatores de transcrição AaERF1 e AaERF2 levaram a um aumento na produção e no acumulo da artemisina.

Embora a síntese de metabólitos secundários seja regulada geneticamente, os fatores ambientais exercem uma importante influência na produção e no acumulo destes compostos. Os níveis de radiação solar pode representar um estresse para as plantas, isso desencadeia efeitos protetivos que alteram a produção de metabólitos secundários pelas plantas (GOBBO-NETO; LOPES, 2007; BORGES et al., 2017). Em um estudo realizado por Khatib et al. (2011) com três espécies de Apiaceae, observou-se que a exposição direta à luz do Sol resultou em um aumento no conteúdo de compostos fenólicos ao comparar com plantas cultivadas sob 50% de sombra. Alqahtani et al. (2011) também concluíram que o conteúdo de flavonoides e de ácido clorogênico em *Centella asiatica* está positivamente correlacionado com as condições de luminosidade do local de crescimento da planta.

A disponibilidade de água e de nutrientes constituem importantes fatores que exercem influência na produção e no acúmulo de compostos bioativos. De acordo com Sampaio et al. (2016) o estresse hídrico provoca um aumento na produção de espécies reativas de oxigênio, resultando em um aumento na síntese de compostos fenólicos como resposta de defesa da planta. Jaafar et al. (2012) também concluíram de maneira semelhante sobre esse mecanismo, em seu estudo realizado com *Labisia pumila*, onde observou-se um aumento no conteúdo de compostos fenólicos, flavonoides e antocianinas após submeterem estas plantas ao estresse hídrico. Kováčik e Bačkor (2007) demonstraram que a deficiência de nutrientes também promove alteração no metabolismo secundário da planta, pois,observaram aumento no conteúdo de compostos fenólicos em *Matriarca chamomilla*, tanto nas folhas quanto nas raízes, ao cultivar essas plantas mediante expropriação de nitrogênio.

A sazonalidade representa um dos fatores abióticos mais expressivos com relação às variações quantitativas e qualitativas dos compostos ativos das plantas (PRINSLOO; NOGEMANE, 2018). Ao analisarem quimicamente as folhas de *Eremanthus mattogrossensis* ao longo das quatro estações do ano, observou-se que as concentrações de compostos fenólicos foram mais elevadas durante a primavera (GOUVEA et al., 2012). Em estudos realizados com *Piper cernuum*, a composição e a produção de metabólitos secundários foram diferentes nas quatro estações analisadas influenciando na atividade antimicrobiana do óleo essencial da planta (GASPARETTO et al., 2016).

Danos causados por patógenos frequentemente levam a uma resposta bioquímica, por consequência altera a produção de compostos bioativos, dessa forma, os metabólitos secundários são importantes elementos que atuam na defesa química das plantas contra os ataques de pragas e patógenos (GOBBO-NETO; LOPES, 2007; BORGES et al., 2017). De acordo com Huang e Backhouse (2004) há um aumento nos níveis de apigenidina e luteolinidina no sorgo quando inoculado com *Fusarium proliferatum* e *Fusarium thapsinum.* Huffaker et al. (2011) observaram um maior acumulo de fitoalexinas, que são terpenoides com ação antifúngica, em tecidos da planta *Zea mays* infectado por *Fusarium graminearum.*

2.3 Compostos fenólicos

Os metabólitos secundários são divididos em três pricipais grupos e esta classificação é baseada na sua origem biossintética, sendo eles: os terpenos, os compostos fenólicos e os compostos nitrogenados (alcaloides, glicosídeos, glucosinolatos e os glicosídeos cianogênicos) (BORGES et al., 2017). Dentre a grande diversidade estrutural de metabólitos secundários, os compostos fenólicos têm atraído considerável interesse e atenção por apresentarem uma ampla variedade de atividades biológicas (HAN et al., 2007).

Os compostos fenólicos são caracterizados por possuírem um ou mais anéis aromáticos com pelo menos um grupo hidroxila (Figura 4). Porém essa descrição que considera apenas a estrutura química não é adequada, visto que, existem substâncias que apresentam anéis aromáticos com hidroxilas substituintes, mas fazem parte de outras classes de metabólitos, dessa forma, é necessário considerar também a rota biossíntética (ZUANAZZI et al., 2017). Portanto, os compostos fenólicos constituem uma classe de metabólitos secundários de plantas derivados exclusivamente da via do ácido chiquímico ou dos policetídeos, apresentando um ou mais anel fenólico e desprovido de qualquer grupo funcional à base de nitrogênio em sua expressão estrutural mais básica (BALASUNDRAM et al., 2006; CHEYNIER, 2012).

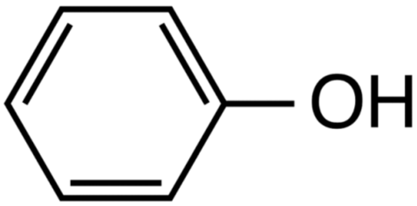
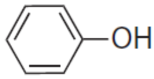
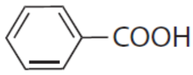
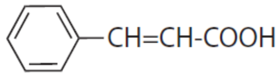
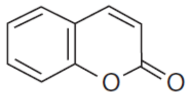
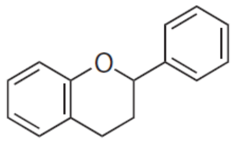
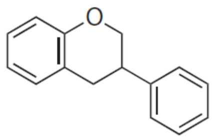


Figura 4 - Estrutura química básica de um fenol.

Compostos fenólicos representam os metabólitos secundários mais amplamente presentes e distribuídos no reino vegetal. Já foram encontrados mais de 8.000 compostos fenólicos bem diversificados em termos de estrutura química e de função nas plantas. Estas substâncias podem ser classificadas de várias maneiras, dentre estas, podemos citar a classificação pelo número de anéis aromáticos, número de carbonos na molécula ou estrutura química básica, sendo alguns deles: fenóis simples, ácidos fenólicos, ácidos cinâmicos e cumarinas, flavonoides e isoflavonoides, ligninas e taninos condensados (Tabela 1) (SANTANA-GÁLVEZ; JACOBO-VELÁZQUEZ*,* 2018).

Tabela 1 - Representação de algumas classes de compostos fenólicos de acordo com o número de anéis aromáticos, esqueleto carbônico e estrutura química básica.
.

Classes	Números de anéis aromáticos	Esqueleto carbônico	Estrutura química básica
Fenóis simples	1	C_6	
Ácido hidroxibenzeno	1	C_6-C_1	
Ácido hidroxicinâmico	1	C_6-C_3	
Cumarinas	1	C_6-C_3	
Flavonoides	2	C_6-C_3- C_6	
Isoflavonoides	2	C_6-C_3- C_6	
Ligninas	3 ou mais	$(C_6$-$C_3)_n$	Múltiplo
Taninos condensados		$(C_6$-C_3- $C_6)_n$	Múltiplo

Fonte: SANTANA-GÁLVEZ; JACOBO-VELÁZQUEZ, 2018).

Os compostos fenólicos são frequentemente associados às respostas de defesa das plantas, entretando, os metabólitos fenólicos têm diversas outras funções nas plantas, como em processos germinativos, pois inibidores de crescimento fenólicos protegem sementesda germinação prematura, de maneira a reduzir a possibilidade de germinação em condições desfavoráveis. Estas substâncias também apresentam um papel importante no desenvolvimento da planta, já que a presença de compostos fenólicos nas partes vegetativas pode inibir ou estimular o seu crescimento (CHALKER-SCOTT; FUCHIGAMI, 1989). Além disso, propriedades sensoriais (cor, aroma, sabor e adstringência) e estruturais, a incorporação de substâncias atraentes para acelerar a polinização, defesa contra herbívoros e patógenos, entre outros, estão associados à presença de compostos fenólicos nos tecidos vegetais (TOMÁS-BARBERÁN, ESPÍN, 2001; LIN et al.,2016).

Cada vez mais têm aumentado o interesse pelos metabólitos fenólicos, tanto pela ciência quanto pela indústria alimentícia e farmacêutica, isso porque estas substâncias exibem uma ampla variedade de propriedades farmacológicas, incluindo efeitos antialérgico, antihiperglicêmico (HOSSAIN et al., 2008), antiaterogênico (LIU et al., 2004; MILES et al. 2005) anti-inflamatório (SERGENTet al., 2010;VILAR et al., 2015), antimicromibiano (MANDAL et al., 2017; OUERGHEMMI et al., 2017), cardioprotetivo(BALEA et al., 2018) e vasodilatador (VICTÓRIO et al., 2005).

Os efeitos benéficos derivados dos compostos fenólicos são atribuídos à sua capacidade de modular certas enzimas do metabolismo celular ou em decorrência da sua atividade antioxidante, sendo que, o potencial antioxidante dos compostos fenólicos está diretamente relacionado com a sua habilidade de eliminar espécies reativas de oxigênio, capacidade de quelar metais e eliminar eletrófilos, inibir a nitrosação ou pelo seu potencial de auto-oxidação, produzindo peróxido de hidrogênio na presença de certos metais (HUANG; FERRARO, 1992). Estruturas moleculares, particularmente o estado de hidroxilação dos seus anéis aromáticos, como o número e as posições dos grupos hidroxila, e a natureza das substituições nos anéis aromáticos, conferem aos compostos fenólicos a capacidade de inativar os radicais livres (BALASUNDRAM et al., 2006; HUANG et al., 2009; OZCAN et al., 2014).

2.3.1 Flavonoides

Os flavonoides constituem um dos mais importantes grupos de compostos fenólicos, e estão amplamente distribuídos por todo o reino vegetal, sobretudo nas angiospermas e praticamente ausentes em algas e fungos, podendo ser encontrados em frutas, folhas, sementes ou em outras partes da planta. Até o presente momento são conhecidas mais de 7000 variedades de flavonoides, sendo que em grande parte são encontrados na forma conjugada com açúcares e na forma oxigenada (ANGELO; JORGE, 2007; ZUANAZZI et al., 2017).

Eles constituem uma classe de polifenois com baixo peso molecular que se encontram em diversas formas estruturais, entretanto a maioria dos representantes desta classe apresentam 15 átomos de carbono em seu núcleo fundamental (C6 –C3 –C6), organizados em dois anéis aromáticos, unidos por uma cadeia de três carbonos que podem ou não formar anel. Os compostos possuem três anéis que são denominados A, B e C, sendo os átomos de carbono dos núcleos A e C numerados com números ordinários, já o núcleo B recebe os mesmos números seguidos de uma linha (’) (Figura 5) (ZUANAZZI et al., 2017).

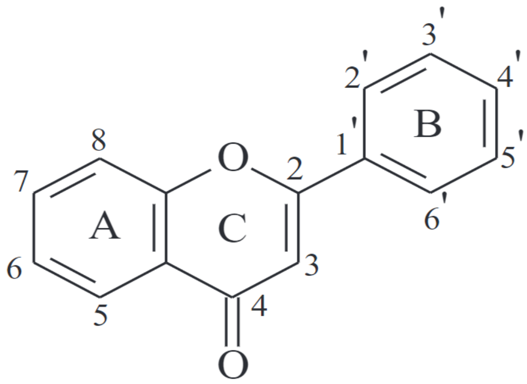


Figura 5 - Estrutura básica de um flavonoide com identificação dos anéis e sua numeração. Fonte: Cheng et al., 2014.

A biossíntese dos flavonóides ocorre através de duas vias diferentes: o anel aromático A é sintetizado a partir de três moléculas de malonil-CoA geradas através das transformações da glicose no ciclo do ácido cítrico, enquanto o anel B é sintetizado a partir de 4-cumaril-CoA produzido a partir da fenilalanina através da via do ácido chiquímico. A condensação dos anéis A e B gera uma chalcona e ao sofrer ciclização catalisada por uma isomerase forma uma flavanona que é utilizada como composto inicial para a síntese de outros flavonoides (Figura 6) (CHENG et al., 2014; NABAVI et al., 2018).

Devido a grande diversidade estrutural dos flavonoides, estes compostos são classificados em diferentes grupos assim como as flavanonas, flavanois, flavonas, flavonóis, isoflavonas, antocianinas, dentre outros. Essas classes diferem nos substituintes dos anéis formados em processos de hidroxilação, metilação, alquilação, glicosilação, acilação e sulfação. As alterações em substituição do anel C originam a principais classes de flavonoides, já as substituições dos anéis A e B originam diferentes compostos dentro de cada grupo de flavonoides (HOLLMAN; KATAN, 1999; PIETTA, 2000; CHENG et al., 2014).

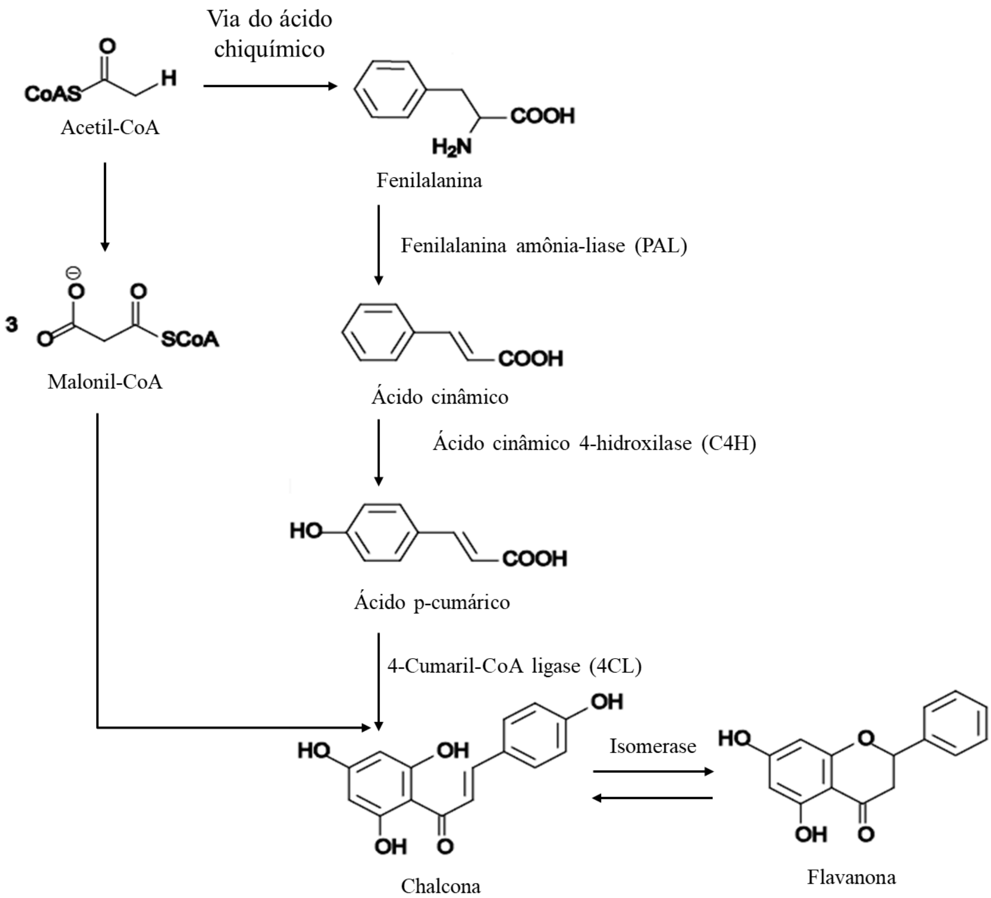


Figura 6 - Representação simplificada da via de biossíntese de flavonoides em plantas. Fonte: Adaptada de Cheng et al. (2014).

Os flavonoides exercem um importante papel ecológico para as plantas, podendo atuar na atração de insetos polinizadores em função da cor atrativa que alguns compostos exibem e devido à adstringência de catequinas e outros flavanois podem representar um sistema de proteção contra insetos prejudiciais à planta. Além disso, os flavonoides protegem os vegetais dos raios ultravioletas e visíveis eliminando as espécies reativas geradas pela radiação e ainda atuam no controle da ação de hormônios vegetais (PIETTA, 2002; ZUANAZZI et al., 2017).

Estudos também têm mostrado que os flavonoides apresentam várias propriedades farmacalógicas que podem ser utilizadas em atividades anticâncer, antibacteriana, antiviral, anti-inflamatória, anti-hipertensiva e também possuem efeitos benéficos no retardo de doenças neurodegenerativas (COWAN, 1999; HAVSTEEN, 2002; HOLLMAN, 2004). Além disso, os flavonoides exibem atividade antioxidante. Os mecanimos de ação antioxidante desses compostos incluem a supressão da formação das espécies reativas de oxigênio através da inibição de enzimas ou neutralização de elementos quelantes envolvidos na produção desses radicais livres, a captura de espécies reativas e o aumento ou proteção de defesas antioxidantes. A eliminação eficiente de radicais livres pelos flavonoides está relacionada com características estruturais, que incluem: (1) a presença da estrutura orto-dihidroxi no anel B; (2) uma ligação dupla C2-C3 com a função 4-oxo no anel C e (3) a presença de um grupo hidroxila em C3 e C5 (Figura 7) (BURDA; OLESZEK, 2001; KUMAR; PANDEY, 2013).

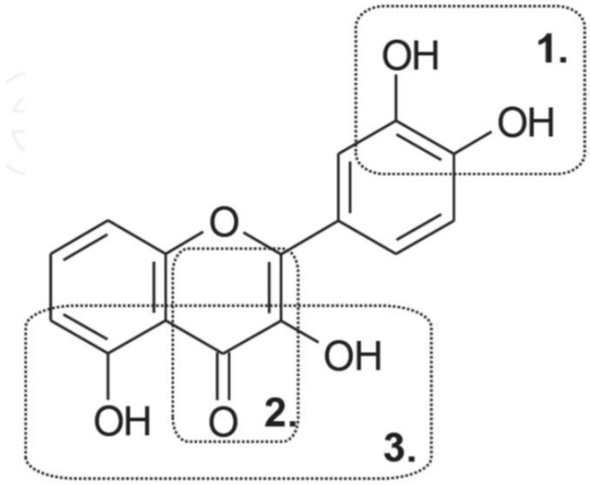


Figura 7 - Principais características estruturais necessárias para a atividade antioxidante dos flavonóides. Fonte: Uivarosi; Munteanu, 2017.

Devido à grande quantidade de flavonoides que as plantas sintetizam estes compostos são classificados em diferentes classes, de acordo com suas características químicas e estruturais (KUMAR; PANDEY, 2013) (Figura 8). As flavonas constituem um dos grupos mais importantes de flavonoides e estão presentes em folhas, flores e frutos, a apigenina e a lutelina são as mais abundantes encontratadas nas plantas que podem estar na livre (aglicona) ou conjungada (glicosídeo). As flavonas diferem de outros flavonoides por possuírem dupla ligação entre C2 e C3, não há substituição na posição C3 e são oxidadas na posição C4(PANCHE et al., 2016; HOSTETLER et al., 2017). Os flavonóis apresentam uma estrutura semelhante das flavonas, com a diferença de possuírem uma hidroxila na posição C3 do anel C, que também pode ser hidroxilada e os flavonóis mais estudados são canferol, quercetina, miricetina e ficetina (PANCHE et al., 2016).

A hesperidina e a narigenina são exemplos de flavanona que uma classse de flavonoide caracterizada pela ligação simples entre os carbonos C2 e C3 do anel C, diferentemente das flavonas em que a ligação é dupla. A distribuição no reino vegetal dos isoflavoides é bastante limitada, sendo encontrado em soja e outras leguminosas, e os representantes mais comuns são a genisteína e a daidzeína. Os flavonóis são encontrados em abundância em bananas, maçãs, mirtilos, pêssegos e peras, e são caracterizados por apresentarem um grupo hidroxila ligado à posição C3 do anel C. Ao contrário de muitos flavonóides, não há ligação dupla entre as posições C2 e C3. As antocianinas são pigmentos responsáveis pela coloração em plantas, flores e frutos, sendo que a colaração depende do pH e também da metilação ou acilação dos grupos hidroxilas nos anéis A e B (PANCHE et al., 2016; ZUANAZZI et al., 2017).

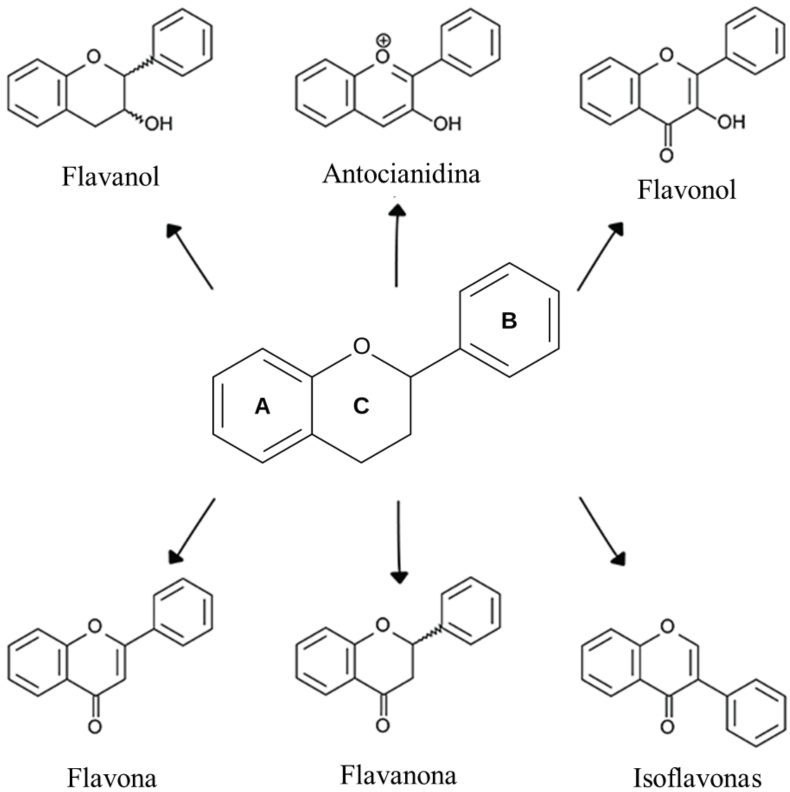


Figura 8 - Estrutura básica de algumas classes de flavonoides. Fonte: Adaptado de Nishiumi et al. (2011).

2.3.2 Ácidos fenólicos

Os ácidos fenólicos constituem uma subclasse de compostos fenólicos que possuem na estrutura um grupo funcional carboxila e um anel aromático com pelo menos uma hidroxila substituinte e são divididos em dois grupos: o primeiro é o dos ácidos hidroxibenzoicos que são os ácidos fenólicos mais simples com sete átomos de carbono (C_6-C_1); o segundo é formado pelos ácidos hidroxicinâmicos, que possuem em sua estrutura nove átomos de carbono (C_6-C_3) (SOARES, 2002; OLIVEIRA; BASTOS, 2011).

Os ácidos hidroxicinâmicos e hidroxibenzóicos (Figura 9), bem como os seus derivados estão amplamente distribuídos entre as plantas, podendo estar presentes nas formas livre ou conjugada através de ligações éster, éter ou acetal com polifenóis, proteínas, taninos, celulose e lignina (ZADERNOWSKI, CZAPLICKI E NACZK, 2009).

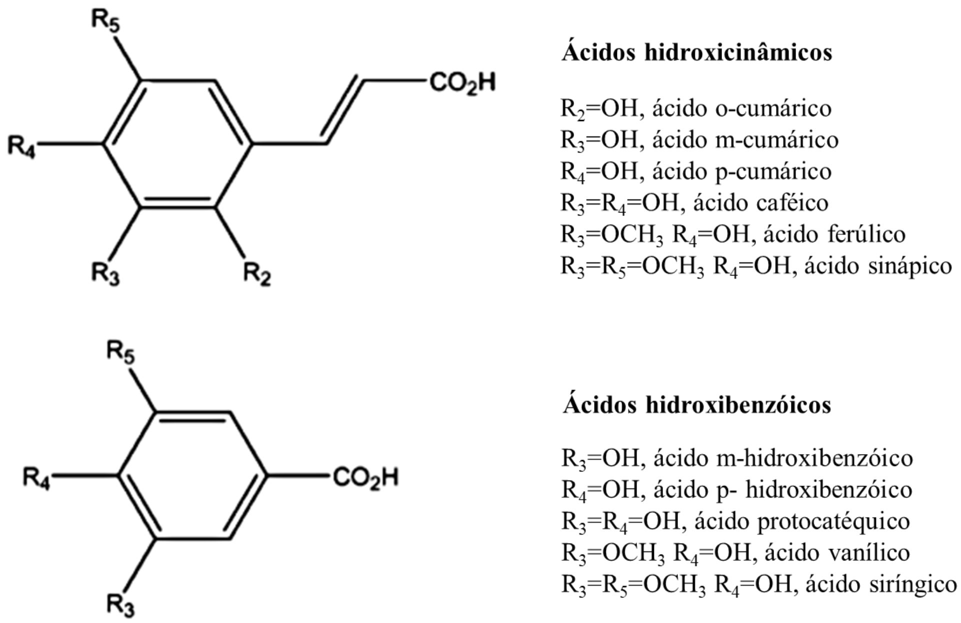


Figura 9 - Estrutura dos ácidos hidroxicinâmicos e hidroxibenzóicos comumente presentes em plantas. Fonte: Paiva, 2014.

Os ácidos hidroxicinâmicos são mais comuns que os hidroxibenzóicos e estão presentes em diversos alimentos e bebidas de origem vegetal, como o café, erva mate, frutas, cereais, entre outros. Dentre os representantes dos ácidos cinâmicos destacam-se os ácidos salicílico, ρ-cumárico, caféico, ferúlico e sináptico, além de seus conjugados esterificados/eterificados, tais como os ácidos clorogênicos. Os ácidos benzóicos são menos abundantes e fazem parte da composição das complexas estruturas dos taninos hidrolisáveis, e dentro deste grupo destacam-se os ácidos protocatecóicos, vanílico, sirínico, gentísico, salicílico, elágico e o gálico (OLIVEIRA; BASTOS, 2011; GOLENIOWSKI et al., 2013; PAIVA, 2014).

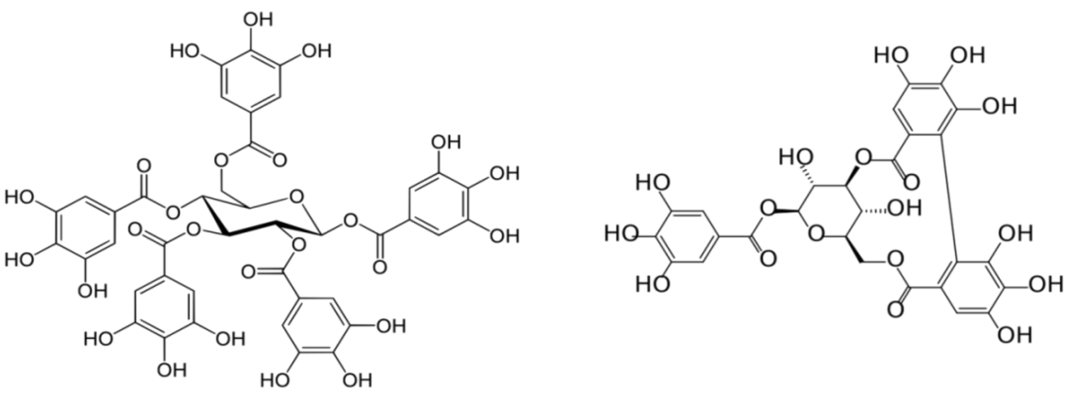
Os ácidos hidroxicinâmicos são formados na via do chiquimato, a partir de reações de hidroxilação e metilação do ácido p-cumárico. No primeiro passo para a síntese desses compostos, a fenilalanina é convertida em ácido transcinâmico pela ação fenilalanina amônia-liase (PAL), que é o precursor do ácido p-cumárico. Já os derivados dos ácidos benzoicos podem ser formados diretamente a partir de intermediários no início da via do chiquimato, esta reação é a principal via para síntese do ácido gálico. No entanto, eles também podem ser produzidos pela degradação de um dos derivados dos ácidos hidroxicinâmicos (GOLENIOWSKI et al., 2013; PAIVA, 2014).

O papel dos ácidos fenólicos nas plantas ainda não está completamente elucidado, entretanto eles têm sido associados a diversas funções, incluindo a maturação e o desenvolvimento de frutos, síntese de proteínas e de componentes estruturais, regulação da atividade enzimática e da fotossíntese e também com a alelopatia (LUY et al., 1999).

Os ácidos fenólicos têm sido reportados com importantes propriedades biológicas e farmacológicas e podem trazer benefícios para a saúde humana, tais como efeitos antibacteriano, antiviral, antitumoral e antialérgico (MADDOX et al., 2010; KING et al., 1999; GOMES et al., 2003; MAGGI-CAPEYRON et al., 2001). Além disso, essas substâncias e seus ésteres possuem alta atividade antioxidante em função da capacidade do grupo fenol de doar o hidrogênio da hidroxila aos radicais livres, estabilizando-os, sendo assim, a habilidade dos ácidos fenólicos em atuar na neutralização de espécies reativas é geralmente determinada pelo número de grupos hidroxilas encontrado em sua molécula. Em geral, os ácidos cinâmicos hidroxilados são mais eficazes que seus equivalentes de ácidos benzóicos (SÁNCHEZ-MORENO, 2002; GOLENIOWSKI et al., 2013).2.3.3 Taninos

Os taninos estão largamente distribuídos nas plantas, sendo comuns tanto em espécies gimnospermas como angiospermas. Apresentam como principais características a solubilidade em água, exceto os de elevados pesos moleculares, que podem variar de 500 a 3000 Dalton, e ainda possuem a habilidade de formar complexos insolúveis com proteínas, celulose, pectina e alcaloides. Embora todos estes compostos fenólicos tenham grupos poli-hidroxifenóis em suas moléculas, eles são classificados em dois grupos distintos: taninos condensados e taninos hidrolisáveis (BATTESTIN et al., 2004; MONTEIRO et al., 2005).

Os taninos hidrolisáveis (Figura 10) são caracterizados por apresentar um poliol, usualmente a D-glucose, cujas hidroxilas são esterificadas com ácidos gálicos (galotaninos) ou ácidos elágicos (elagitaninos), sendo que os taninos elágicos são muito mais freqüentes que os galotaninos (BATTESTIN et al., 2004 et al., DEGÁSPARI et al., 2004; MELLO; SANTOS, 2017).Os galotaninos são caracterizados por possuírem unidades de ácido gálico (grupo galoil), dos quais os seus grupos fenólicos esterificam o núcleo glicosídico. As moléculas deste tipo de tanino são usualmente compostas por um núcleo de glicose com 6 a 9 grupos galoil, sendo o mais comum o ácido tânico. Nos elagitaninos os grupos fenólicos utilizados são moléculas de ácido hexahidroxidifênico, que após a hidrólise ácida das ligações ésteres, ocorre a liberação do ácido difênico, que se rearranja para formar o ácido elágico (MELLO; SANTOS, 2017).



Galotanino **Elagitanino**

Figura 10 - Estruturas de taninos hidrolisáveis galotanino e elagitanino.

Os taninos condensados (Figura 11) são constituídos por unidades de flava-3-ols (catequina) ou flavan-3,4-diols (leucoantocianinas), produtos do metabolismo dos fenilpropanoides. Eles estão presentes em maior quantidade nos alimentos normalmente consumidos. Essa classe de taninos também é conhecida como proantocianidinas, provavelmente pelo fato de produzirem um vasto conjunto de pigmentos avermelhados do grupo das antocianidinas (MELLO; SANTOS, 2017).

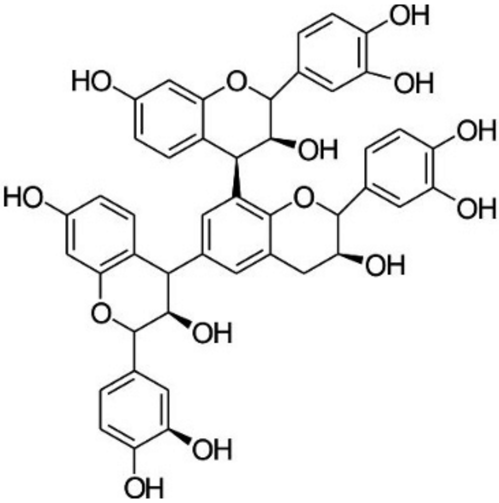


Figura 11 - Estrutura básica de um tanino condensado. Fonte: Raja et al. (2014).

Os taninos desempenham um importante papel na defesa das plantas contra fungos patogênicos, bactérias e vírus e também contra insetos herbívoros. Além disso, estes compostos podem atuar na eliminação de radicais livres e na defesa das plantas contra estresses ambientais, tais como baixa fertilidade do solo e seca (BATTESTIN et al., 2004; MONTEIRO, 2005). Os taninos têm sido relatados na literatura por possuir propriedades medicinais trazendo benefícios para a saúde humana, dentre as quais, podemos citar as atividades antioxidante, anticancer e antimutagênica, antimicrobiana, além de estarem associados a outros efeitos fisiológicos como a coagulação do sangue, redução da pressão arterial e dos níveis de lipídios no sangue e modulação das respostas imunitárias. Essas propriedades apresentadas pelos taninos, provavelmente estão relacionadas com a capacidade desses compostos de complexar íons metálicos, capturar radicais livres e de se associar com macromoléculas (CHUNG et al., 1998; SIMÕES et al., 2017).

2.4 Marcadores ISSR e a caracterização genética de plantas medicinais

A utilização de plantas medicinais para a prevenção, tratamento e cura de doenças depara-se com a dificuldade de obtenção de matéria-prima, uma vez que este tipo de material ainda é muito pouco cultivado, sendo o extrativismo a principal forma de obtenção de plantas medicinais (HOELTGEBAUM et al., 2015). O extrativismo quando realizado sem critérios de manejo adequados torna-se preocupante, pois, pode resultar em reduções drásticas de variabilidade genética, fato que já vem sendo relatado para várias espécies, como a espinheira-santa (*Maytenus ilicifolia),* espécies cultivadas na Amazônia, dentre outras (SEBBENNet al., 2008; RIBEIRO et al., 2010). Dentro deste contexto, a caracterização genética de plantas medicinais torna-se muito importante na identificação de genitores para programas de domesticação e melhoramento genético e também para sua conservação, e nesse sentido os marcadores moleculares são ferramentas muito úteis para essas análises genéticas (CARVALHO et al., 2009).

Os marcadores moleculares são amplamente difundidos no campo da genética devido a sua grande capacidade de acessar as variações genômicas, sendo que estes marcadores podem ser do tipo bioquímico, enzimático ou de DNA, estes últimos, com os avanços das técnicas de biologia molecular, passaram a ser mais comumente utilizados, pois são capazes de detectar uma maior variação entre os indivíduos e ainda evitam o efeito ambiental e consequentemente erros de identificação (GROVER; SHARMA, 2016; TURCHETTO-ZOLET et al., 2017).

Os marcadores de DNA podem ser classificados como: (I) baseados em hibridização, exemplo são os RFLPs (*Restriction Fragment Length Polymorphism*); (II) os baseados na reação em cadeia da polimerase (PCR), dos quais se pode citar o RAPD (*Random Amplified Polymorphic DNA*), AFLP (*Amplified Fragment Length Polymorphism*), ISSR (*Inter Simple Sequence Repeats*), SSR (*Simple Sequence Repeat*) ou microssatélites; e por fim (III) os baseados em sequenciamento. Estes marcadores também podem ser distinguidos em dominantes ou codominantes, nos dominantes é possível apenas identificar a presença ou ausência de um determinado alelo, já nos codominantes há a possibilidade de diferenciar indivíduos homozigotos e heterozigotos (VARSHNEY et al., 2007; TURCHETTO-ZOLET et al., 2017).

Os marcadores de natureza codominante, assim como os microssatélites e os RFLPs, são mais vantajosos quando comparados com os de natureza dominante, uma vez que fornecem mais informações, especialmente no que diz respeito à diferenciação de genótipos. Entretanto, as técnicas que se utilizam desses marcadores geralmente são mais onerosas e demandam mais tempo para sua realização, pois há a necessidade de conhecimento prévio das sequências de DNA a serem analisadas e precisam ser desenvolvidos separadamente para cada espécie. A rapidez, a simplicidade, a natureza hipervariável, o grande número de locos que podem ser analisados e a economia de reagentes nas técnicas que se utilizam de marcadores dominantes, assim como RAPD e ISSR, tornaram estes marcadores muito populares em estudos genéticos de plantas nos últimos anos (KREMER et al., 2005).

Os marcadores ISSR (*Inter Simple Sequence Repeats*) combinam os benefícios do RAPD, pois não há a necessidade do conhecimento prévio do da sequência de DNA da espécie-alvo, além de ser simples, rápida, eficiente e gerar altos índices de polimorfismo, aliado com o aumento na reprodutibilidade e especificidade, decorrente do fato de se utilizarem primers mais longos para amplificação e por isso apresentam maior superfície de ancoragem e ainda permite a aplicação de temperatura de anelamento mais altas na reação de PCR, gerando produtos mais específicos que os gerados pelos marcadores RAPDs (ZIETKIEWICZ et al., 1994; REDDY et al., 2002).

O ISSR envolve amplificações de fragmentos de DNA localizados entre regiões de microssatélites e orientados em direções opostas, sendo que os microssatélites consistem em sequências simples de 2 a 4 nucletídeos repetidas em *tandem* (Figura 12). A técnica utiliza de um único *primer* composto por uma sequência de microssatélite selecionada aleatoriamente com um comprimento usualmente de 16-25 pares de bases (pb), para amplificação de sequências entre as regiões microssatélites, gerando produtos de amplificação que podem variar de 200 a 2000 pb de comprimento sendo visualizadas em gel de agarose ou poliacrilamida (Figura 13).

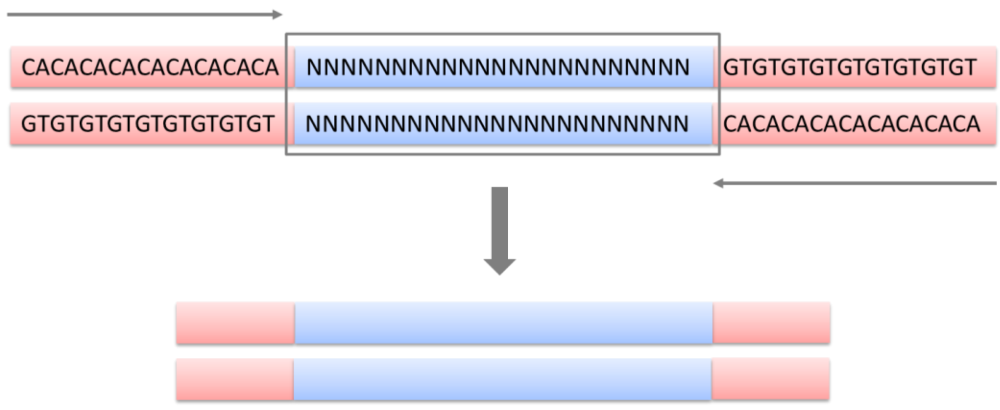


Figura 12 - Esquema da amplificação do ISSR.

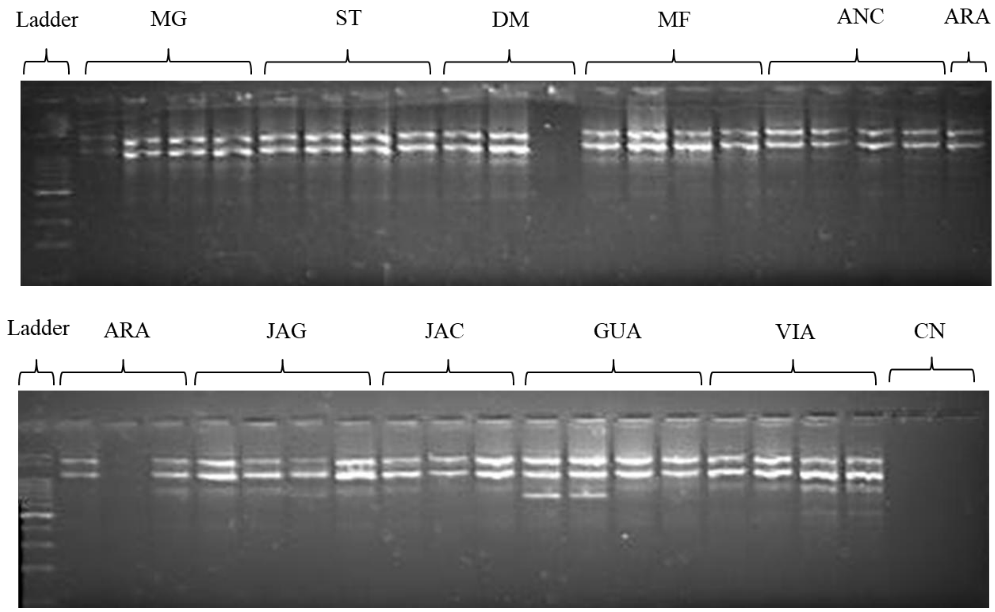


Figura 13 - Gel de agarose dos produtos da amplicação ISSR de 10 populações de diferentes localidades de *Varronia curassavica* gerados por um único primer.

Diversas pesquisas têm sido realizadas para caracterização genética de plantas medicinais utilizando os marcadores moleculares , que tem se mostrado muito eficaz nas análises de variabilidade genética, em avaliações de DNA *fingerprint* (impressão digital do DNA), seleção assistida por marcadores, estabelecimento de relações filogenéticas e mapeamento genético (ARCHAK et al., 2003; RAKOCZY-TROJANOWSKA; BOLOBOK, 2004; BRITO et al., 2016). Brito et al (2016) empregaram marcadores ISSR para analisar a diversidade genética entre acessos de *Varronia curassavica* do banco de germoplasma da Universidade Federal de Sergipe e observaram que a variação entre esses acessos era de baixa a média, alertando para a necessidade de ampliação do banco de germoplasma de maneira a contribuir para programas de conservação da espécie. A variabilidade genética de 38 acessos de *Momordica charantia* foi analisada por Behera et al., (2008) por meio de marcadores RAPD e ISSR, que observou padrões semelhantes de variabilidade entre os acessos com os dois marcadores.

A análise genética dos marcadores moleculares de natureza dominante, como os do tipo ISSR, é realizada através da leitura de géis de eletroforese, que fornecem variáveis qualitativas binárias caracterizadas pela presença ou ausência de banda. A partir daí constrói-se uma matriz de dados binários atribuindo os números 1 e 0, sendo o número 1 para presença de banda e o número 0 para ausência de banda. E posteriormente essa matriz é analisada utilizando-se coeficientes de similaridade que possibilitam a determinação das distâncias genéticas (MEYER, 2002; CARVALHO et al., 2009). Podem ser utilizados diferentes índices de similaridade, dentre os quais se destaca o índice de similaridade de Jaccard devido as suas propriedades matemáticas por não considerar a ausência conjunta de bandas como sinônimo de similaridade genética, sendo uma característica muito importante quando se utilizam marcardores como ISSR, pois a ausência de banda em dois genótipos não significa, necessariamente, similaridade entre eles (ARRIEL et al., 2006).

Feito os cálculos das distâncias genéticas a partir do índice de similaridade, procede-se com agrupamento dos itens análisados. A análise de agrupamentos é uma técnica estatística que permite classificar os itens com maior proximidade em grupos ou conglomerados (clusters), o que resulta em um dendrograma de similaridade, permitindo assim uma visualização mais fácil e rápida dos resultados (DIAS, 1998; CARVALHO et al., 2009).

Os métodos de agrupamento podem ser classificados, de modo geral, em métodos hierárquicos e não-hierárquicos, sendo os hierárquicos os mais utilizados para análise de caracterização genética de espécies vegetais (Dias, 1998). Os métodos hierárquicos apresentam formas distintas de representar a estrutura de agrupamento e dentre estes, o UPGMA (*Unweighted Pair-Group Method Using Arithmetic Averages*) é amplamente utilizado em diferentes áreas de pesquisa e ainda consiste no método mais utilizado para caracterização de diversidade genética em plantas medicinais. O UPGMA é um método aglomerativo baseado na média das distâncias entre todos os pares de genótipos para formação de cada grupo, de maneira que as relações são identificadas pela similaridade e o dendograma é construído a partir das duas unidades mais similares (BERTAN et al., 2006).

2.5 Varronia curassavica

Varronia curassavica Jacq. (Figura 14) (sinonímia *Cordia verbenacea* DC) era classificada como pertencente à família Boraginaceae, esta família na antiga classificação era dividida em quatro subfamílias: Ehretioideae, Cordioideae, Helitropioideae e Boraginoideae (NOWICKE e MILLER, 1990; MILLER, 2007). No entanto, estudos filogenéticos recentes sustentados por dados moleculares elevaram estas subfamílias ao nível de famílias (GOTTSCHLING et al., 2001 e 2005; MILLER, 2007; MILLER; GOTTSCHLING, 2007). Dessa forma, nessa nova classificação a espécie *Varronia curassavica* passou a pertencer à família Cordiaceae (MISSOURI BOTANICAL GARDEN, 2017).

O gênero *Varronia* inclui aproximadamente 100 espécies arbustivas, com inflorescências, e folhas serrilhadas distribuídas ao longo das regiões subtropicais e temperadas do mundo (MILLER; GOTTSCHLING, 2007). Os registros da literatura apontam que existem cerca de 30 espécies do gênero no Brasil, que podem ocorrer em diferentes habitats desde florestas, como em vegetação de cerrado e caatinga (STAPF, 2010).

V. curassavica está amplamente distribuída na América Central e do Sul e é comumente denominada como erva-baleeira, maria-preta, maria-milagrosa, salicina, catinga-de-barão, balieira-cambará, erva-preta, catinga-preta, maria-rezadeira, camarinha, pimenteira (MONTANARI JÚNIOR, 2000; CARVALHO et al., 2004; LORENZI; MATOS, 2008). Dentre estes, o nome pelo qual é mais conhecida é erva-baleeira, que segundo Montanari Júnior (2011), esse nome está associado à caça de baleias, pois, ao se ferirem, os caçadores eram orientados pelos nativos a utilizarem a planta na cura dos ferimentos ocasionados pela atividade.

V. curassavica é uma espécie arbustiva que cresce de forma abundante sobre solos arenosos e pedregosos, por isso ocorre espontaneamente na costa brasileira, desde o Ceará ao Rio Grande do Sul, entretanto, apesar dessa característica marcante é comum também encontrá-la em regiões afastadas da costa, em terrenos com baixa fertilidade e baixo escoamento de água (MONTANARI JÚNIOR, 2011; BOLINA, 2015).

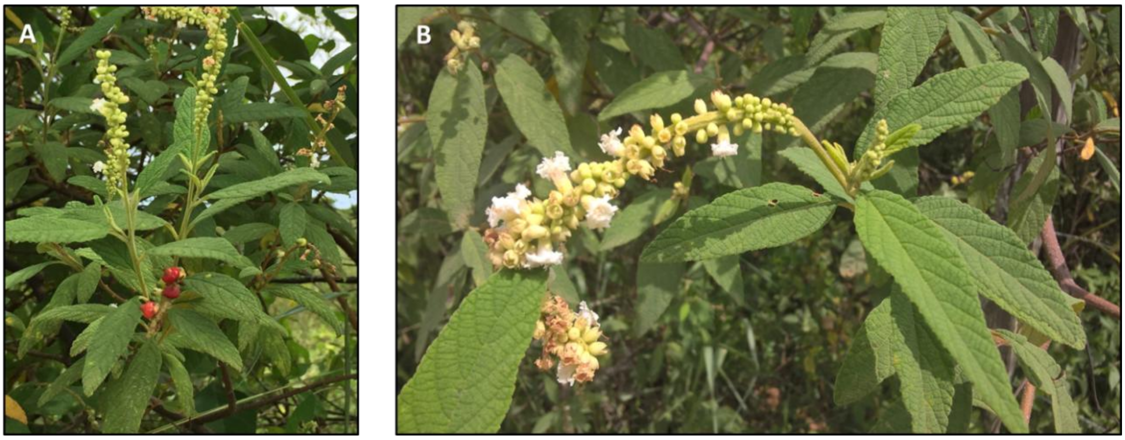


Figura 14: *Varronia curassavica*: Visão geral da planta (A) e detalhe da inflorescência (B). Fotos: Mainã Mantovanelli da Mota.

Essa espécie apresenta arbustos de 0,5 a 4 metros de altura, ereto, perene, com ramos dispostos helicoidalmente, possui folhas (Figura 14A) simples, alternas, coriáceas, a margem denteada, com 5-9 cm de comprimento e são aromáticas por apresentar óleo essencial sintetizado e armazenado em seus tricomas glandulares globulares (LORENZI; MATOS, 2008; VENTRELLA; MARINHO, 2008).

Apresenta inflorescência terminal em espiga com flores branca, tubulares, com antese diurna e órgãos sexuais expostos (Figura 14B; 15A). A erva-baleeira pode florescer durante todos os meses do ano, porém a floração ocorre em maior intensidade durante os meses mais quentes da primavera e do verão. Possui polinização entomófila, sendo realizada principalmente por insetos voadores como as abelhas europeias, himenópteras, moscas e borboletas (MONTARI, 2000; BRANDÃO et al., 2015). Apesar de *V. curassavica* ser hermafrodita, a espécie apresenta mecanismos que impedem a autofecundação, como a heterostilia, característica bem descrita nas espécies da família Cordiaceae (GASPARINO; BARRO, 2005; TAISMA & VARELA 2005; HOELTGEBAUM, 2017).

A heterostilia consiste em um sistema de autoincompatibilidade genética que determina a presença de morfos florais distintos, se diferenciando quanto ao tamanho do estilete (VUILLEMIER, 1967; GANDERS, 1979; BARRETT; RICHARDS 1990; TEIXEIRA; MACHADO, 2004; CONSOLARO et al., 2005). *V. curassavica* é considerada uma espécie distílica, nas quais se encontram flores com morfo brevistilo (Figura 15B e 15C) e flores com morfo longistilo (Figura 15D e 15E) e espécies com esta característica apresentam incompatibilidade intramorfos, sendo assim, geralmente, apenas os cruzamentos entre flores de morfos distintos resultam em uma progênie viável (TAISMA; VARELA 2005; HOELTGEBAUM, 2017).

Os frutos são cariopses esféricas, possuem coloração avermelhada quando maduros e medem aproximadamente 0,4 cm (Figura 2F e 2G). Por serem comestíveis, os frutos são muito procurados por pássaros de diversas espécies que acabam por fazer a dispersão das sementes (LORENZI e MATOS, 2008; MONTANARI JÚNIOR, 2000).

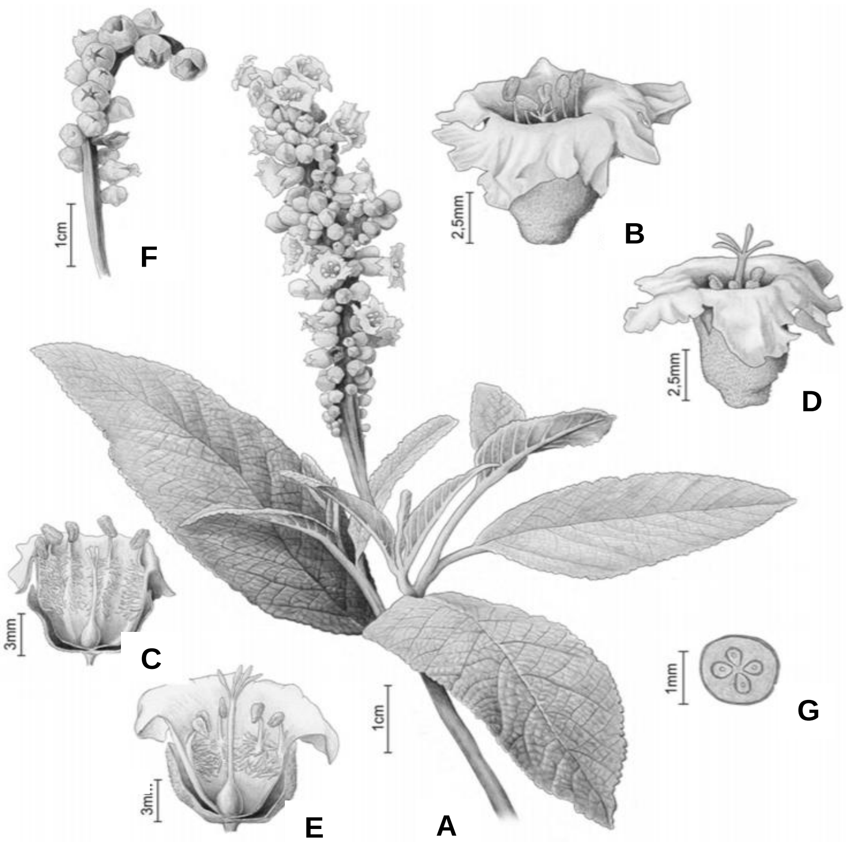


Figura 15 - *Varronia curassavica* Jacq.- A. ramo com inflorescência; B. flor brevistila; C. seção lateral da flor brevistila mostrando as estruturas reprodutivas; D. flor longistila; E. seção lateral da flor longistila mostrando as estruturas reprodutivas; F. Infrutescência; G. gineceu. Fonte: Hoeltgebaum et al., 2018.

A espécie é amplamente utilizada na medicina popular devido às suas propriedades anti-inflamatória, analgésica e cicatrizante (LORENZI & MATOS, 2008). A sua ação anti-inflamatória foi comprovada por diversos estudos (SERTIÉ et al., 1988; SERTIÉ et al., 2005; FERNANDES et al., 2007; PASSOS et al., 2007) e em função dessa propriedade, no ano de 2005 começou a ser produzido o Acheflan®, um fitoterápico fabricado pelo Laboratório Farmacêutico Aché utilizado para o tratamento de tendinite crônica e dores musculares e que contém na sua composição o α-humuleno (Figura 16A) e o β-cariofileno (Figura 16B) obtido a partir do óleo essencial da erva-baleeira (GILBERT e FAVORETO, 2012).

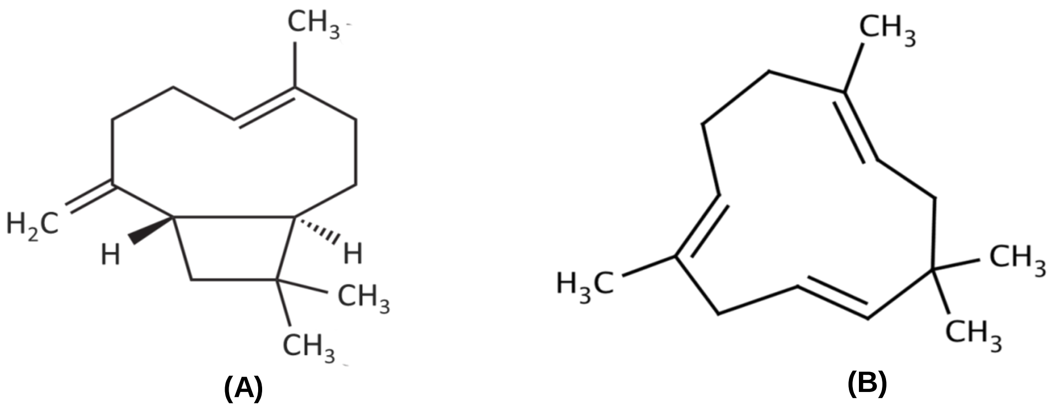


Figura 16 - Estrutura do α-humuleno (A) e o β-cariofileno (B) encontrados no óleo essencial de *Varronia curassavica.* Fonte: (GILBERT e FAVORETO, 2012).

Em um trabalho desenvolvido por Santo et al. (2006) foram encontrados monoterpenos e sesquiterpenos no óleo essencial das folhas de *V. curassavica*, dentre os quais o α-pineno foi o composto com maior predominância (Figura 17A). Um flavonóide encontrado na folha dessa espécie por Sertié et al. (1990) e Bayeux et al. (2002) foi a artemetina, substância com propriedades antiinflamatórias e cicatrizantes (Figura 17B). Também há outros flavonóides presentes no extrato de folhas de *V. curassavida* descritos por Matias et al., (2013), como o ácido cafeico (Figura 17C) e o ácido gálico (Figura 17D).

Ticli et al. (2005) isolaram o ácido rosmarínico (Figura 17E) do extrato metanólico da erva-baleeira e o apontaram como sendo responsável pela inibição do edema induzido por veneno de cobra. O ácido rosmarínico também foi encontrado no extrato hidroalcoólico das folhas de erva-baleeira por HAGE-MELIM, 2009.

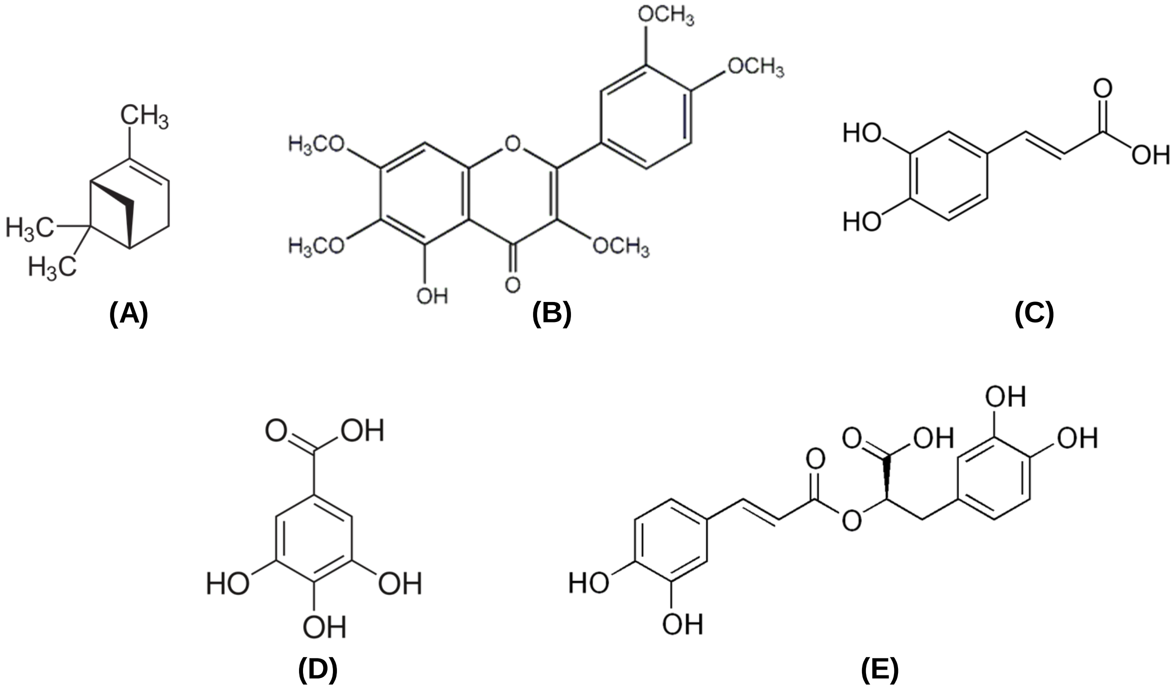


Figura 17 - Compostos químicos isolados de *Varronia curassavica*. A. α-pineno; B. artemetina; C. ácido cafeico; D. Ácido gálico; E. ácido rosmarínico. Fonte: Matias e colaboradores (2015).

As ações terapêuticas de *V. curassavica* estão geralmente associadas à sua propriedade anti-inflamatória, entretanto estudos farmacológicos têm demonstrado que essa espécie também exibe atividade antimicrobiana. Carvalho et al. (2004) confirmaram a eficácia do óleo essencial na inibição do crescimento de cepas de bactérias gram-positivas. Matias et al. (2013) observaram que o extrato metanólico da erva baleeira foi capaz de inibir o crescimento de bactérias. Em adição, o extrato da planta apresenta a capacidade de combater fungos e protozoários (NIZIO et al., 2015).

Parisoto et al. (2012) investigaram o efeito citotóxico e antitumoral do extrato de *Cordia verbenacea* em linhagem celular cancerosa MCF-7. Os autores observam que o extrato foi citotóxico para as células cancerosas, sendo que o mecanismo antitumoral está relacionado com o bloqueio da sobrevivência das células pela indução da apoptose celular. Michiellin et al.

(2011) confirmou que a erva baleeira apresenta também apresenta potencial antioxidante.

2.6 Momordica charantia

Momordica charantia L. (Figura 18A) é uma planta trepadeira originária da Ásia e África e pertence à família Curcubitaceae. A família Cucurbitaceae inclui aproximadamente 90 gêneros e mais de 800 espécies presentes nas regiões tropicais e subtropicais do mundo. No Brasil ocorrem cerca de 30 gêneros e 200 espécies, muitas das quais apresentam grande importância econômica e social, pois são comestíveis e possuem propriedades medicinais especialmente aquelas dos gêneros *Cucurbita, Momordica, Fevillea e Sechium* (WUNDERLIN, 1978; LIMA, 2010; ASSIS et al, 2015; AMARO et al., 2016).

No Brasil, essa planta é mais conhecida como melão-de-são-caetano, pois, inicialmente, as plantas foram cultivadas por escravos, vindos da África, ao redor da capela de São Caetano localizada no estado de Minas Gerais (SANTOS, 2014). Além desse nome popular, também é chamada de erva-das-lavadeiras, erva-de-são-caetano, fruto-de-cobra, melão-de-são-vicente, melãozinho, fruta-de-sabiá (LORENZI; MATOS, 2008).

Trata-se de uma espécie ruderal, descrita como herbácea anual, bastante ramificada, podendo medir de 2 a 3 metros de comprimento e que cresce sobre algum suporte como cercas, muros ou outras plantas (CIRINO et al., 1991; CORREIA; ZEITOUM, 2010). Apresenta o caule do tipo herbáceo fino, sulcado e com coloração esverdeada. Suas folhas (Figura 18B) são de consistência membranácea, alternas, lisas, pilosas e lobadas com cinco a sete lobos. A presença de gavinhas simples, longas, delicadas e pubescentes é bem característica nessa planta (JORGE et al., 1992; ZOCOLER et a., 2006; MARCELLINO, 2018).

Suas flores (Figura 18C) são amarelas e saem das axilas das folhas, apresentam cinco pétalas arredondadas ou recortadas nas pontas e possuem pequenos pistilos alaranjados brilhantes com o estame no centro (WALTERS; DECKER-WALTERS, 1988; JORGE et al., 1992; ZOCOLER et a., 2006). *Momordica charantia* é uma espécie monóica, entretanto suas flores são diclinas, ou seja, são unissexuais de maneira que as flores masculinas estão separadas das femininas (LENZI et al., 2005). A flor masculina é solitária, em pedúnculo com bráctea reniforme e ligeiramente pubescentes. Já a flor feminina apresenta o pedúnculo longo e delgado com brácteas geralmente perto da base (ZOCOLER et al., 2006; TCHEGHEBE et al., 2016).

Os frutos (Figura 18D) de *M. charantia* apresentam um formato cônico com extremidade final pontiaguda, são carnosos e com uma superfície verrugosa, tem uma coloração verde escura quando imaturos e amarelo-alaranjado quando maduros. Os frutos têm um sabor extremamente amargo, exibem deiscência irregular e ao se abrir expõem suas sementes (Figura 18E) envolvidas por apêndice carnoso avermelhado e comestível. (YANG; WALTERS, 1992; ZOCOLER et a., 2006; LORENZI; MATOS, 2008; SANTOS, 2014). A dispersão das suas sementes é realizada por répteis, pássaros e pequenos mamíferos (WALTERS; DECKER-WALTERS, 1988; PASSOS et al., 2013; NOGALES et al., 2017).

Figura 18 - *Momordica charantia*. Visão geral da planta (A). Detalhe da folha (B), flor (C), fruto (D) e do fruto aberto com sementes (E). Fotos: Mainã Mantovanelli da Mota.

O uso popular do melão-de-são-caetano é bastante difundido, sendo empregado como hipoglicemiante, no tratamento de diabetes, da obstipação intestinal e de hemorroidas, para regular o fluxo menstrual e aliviar cólicas abdominais e é utilizado também no combate a lesões da pele, como cicatrizante, anti-reumática e para tratar inflamações que acometem o fígado

(PIO CORREA, 1984; PENALBA; RITA, 1988; MATOS, 1997; DINIZ et al. 1997). Os benefícios para a saúde que essa planta fornece, estão relacionados com a presença de compostos bioativos, sendo que *M. charantia* apresenta uma variedade desses compostos, especialmente compostos fenólicos, saponinas, peptídeos e alcaloides (TAN et al., 2015; JIA et al., 2017; OLIVEIRA et al., 2018).

Diversos compostos encontrados nessa espécie têm sido associados com o potencial hipoglicemiante da planta, dentre os quais podemos citar a charantina (Figura 19). Trata-se de um triterpenoide extraído de sementes, folhas e frutos que produz uma redução significante nos níveis de açúcar do sangue, estimulando o estoque de glicogênio pelo fígado e a produção de insulina no pâncreas (EL-SAID; AL-BARAK, 2011; SINGH et al., ZHU et al., 2012).

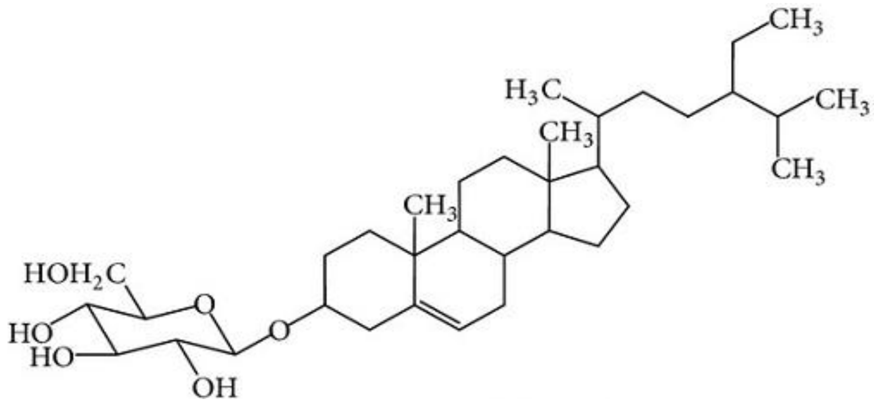


Figura 19 - Estrutura da charantina isolada a partir da planta *Momordica charantia*. Fonte: Raina et al., 2016.

Outras substâncias bioativas isoladas da planta *M. charantia* também demonstram potencial antidiabético, incluindo o grupo de terpernoides chamados de momordicinas e momordicosídeos. Para Singh et al. (2011) a capacidade hipoglicemiante da momordicina (Figura 20A) é devido ao fato desta substância apresentar estrutura e propriedades químicas semelhantes à insulina. Segundo Tan et al. (2008), o momordicosídeo S (Figura 20B) isolado do melão-de-são-caetano estimula os receptores de membrana GLUT-4 induzindo a entrada de glicose para dentro das células.

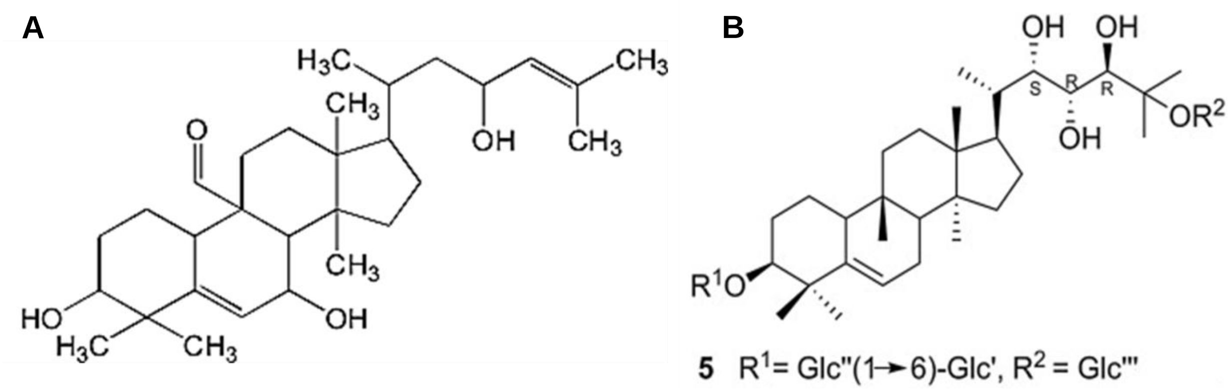


Figura 20 - Estrutura química dos terpenoides momordicin (A) e mormodicosídeo S (B) identificados em *M. charantia*. Fonte: Singh et al. (2011) e Tan et al. (2008).

Os compostos fenólicos, assim como os fenilpropanoides e os flavonoides, são metabolitos secundários amplamente produzidos pelas plantas, sendo conhecidos por apresentarem atividade antioxidante e proteger as plantas dos estresses bióticos e abióticos. Como descrito por diversos autores os compostos fenólicos (Figura 21) constituem importantes componentes de *M. charantia* (CHOI et al., 2012; KENNY et al., 2013; THIRUVENGADAM et al., 2014; LEE et al., 2016; SHODEHINDE et al., 2016; CUONG et al., 2018).

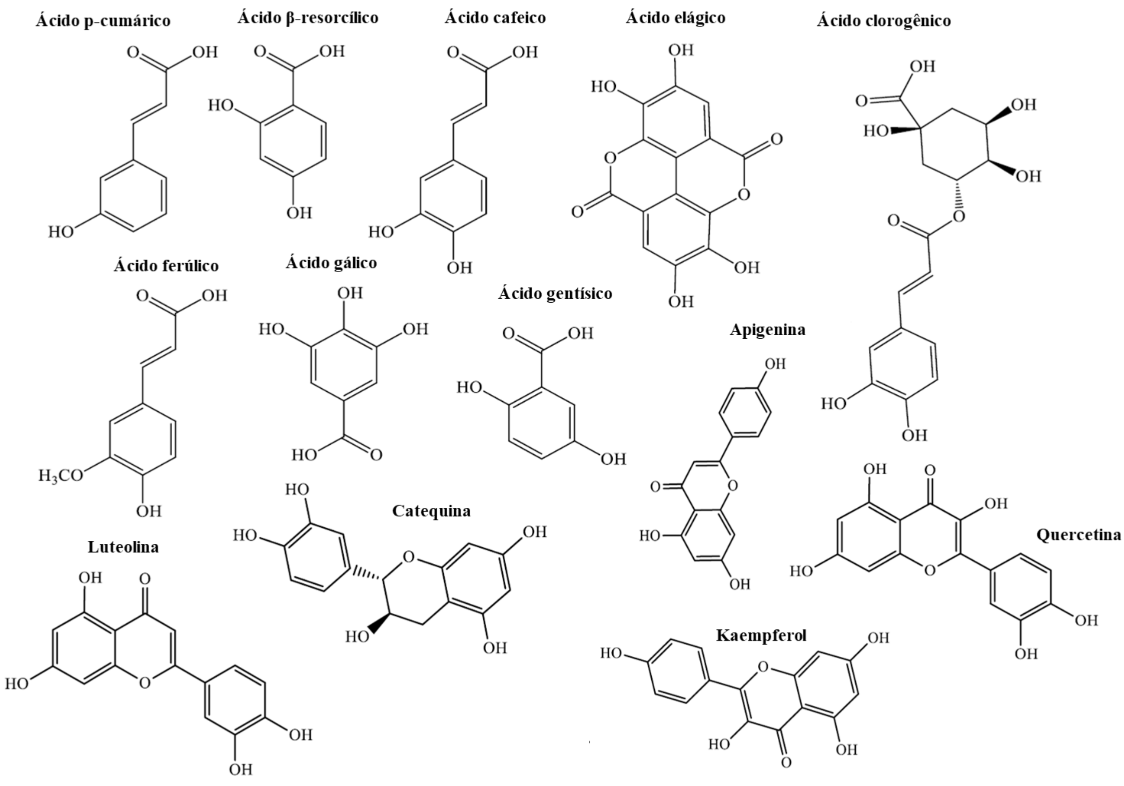


Figura 21 - Estrutura química dos compostos fenólicos encontrados em *Momordica charantia*. Fonte: Oliveira et al., 2018.

Estudos farmacológicos têm demonstrado que além de atuar como um potente hiploglicemiante *M. charantia* apresenta uma série de outras propriedades, que incluem efeitos antioxidantes, antitumoral, antiinflamatório, antimicrobiano, hepatoprotetivo e neuroprotetivo (WEI et al., 2013; KWATRA et a., 2013; CHAO et al., 2014; COSTA et al., 2010; SANTOS et al., 2010; LU et al., 2014; MALIK et al., 2011).

Rezaeizadeh e colaboradores (2011) confirmaram o efeito no combate aos radicais livres da planta ao comparar a atividade antioxidante do extrato metanólico e clorofórmico do fruto de *M. charantia* e observaram que o extrato metanólico apresentou um potencial antioxidante maior quando comparado com o extrato clorofórmico, sendo que os autores atribuíram essa melhor desemprenho ao fato do extrato metanólico conter maiores concentrações de compostos fenólicos e flavonoides.

A atividade antitumoral da espécie foi avaliada por Li et al. (2012), sendo que estes verificaram que o extrato das folhas de *M. charantia* foi capaz de induzir a apoptose em diversas linhagens de células de câncer humano. Os estudos de Shobha et al. (2015) indicaram atividade anticâncer dessa planta contra as linhagens de célula de câncer de mama e cervical. Alshehri (2016) também confirmou a propriedade antitumoral de *M. charantia* ao testar o efeito citotóxico do extrato da planta contra três linhagens de células de carcinoma humano.

Manabe et al. (2003) observaram que *M. charantia* apresenta a capacidade de induzir respostas atiinflamatórias. Resultados semelhantes foram observados por Sin et al. (2012). Estudos realizados por Chao et al. (2014) comprovaram os efeitos antiinflamatórios do melão-de-são-caetano, onde foi observado que a planta é capaz de reduzir marcadores bioquímicos inflamatórios e as citoquinas inflamatórias do corpo de camundongos.

Em relação ao efeito antimicrobiano, Maia et al. (2008) observaram que o extrato do melão-de-são-caetano demonstrou potencial efeito antibacteriano tanto em amostras bovinas quanto em amostras humanas, inclusive contra linhagens multirresistentes de *Staphylococcus aureus*. Lucena Filho et al.

(2015) também comprovaram a atividade antimicrobiana de *M. charantia*, chegando a conclusão que esta planta apresenta perfil bactericida e fungicida.

2.7 Atividade antioxidante de produtos naturais

Em sistemas biológicos, as espécies reativas de oxigênio (ROs) e as espécies reativas de nitrogênio (ERNs) são constantemente geradas pelo metabolismo celular normal ou e em vários eventos patológicos. A manutenção dos níveis normais de espécies reativas é muito importante para mecanismos fisiológicos diversos, assim como a regulação de vias de sinalização celular, ativação de genes e respostas aos agentes infecciosos. Os sistemas antioxidantes atuam para eliminar o excesso de radicais livres, mantendo o equilíbrio entre a oxidação e a anti-oxidação. Porém, a produção excessiva pode conduzir a danos oxidativos (BARBOSA et al., 2010; XU et al., 2017).

Quando em excesso, os radicais livres causam danos ao DNA e levam a oxidação de lipídios e proteínas, podendo resultar em doenças crônicas e degenerativas, sendo que, os antioxidantes apresentam como função inibir ou reduzir os danos causados pela ação deletéria dessas espécies reativas. Os antioxidantes são definidos como sendo qualquer molécula que apresenta capacidade de atrasar ou inibir a oxidação de outra molécula de maneira eficaz (MOHARRAM, YOUSSEF, 2014; ABDEL-LATEIF et al., 2016).

De acordo com Moharram e Youssef (2014) os sistemas antioxidantes podem ser classificados em enzimático e não-enzimático (Figura 22). Muitos desses antioxidantes podem ter origem endógena incluindo enzimas, moléculas de pequeno peso molecular e cofatores enzimáticos, e entre os antioxidantes não enzimáticos, muitos são obtidos através da dieta. Os antioxidantes exógenos são principalmente derivados de alimentos e plantas medicinais, como frutas, vegetais, cereais, cogumelos, bebidas, especiarias e ervas medicinais tradicionais (NIMSE; PAL, 2015; XU et al., 2017).

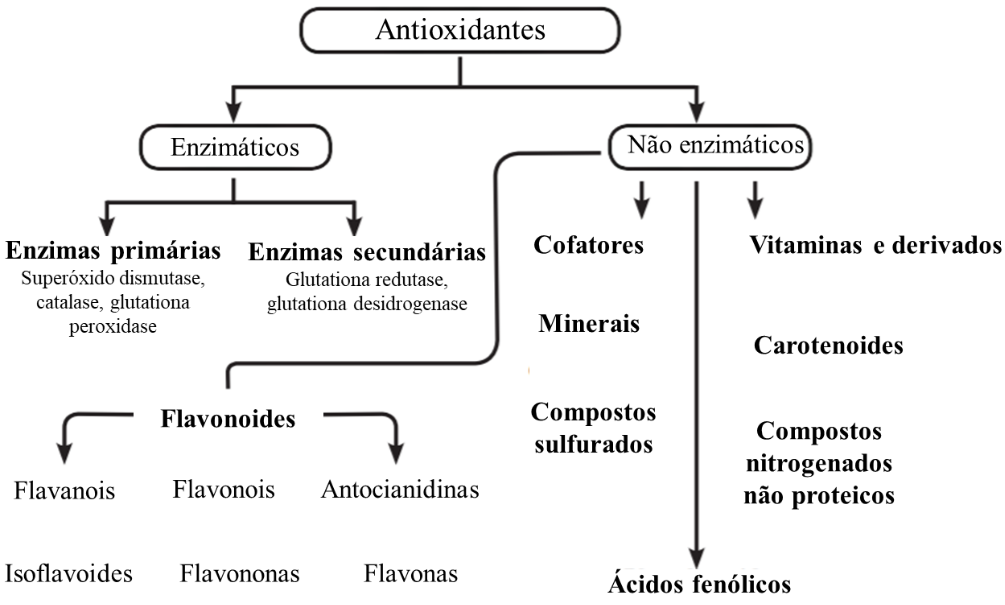


Figura 22 - Classificação dos antioxidantes naturais. Fonte: Adaptado de Moharram; Youssef (2014).

A superóxido dismutase (SOD), catalase (CAT) e a glutationa peroxidase (GPx) constituem as principais enzimas do sistema antioxidante enzimático (Figura 23). Estas enzimas atuam por meio de mecanismos de prevenção, neutralizando EROs como o radical hidroxil($OH^{\bullet}$), ânions superóxido ($O_2^{\bullet}$) e o peróxido de hidrogênio (H_2O_2) (CERON-GÁRCIA et al., 2012;YANG; LEE, 2015). A superóxido é uma das principais enzimas de eliminação de EROs encontradas em organismos aeróbicos e é responsável por converter o ânion superóxido em peróxido de hidrogênio. Existem dois tipos principais de SOD, uma é a CuZnSOD (SOD 1) tendo o cobre e o zinco como cofatores e está presente principalmente no citoplasma, e a outra é a MnSOD (SOD2), localizada na matriz mitocondrial e tem o manganês em seu sítio ativo(SHIM; KIM, 2013; PENG et al., 2014). Já a CAT é responsável pela conversão do H_2O_2 em oxigênio e água e a GPx, assim como a catalase protege as células e o tecido contra os danos causado pelo peróxido de hidrogênio, entretanto a GPx remove essa espécie reativa pela oxidação da glutationa que retorna para a forma reduzida pela ação da glutationa redutase (GR). A ação conjunta destas enzimas é de grande importância, uma vez que o H_2O_2 mediante a participação dos íons metálicos, podem gerar o radical $OH^{\bullet}$, contra o qual não há sistema enzimático de defesa (HUANG et al., 2005; FLORA, 2009; BARBOSA et al., 2010; YANG; LEE, 2015).

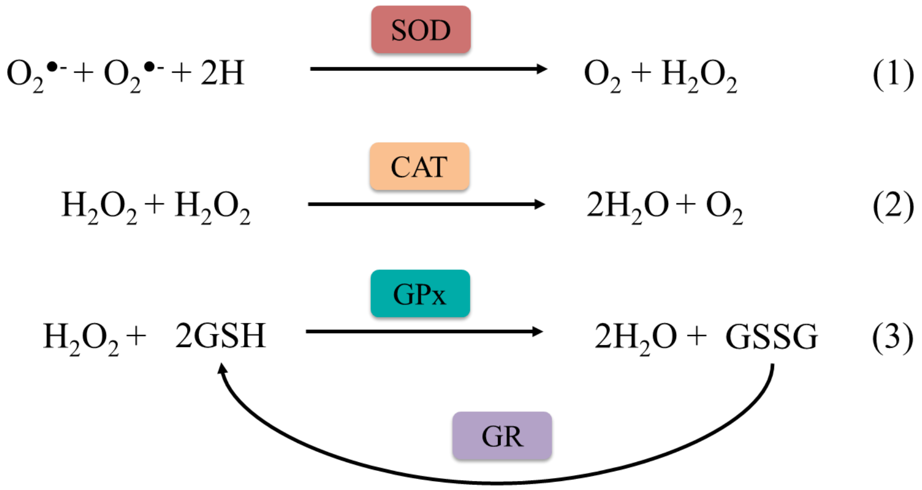


Figura 23 - Principais enzimas do sistema antioxidante e suas reações para eliminar espécies reativas de oxigênio. SOD, superóxido dismutase; CAT, catalase; GPx, glutationa peroxidase; GR, glutationa redutase; GSH, glutationa reduzida; GSSG, glutationa oxidada. Fonte: Adaptada de PENG et al. (2014).

Considerando os antioxidantes não enzimáticos, podemos citar vitaminas, minerais, cofatores, compostos nitrogenados e sulfurados, além dos compostos fenólicos. Estes antioxidantes além de atuarem diretamente na proteção contra o dano oxidativo, favorecem o desempenho dos antioxidantes enzimáticos, atuando sinergicamente na eliminação dos radicais livres (BARBOSA et al., 2010; NIMSE; PALB,2015). Os antioxidantes não enzimáticos podem ser sintéticos, amplamente utilizados pela indústria alimentícia, cosméticos, bebidas e também na medicina, ou naturais, que fazem parte da composição de produtos naturais de diferentes origens (BIANCHI; ANTUNES, 1999; OLIVEIRA et al., 2009; DEL RE; JORGE, 2012).

O interesse por antioxidantes naturais cresce a cada ano, isso tem incentivado pesquisas que visam à caracterização de matérias-primas e identificação de novas substâncias capazes de atuarem na eliminação, neutralização dos radicais livres no reparo aos danos causados por estes. Neste contexto, destacam-se as fontes de origem vegetal, pois os alimentos e as plantas medicinais apresentam uma ampla diversidade de compostos bioativos com um grande potencial antioxidante (MELO; GUERRA, 2002; DEL RE; JORGE, 2012).

Nas últimas décadas, estudos têm evidenciado a ação antioxidante atribuída às plantas medicinais. Recentemente, Nunes e seus colaboradores (2018) demonstraram que diversas espécies de plantas medicinais do cerrado brasileiro apresentam uma potente atividade antioxidante, correlacionando esta atividade biológica ao conteúdo de fenóis e flavonoides encontrados nos extratos dessas plantas. Pesquisas realizadas com extrato aquoso de *Stryphnodendron adstringens*, conhecida popularmente como barbatimão, também mostraram a capacidade de compostos biotivos presentes na planta em eliminar radicais livres (BALDIVIA et al., 2018). Cunha et al. (2016) apontaram que a capacidade de capturar radicais livres da pitanga (*Eugenia uniflora*), deve-se ao seu conteúdo de compostos fenólicos.

Existem diversos métodos para determinar a atividade antioxidante *in vitro* de substâncias biologicamente ativas presentes em plantas medicinais. Os principais testes de avaliação da capacidade antioxidante podem ser divididos em duas categorias, de acordo com as reações químicas envolvidas, podendo ser: (1) ensaios baseados em reação de transferência de átomo de hidrogênio (TAH), (2) ensaios que se baseiam na reação de transferência de elétrons (TE) e (3) testes que avaliam a capacidade quelação de metais de transição, como o Fe^{+2} (HUANG at al., 2005; GÜLÇIN, 2012; AMATATONGCHAI et al., 2012). Dentre os testes *in vitro* desenvolvidos para determinar a capacidade antioxidante dos produtos naturais, destacam-se o $ABTS^{\bullet+}$ (2,2'-azino-bis (3-etilbenzotiazolina-6-sulfônico)) e $DPPH^{\bullet}$ (2,2-difenil-1-picril-hidrazila), FRAP Ferric Reducing Antioxidant Power) e atividade quelante de íons Fe^{2+}.

Entre os métodos mais comumente utilizados destacam-se aqueles relacionados à habilidade das substâncias em neutralizar radicais como os testes $ABTS^{\bullet+}$(2,2'-azino-bis (3-etilbenzotiazolina-6-sulfônico)) e $DPPH^{\bullet}$ (2,2-difenil-1-picril-hidrazila), sendo que ambos os ensaios apresentam um mecanismosimilar para determinar a capacidade antioxidante, nestes casos o espectro de absorção do radical livre estável muda devido a sua redução por umantioxidante (TEOWet al., 2007; LÓPEZ-ALARCÓN; DENICOLA, 2013).

O DPPH é um radical estável com uma coloração violeta e este ensaio é baseado na doação de elétrons de uma determinada substância para sequestrar o radical DPPH, reduzindo-o a hidrazina. Esta reação é acompanhada por uma com mudança simultânea na coloração de violeta a amarelo que pode ser estimada a 517nm, portanto a descoloração atua como um indicador de eficácia antioxidante (ALVES et al., 2010; ZHONG;

SHAHIDI,2015). O ensaio do DPPH é considerado um método rápido, simples, barato, sendo queo radical DPPH está comercialmente disponível e não precisa ser gerada antes do ensaio, dessa forma, a reação envolve somente o radical e a substância a ser testada (KEDARE; SINGH, 2011; OLIVEIRA, 2015; ZHONG; SHAHIDI, 2015). Segundo Prior e Schaich (2005), principal mecanismo envolvido neste teste é a transferência de elétron, sendo a transferência de hidrogênio apenas uma via marginal de reação (Figura 24).

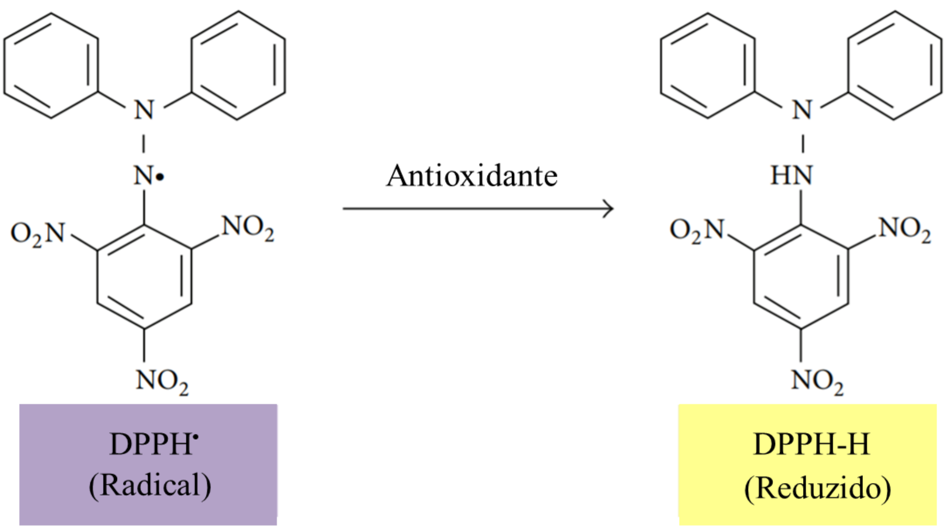


Figura 24 - Mecanismo de reação entre o radical DPPH• e um antioxidante através da transferência de um átomo de hidrogênio. Fonte: Adaptado de Teixeira et al. (2013).

O ensaio do ABTS avalia a capacidade de compostos em capturar o cátion ABTS$^{\bullet +}$(2,2’-azino-bis (3-etilbenzotiazolina-6-sulfônico)). O radical ABTS$^{\bullet +}$ é um cromóforo azul-esverdeado quimicamente estável com um máximo de absorção de 734nm e pode ser gerado por reações enzimáticas ou químicas sendo que a reação com persulfato de potássio é a mais comumente utilizada para a produção do radical. Antioxidantes podem neutralizar o radical ABTS$^{\bullet +}$ gerando o ABTS e esta captura provoca um decréscimo na absorbância um indicando a eficácia do composto antioxidante (Figura 25) (SUCUPIRA et al., 2012; MOHARRAM; YOUSSEF, 2014;ZHONG; SHAHIDI, 2015). Para Prior e Schaich (2005) a redução do radical ABTS$^{\bullet +}$pode ocorrer pela transferência de elétrons ou pela transferência de átomo de hidrogênio. Este método consiste em um dos testes mais rápidos dentre aqueles utilizados para avaliar a atividade antioxidante *in vitro* e que oferece resultados altamente reprodutíveis, além disso, tem como vantagem o fato de permitir análises de substâncias tanto de natureza lipofílica como hidrofílica (SUCUPIRA et al., 2012; MOHARRAM; YOUSSEF, 2014; ZHONG; SHAHIDI, 2015).

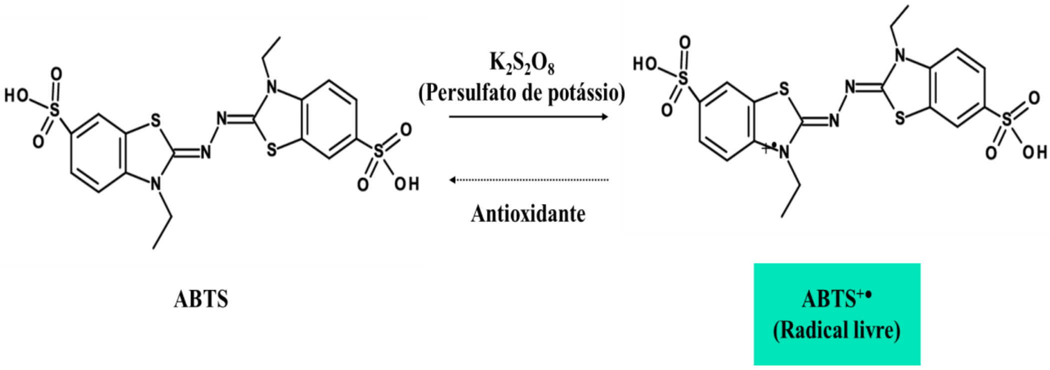


Figura 25 - Oxidação do ABTS pelo persulfato de potássio para gerar o radilcal ABTS$^{+\bullet}$ e a sua reação com um composto antioxidante.

O método FRAP (*Ferric Reducing Antioxidant Power*) também é utilizado para avaliar a capacidade antioxidante de produtos naturais. Este teste é baseado na transferência de elétrons e determina a redução do íon férrico (Fe^{3+}) a íon ferroso (Fe^{2+}) no solvente TPTZ (2,4,6- tripiridil-s-triazina), formando um complexo $[Fe(II)(TPTZ)_2]^{3+}$que possui uma coloração azul intensa (Figura 26), portanto a atividade antioxidante é determinada pelo aumento da absorbância a 593 nm. Esta reação ocorre em condições ácida, a fim de manter a solubilidade do ferro e, mais importante, possibilitar a transferência de elétrons (BENZIE; STRAIN, 1996, SUCUPIRA et al., 2012; ZHONG; SHAHIDI, 2015; BENZIE; DEVAKI, 2017).

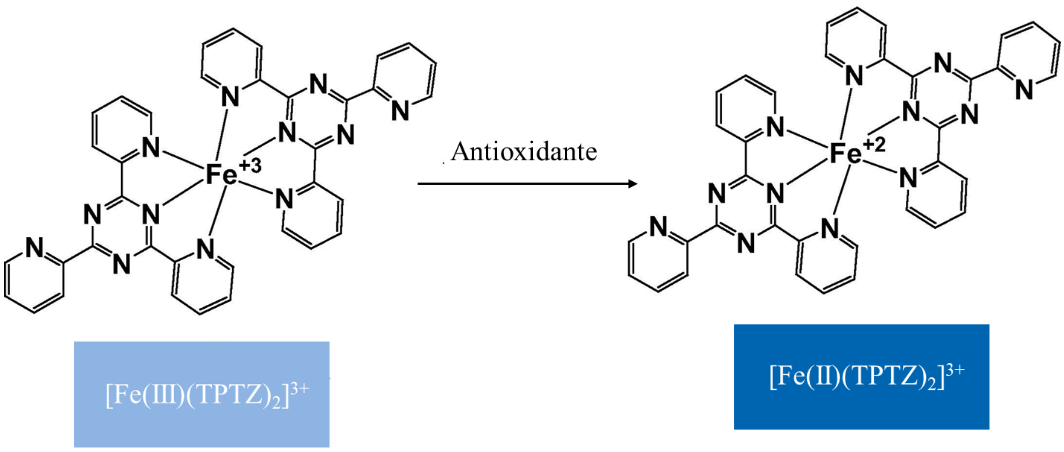


Figura 26 - Redução do complexo TPTZ (2,4,6-tri(2-piridil)-1,3,5-triazina) com o íon Fe^{3+} a Fe^{2+} pela ação de um antioxidante. Fonte: Adaptado de Rufino et al. (2006).

Os íons metálicos livres, assim como o Fe^{2+} e Fe^{3+}, são importantes catalizadores em diversas reações celulares que produzemespécies reativas tanto em sistemas *in vivo* quanto *in vitro*. Os íons livres de Fe^{2+}, por exemplo, podem reagir com o peróxido de hidrogênio (H_2O_2) via reação de Fenton e gerar radicais hidroxilas ($OH^{\bullet}$), que são moléculas altamente reativas (Equação 1). Substâncias que se ligam a íons metálicos alteram o potencial redox desses íons, reduzindo o seu poder redutor, por isso, ensaios que avaliam a capacidade de extratos vegetais em quelar metais tem sido bastante utilizados, assim como o teste da atividade quelante do Fe^{2+}(BARREIROS et al., 2006; WONG et al., 2006; JOMOVA et al., 2010).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-} + OH^{\bullet} \qquad (1)$$

Neste método, a ferrozina (3-(2-piridil)-5,6-bis-(4-ácido fenil sulfônico)-1,2,4-triazina)que, na presença do íon Fe^{2+}, forma um complexo estável de coloração roxo, cuja absorbância pode ser medida a 562 nm. A presença de agentes quelantes dificulta ou impedem a formação desse complexo, ocasionando uma descoloração da reação, dessa forma, quanto menor a absorbância, maior será o potencial antioxidante da amostrapela sua capacidade em quelar os íons ferro (BARREIROS et al., 2006; LIMA et a., 2010; HARB et al., 2016).

2.8 Atividade antitumoral de plantas medicinais e a busca por novos medicamentos

O câncer é uma doença que surge a partir transformação de células normais em células tumorais que passam a crescer de forma desordenada e descontrolada. A Organização Mundial de Saúde (OMS) aponta o câncer como a segunda causa de mortes no mundo, sendo responsável por levar a óbito aproximadamente 9,6 milhões de pessoas no ano de 2018 e os cânceres de pulmão, próstata, colorretal e de mama foram os mais prevalentes. Essa enfermidade pode afetar pessoas de todas as idades, porém a incidência de câncer aumenta drasticamente quando o envelhecimento, provavelmente em função da redução da eficiência de mecanismo de reparo celular (WHO, 2018).

Segundo estimativas do Instituto Nacional de Câncer (INCA), serão diagnosticados no Brasil, entre os anos de 2018 e 2019, 600 mil novos casos de câncer, e os tipos de cânceres mais frequentes entre homens e mulheres, serão o de próstata (68 mil) e o de mama (60 mil), respectivamente (BRASIL, 2018). Aproximadamente 70% das mortes por câncer ocorrem em países de média e baixa renda, por isso é essencial que se busque novas alternativas ao tratamento desta doença para aumentar o acesso da população a terapias de combate a essa doença (WHO, 2018).

A modificação de células normais até o surgimento de células tumorais é o resultado da interação entre os fatores genéticos de uma pessoa com agentes externos, que podem ser físicos, químicos ou biológicos. (ALMEIDA et al., 2005; AGUIAR JUNIOR, 2016; WHO, 2018). O câncer é o resultado de mutações em genes que estão envolvidos com o controle do ciclo celular, reparação do DNA danificado e apoptose e esses genes relacionados ao câncer podem ser divididos em três grupos: oncogenes, proto-oncogenes e genes supressores tumorais (LOPES et al., 2002; ZHU et al., 2015). Os proto-oncogenes referem-se a uma família de genes que atuam de forma positiva no controle do ciclo celular normal, porém ao sofrerem mutações se tornarão oncogenes induzindo ou estimulando excessivamente a proliferação celular. Por fim, as proteínas que inibem o crescimento de células tumorais são codificadas pelos genes supressores tumorais (WARD, 2002; AUDIC; HARTLEY, 2004).

Diversos estudos tem demonstrado que a inflamação é um importante componente na progressão tumoral, uma vez que células e mediadores do sistema imune inato são detectados na maioria dos casos de câncer e essa alta prevalência de mediadores do sistema imune inato leva à indução de vias inflamatórias em células cancerígenas (SETIA; SANYA, 2012).

Os agentes químicos inflamatórios atuam para modifcar os tecidos adjacentes a uma determinada lesão, tornando-os permeáveis para que as células imunológicas possam penetrar e chegar ao local para destruir os agentes agressores, induzindo no local uma nova formação de pequenos vasos sanguíneos, de forma a permitir a chegada de oxigênio e nutrientes no local da lesão a ser reparada, e as células tumorais se utilizam dessas substâncias para induzir sua própria proliferação e torna-las permeáveis as barreiras que o cercam. A inflamação regula a tumorigênese por diversos mecanismos como alteração do ciclo celular e proliferação celular, migração de células, estímulo à angiogênese e inibição da apoptose. Assim, a composição do microambiente inflamatório do tumor tem uma influência fundamental no resultado da doença (PAUL et al., 2013; CRUSZ; BALKWILL, 2015).

A enzima ciclooxigenase-2 (COX-2) constitui um ponto comum entre a inflamação e o câncer, pois, vários tipos de tumores expressam essa enzima em níveis elevados, o que aponta para um papel potencial da COX-2 na promoção e progressão tumoral (KUNDUH; SURH, 2008; CRUSZ; BALKWILL, 2015). A enzima ciclooxigenase apresenta duas isoformas denominadas de ciclooxigenase-1 (COX-1) e ciclooxigenase-2 (COX-2). A pimeira é considerada como fisiologicamente constitutiva, pois é expressa na maioria dos tecidos de maneira constante e apresenta papel regulador chave na fisiologia normal desses tecidos. Já a segunda é indutiva, sendo expressa apenas em situação de trauma tissular, inflamação, etc (KUMMER; COELHO, 2002; GARCÍA-BUENO; LEZA, 2008).

A COX-2 exerce uma importante função na produção das prostaglandinas, que por sua vez estão envolvidas em diversos processos fisiológicos e patológicos, incluindo os processos inflamatórios. Diversos estudos demonstram que as prostaglandinas apresentam a capacidade de promover a proliferação celular, a angiogênese associada ao tumor, inibir a morte celular de maneira a favorecer o crescimento tumoral, inibir a resposta antitumoral e modular a invasão do tumor (BOUGHTON-SMITHet al., 1983; SHENG et al., 1998; CAMACHO et al., 2008). Por isso, diversos agentes terapêuticos direcionados a mediadores da resposta inflamatória tem sido utilizados para a prevenção e tratamento do câncer (CRUSZ; BALKWILL, 2015).

Dentre as principais formas de tratamento do câncer estão excisão cirúrgica, radioterapia e quimioterapia. A cirurgia e a radioterapia são recomendadas para tratamento da doença localizada, sendo utilizadas nos estágios iniciais da doença e em geral possuem um papel limitado em cânceres mais avançados e por isso, atualmente, a quimioterapia é considerada o método mais efetivo no tratamento contra o câncer, porém, as células cancerígenas podem apresentar alta resistência aos fármacos aplicados e ainda a elevada toxicidade e inespecificidade desses medicamentos é um problema muito comum, acarretando em uma alta incidência de efeitos colaterais (AJITH; JANARDHANAN; 2003; VON MINCKWITZ; MARTIN, 2012). Em função disso, a procura por novos medicamentos anticancerígenos têm-se aumentado cada vez mais, a fim de se encontrar fármacos mais eficazes e específicos que impeçam o avanço da doença (BRANDÃO et al., 2010).

As plantas constituem uma importante fonte de compostos com potencial para prevenção e cura do câncer, e de fato muitos fármacos utilizados no tratamento desta patologia são de origem exclusivamente vegetal ou obtidos a partir de precursores naturais, sendo que mais de 3000 espécies de plantas em todo o mundo exibem propriedades anticarcinogênicas (ALVES-SILVA et al., 2017; TARIQ et al., 2017;SECA; PINTO, 2018). Como exemplo podemos citar vincristina, paclitaxel, homoharringtonina, entre outros. A vincristina é um alcaloide isolado da vinca (*Catharantus roseus* - Apocynaceae) que foi um dos primeiros agentes anticancerígenos derivados de plantas aprovados pela agência norte-americana que regula os alimentos e medicamentos FDA (Food and Drug Administration) (MESQUITA et al., 2009; NEWMAN; CRAGG, 2016). Paclitaxel é uma molécula complexa que se tornou uma das drogas quimioterápicas para câncer mais conhecidas e é isolado da casca de *Taxus brevifolia* (WEAVER, 2014). A homoharringtonina é um alcalóide que foi inicialmente isolado de *Cephalotaxus harringtonii* e *Cephalotaxus fortunei*, espécies utilizadas na medicina tradicional chinesa, atualmente sua forma semissintética é usada para a produção industrial (KANTARJIANet al., 2013).

Além dos fármacos já utilizados para o tratamento da doença, pesquisas têm demonstrado que diversas plantas medicinais apresentam potencial antitumoral com perspectiva para utilização na terapia contra o câncer, dentre as quais podemos citar as espécies que são objetos de estudo no presente trabalho, a *Varronia curassavica* (PARISOTTO et. al, 2012) e a *Momordica charantia* (DIA; KRISHNAN, 2016). Bendaoud e seus colaboradores (2010) também demonstraram que o óleo essencialdas espécies do gênero *Schinus*, *S. molle* L. e *S. terebinthifolius*, apresentam atividade antiproliferativa contra células de câncer de mama humano. Dentre tantas outras espécies com propriedades anticâncer, podemos cita ainda a *Annona muricata*, conhecida popularmente como graviola (YAJIDet al., 2018).

Os métodos colorimétricos para avaliar a proliferação celular em cultura, que possilitam inferir sobre a atividade antitumoral de produtos naturais, são bastante utilizados, por serem rápidos e baratos (BETANCUR-GAVIS et al, 1999). O método colorimétrico MTT (3(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio brometo) é um dos ensaios mais utilizados para a avaliação da citotoxidade. Foi descrito inicialmente por Mosmamm (1983) e é baseado no princípio de redução do MTT resultando de coloração roxa. O MTT, que é um sol solúvel em água de cor amarela, é convertido em formazan, o seu produto de coloração roxa, por enzimas desidrogenases mitocondriais, dessa forma, apenas as células metabolicamente ativas conseguem reduzir o MTT permitindo quantificar as células viáveis (Figura 27).

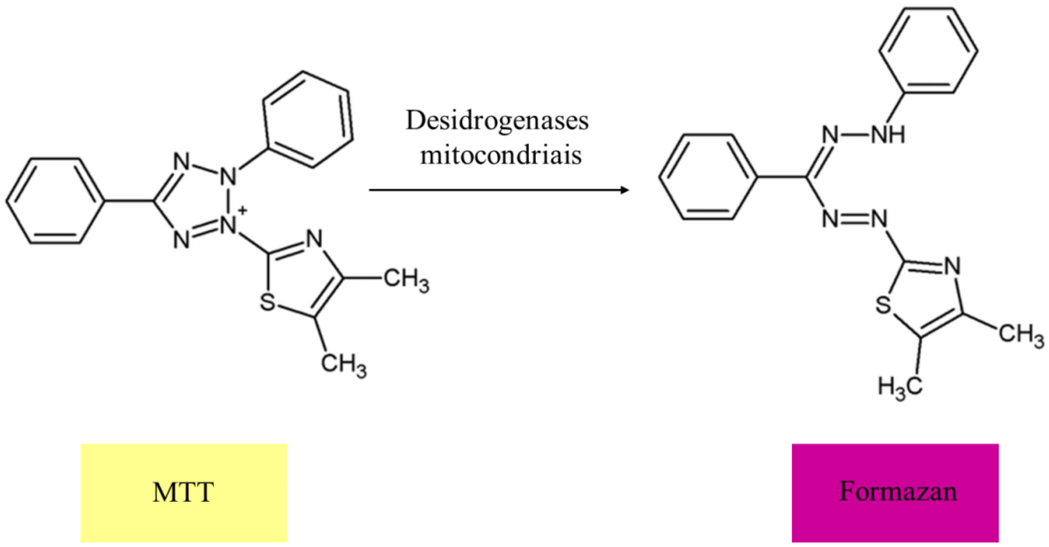


Figura 27 - Redução do MTT catalisada por desidrogenases mitocondriais gerando o seu produto reduzido, o formazan.

3 OBJETIVO GERAL

Avaliar a influência dos fatores ambientais e genéticos na composição fitoquímica e nas atividades antioxidante, citotóxica e antitumoral de *Varronia curassavica* Jacq. e *Momordica charantia* L. oriundas de populações de diferentes regiões do Espírito Santo.

4 OBJETIVOS ESPECÍFICOS

- Caracterizar, por meio de marcadores ISSR, a estrutura genética de diferentes populações naturais de duas espécies de plantas medicinais (*Varronia curassavica Jacq*. e *Momordica charantia L.)* no Espírito Santo;
- Analisar as diferenças nos teores de compostos fenólicos dos extratos de *Varronia curassavica Jacq.* e *Momordica charantia L.* obtidos a partir de plantas coletadas em diferentes localidades do Espírito Santo.
- Avaliar o potencial antioxidante através dos testes DPPH, ABTS; FRAP; atividade quelante do Fe^{+2} das duas espécies coletadas em diferentes regiões.
- Quantificar os compostos fenólicos (luteolina, quercetina, rutina, ácido clorogênico e ácido gálico) presentes em diferentes frações do extrato hidroalcoólico das populações de *Varronia curassavica Jacq.* e *Momordica charantia L.,* utilizando cromatografia líquida de alta eficiência (HPLC);
- Avaliar e comparar a atividade citotóxica *in vitro* do extrato hidroalcoólico das diferentes populações, das duas espécies estudadas, em linfócitos humanos,
- Avaliar e comparar a atividade antiproliferativa *in vitro* do extrato hidroalcoólico das diferentes populações, em linhagem tumoral Sarcoma 180 de roedores, por meio do ensaio do MTT.
- Analisar a atividade antitumoral *in vivo* do extrato hidroalcóolico de *Varronia curassavica Jacq.* e *Momordica charantia L.,*
- Verificar e comparar resultados obtidos por meio de análises de correlação.

CAPÍTULO 1 – Phytochemical variation in *Varronia curassavica* Jacq. populations is influenced by environmental factors.

Após revisão da língua inglesa, e considerações da banca examinadora, este artigo será submetido à revista: Genetics and Molecular Biology (ISSN: 415-4757).

Phytochemical variation in *Varronia curassavica* Jacq. populations is influenced by environmental factors

Mainã Mantovanelli da Mota[*], Anny Carolyne da Luz[a], Irany Rodrigues Pretti[a], Jean Carlos Vencioneck Dutra[a], Maria do Carmo Pimentel Batitucci[a].

[a]Departamento de Ciências Biológicas, Centro de Ciências Humanas e Naturais, Universidade Federal do Espírito Santo, Vitória, Brazil.

*Corresponding author: Mainã Mantovanelli da Mota
Departamento de Ciências Biológicas
Laboratório de Genética Vegetal e Toxicológica
Universidade Federal do Espírito Santo
Av. Fernando Ferrari 514, Goiabeiras, 29075-910, Vitória, ES, Brazil
Phone: 55 27 998311735
Email address: maina.mantovanelli@gmail.com

Abstract

Varronia curassavica Jacq. is a medicinal plant, widely distributed along the Brazilian coast and commonly used to treat inflammation. However, the quality of medicinal plant is influenced for genetic and enviromental conditions. Then, the aim of this work was evaluated genetic and phytochemical variability of *V. curassavica* and determine factors that influence na accumulation of chemical compounds. Genetic diversity among *V. curassavica* populations growing wild in the Southeast Region of Brazil were assessed by ISSR markers and analysis of chemical components of extracts from these populations as well as its antioxidant actions were performed in order to determine its phytochemical variability. Twenty selected ISSR primers produced 136 discernible bands, of them 110 (80.8%) being polymorphic. Genetic similarity values among populations ranged between 0.402 and 0.720 which indicates a low level of genetic variation. UPGMA grouped *V. curassavica* into three main clusters. Cluster analysis and principal component analysis of phenolic compounds content and their antioxidant activity and that clustering it is not positively correlacioned with the genetic cluster. These findings suggest that phytochemical variation was strongly related with environmental conditions.

Keywords: Genetic diversity, ISSR, phenolics compounds, antioxidant activity

1. Introduction

Varronia curassavica Jacq. (*Cordia verbenacea* DC. synonymy), belongs to the family Cordiaceae and is a medicinal species native to Central and South America (Gasparino and Barros, 2009). In Brazil, this species has a wide distribution and can be found along the coastal regions associated with the Atlantic Forest (Feijó et al., 2014; Matias et al., 2013). It is a medicinal plant traditionally used to inflamation and ulcers treatment. Furthermore, pharmacological studies have demonstrated several properties of *V. curassavica* such as antibacterial, antifungal, anti-allergic, antitumor and antioxidant activities (Passos et al., 2007; De Carvalho et al., 2004; Matias et al., 2016; Nizio et al., 2015; De Oliveira et al., 2011; Parisotto et al., 2012; Michielin et al., 2011).

The biological and pharmacological properties of some plants are mainly related to the content of secondary metabolites, such as the phenolics compounds that acts as effective antioxidant and their beneficial effect is attributed to their donating electrons able to scavenging and neutralize reactive oxygen species (ROS) (Kumar et al., 2015; Rice-Evans et al., 1997). *ROS* produced by cellular metabolism have crucial roles in maintaining their redox homeostasis by modulating a numerous process that occur into the cell. However, when the cell is unable to eliminate excess ROS, oxidative stress is generated, leading to damage which is associated with cardiovascular and neurodegenerative disorders and cancer (Lipina and Hundal, 2016).

Investigations have demonstrated that the total content and biological activities of secondary metabolites in plants can be affected by environmental factors, such as light intensity? photoperiod?, temperature, water availability, soil composition (Borges et al., 2013; Liu et al., 2015; Sampaio et al., 2016; Liu et al., 2016) and genetics (Samec et al., 2016; Moustafa et al., 2016). Additionally, the understanding of the genetic structure and diversity of natural populations are crucial for their preservation and genetic improvement programs (Facanali et al., 2015). Among the various molecular techniques that have been used to analyze the genetic diversity of wild plant populations, the inter-simple sequence repeats (ISSR) technique is particularly simple since it doesn´t require previous knowledge of DNA sequence and have good stability and large polymorphism (Godwin et al., 1997). In the last years, ISSR has been successfully used to investigate the genetic diversity and relationships at species, population and cultivar levels in many plants, including some medicinal species (Chen et al., 2013; Simplicio et al., 2015 and Alansi et al., 2016)

Phytochemical and molecular markers have been simultaneously used to characterize the level of variation in several medicinal plant species, such as *Teucrium arduini* (Sampaio et al., 2015); *Ocimum selloi* (Facanali et al., 2015); *Astragalus mongholicus* (Li et al., 2017) and *Uncaria tomentosa (Honório et al., 2017).* However, there is no report relating *V. curassavica* genetic characterization with its environmental growth conditions and the influence of these factors in the accumulation and production of bioatives compounds. In the present study, ISSR markers were used to evaluate the genetic diversity of ten *V. curassavica* wild populations growing in the state of Espírito Santo, located in the Brazil southeastern. To determine the phytochemical diversity we mensured the total phenol, total flavonoid, total tannins and individual phenolic acids contents of the extracts of *V. curassavica* populations and we also evaluated their antioxidant activitie by ABTS assay. In addition, multivariate analyses were carried to determine the relationship between genetic and phytochemical variability of *V. curassavica* populations.

2. Material and methods

2.1. Plant material

Arbitrarily selected samples of wild growing *V. curassavica* plants were collected from the June to September, in 2015 from 10 localities of Southeastern of Brazil: Anchieta (ANC) (20°47'30.6"S; 40°34'58.0"W), Aracruz (ARA) (19°57'32.3"S; 40°08'15.6"W), Domingos Martins (DM) (20°23'01.3"S; 40°38'27.5"W), Guarapari (GUA) (20°36'08.6"S; 40°25'35.7"W), Jaguaré (JAG) (18°57'19.6"S; 40°08'43.5"W), Marechal Floriano (MF) (20°24'37.0"S; 40°40'32.8"W), Serra (MG) (20°12'04.3"S; 40°11'42.9W), Serra (JAC) (20°10'24.4"S; 40°11'11.3"W) and Viana (VIA) (20°23'05.7"S; 40°29'52.9"W). Voucher specimen (41243) was deposited in the Central Herbarium of Universidade Federal do Espírito Santo/VIES, Brazil.

Aerial part samples were collected for phytochemical extraction and the samples were air-dried at room temperature and then ground for further analysis. For each of ten populations, the soil and leaf samples were collected and their chemichal compositions were analized, as presented in Table 1A and 1B, respectively. Leaves were also collected and immediately cooled for DNA extraction from leaf tissues.

2.2 DNA extraction and ISSR genotyping

The genomic DNA was based on the protocol described by Doyle (1987) with modifications. The samples from the fresh and young leaves of ten plants of each locality were ground in liquid nitrogen in presence of polyvinylpolypyrrolidone to obtain a fine powder. To this powder, 1 mL of CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH 8, 20 mM EDTA pH 8), 20 μL of β-mercaptoethanol and 10 μL of proteinase-K were added and incubated at 65°C, for 30 minutes. Subsequently, 500 µL chloroform:isoamyl alcohol (24:1 v/v) were added to samples and centrifuged for 5 min at 12000 RPM, this procedure was repeated twice. The supernatant was treated with RNAse A (10 µg/µL), to 37 ºC for 30 min. DNA precipitation was performed with 0.6% by volume of cold isopropanol. The formed pellet was washed with 70% ethanol, dried at room temperature and resuspended in 100 µL of Tris–EDTA pH 8.0 (TE Buffer). The quantity and purity of the extracted DNA were evaluated spectrophotometrically by NanoDrop 3300 (Termo Scientific). The purity of the DNA was also confirmed by gel electrophoresis system using 1.0% agarose stained with GelRedTM (BIOTIUM^{TM}).

Twenty ISSR primers (Eurofins, Operon Technologies, Louisville, KY, USA) (Table S3 in Supplementary material) were selected for this study based on the presence of clear, reproducible and polymorphic amplified bands. The amplification was carried out in 25 µL reaction volume containing 25 ng DNA template, 5 µL de 5x reaction buffer, 2.5 µL 25 mM $MgCl_2$, 0.5µL 10mM dNTP, 1.25 U Taq and 0.5 µL (10 µM) of specific primer, in Veriti® 96-Well Thermal Cycler (Apllied Biosystems^{TM}). The PCR cycle was performed as follows: 94 ºC for 5 min, 35 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 2 min and elongation at 72 ºC for 2 min after which the program was finished with final elogation of 5 min at 72 ºC. The amplified DNA products were separated on 2% agarose gel in TBE buffer (1X), stained with GelRedTM (BIOTIUM^{TM}), visualized under UV light and documented using a Gel Documentation System LPIX-TOUCH (Loccus Biotecnologia, Brazil).

2.3. Hydroalcoholic extract preparation

The powder of dried aerial parts of *V. curassavica* were submitted to exhaustive extraction with ethanol-water solution (70:30 v/v) in a ratio 5:1 (v/w) by 72h, protected from light, at room temperature. This process was repeated twice with same power to extract the maximum of constituents. After the maceration, resulting solutions were filtered and the solvent recovered in a rotatory evaporator under vacuum to obtain the crude hydroalcoholic extracts (HAE) from each population of *V. curassavica*.

2.4. Phytochemical analysis

2.4.1. Total phenolic content (TPC)

The total phenolic content of the extract was determined by the Folin–Ciocalteu method (Zhang et al. 2006). Ethanol solution of HAE 500µg/mL (20 µL) was mixed to 100 µL of Folin-Ciocalteu reagent diluted in distilled water (1:10) and incubated for 5 min at room temperature. Then, 80 µL of Na_2CO_3 (7.5%) were added to the plate, wich was kept in the dark for 2 hours. After incubation, absorbance at 750 nm was measured with a spectrophotometric microplate reader (Epoch Microplate Spectrophotometer – BioTek). TPC was expressed as milligrams gallic acid equivalent per gram of dry weight of the extract (mg GAE.g^{-1} d.w.) from a calibration curve with gallic acid. The concentrations of gallic acid were 15.62, 31.25, 62.5, 125, 250, 500 µg/mL. Ethanol was used as blank. All samples were analyzed in three replicates.

2.4.2. Total tannins content (TTC)

The total tannin content (TTC) was carried using Folin-Denis method which is based on the reducing power of the phenolic hydroxyl group of tannins (Pansera et al., 2003) with a few modifications. To 500 µL of the ethanol solution of HAE (500 µg/mL) was added an equal volume of Folin-Denis reagent. The contents were mixed thoroughly and, after 3 min, 500 µL of Na_2CO_3 solution (8%) were added. The prepration was mixed and incubated for 2 hour. Subsequently, the material was centrifuged at 2000 rpm for 5 min and the absorbance measured at 725 nm. To construct the standard curve, different concentrations of tannic acid (15.62, 31.25, 62.5, 125, 250, 500 µg/mL) were used and the result was expressed as tannic acid equivalents per gram of dry weight (mg $TA.g^{-1}$ d.w.). Ethanol was used as blank. For each sample, three replications were performed.

2.4.2. Total flavonoid content (TFC)

Total flavonoid content (TFC) was determined following a method by using the colorimetric method with aluminum chloride (Mraihi et al., 2013). In sealed tubes, 1.5 mL of a 2% methanol solution of $AlCl_3{\cdot}6H_2O$ was added to 0.5 mL of methanol solution of HAE 500 µg/mL and then kept in dark for 10 min at room temperature. Absorbance was measured at 430 nm, methanolic $AlCl_3$ was used as blank, and each measure was made in triplicate. Concentrations of rutin used to establish the standard curve of flavonoids were 15.62, 31.25, 62.5, 125, 250, 500 µg/mL. The TFC in extract was expressed as rutin equivalent per gram of dry weight (mg $RE.g^{-1}$ DW).

2.5. Antioxidant assay

2.5.1 Free radical scavenging activity by ABTS assay

The free radical scavenging effects of the extracts on $ABTS^{\bullet+}$ (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) was performed using the method described by Re et al. (1999), with modifications. $ABTS^{\bullet+}$ radical cation was obtained by reaction of 5 mL of an ABTS aqueous solution (7 mM) with 88 µL potassium persulfate (2.5 mM). It was incubated before use, in the dark, for 16 h at room temperature, until the reaction has been completed. The absorbance of the ABTS solution radical was equilibrated to 0.70 (70%) by dilution in ethanol. Then, 200 µL of $ABTS^{\bullet+}$ solution were mixed with 40 µL of the test solution (15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL), samples of the HAE and standard. Trolox was used as an antioxidant standard. The absorbance was measured in 734 nm, after 6 min incubation in the dark. The control was prepared using 40 µL mL of ethanol and 200 µL of ABTS radical solution. The test was performed in triplicate and the scavenging activity was determined by the following formula: % scavenging of $ABTS^{•+}$ = [(Abs0 – Abs1) / Abs0] x 100, where Abs0 = absorbance of control and Abs1 = absorbance of the sample. Results were expressed as IC50 value (µg/mL).

2.6. High-performance liquid chromatography (HPLC) analysis

2.6.1 Prepraration of extract fractions

In order to prepare the fractions were used solvents with increasing polarity (Shahraki et. al, 2016). The obtained HAE from *V. curassavica* (0.5 g) was suspended in 50 mL of water and transferred to a decanter funnel. The n-hexane (50 mL) solvent was added to the funnel, and the hexanic fraction was then extracted. In the next step, the remainder of the solvent in the decanter funnel was combined with dichloromethane solvent, and this fraction was then extracted. Subsequently, the remaining solvent from the previous steps was mixed with ethyl acetate, and the ethyl acetate fraction was extracted. At the end, n-butanol solvent was added to the funnel for obtain butanolic fractions. All fractionates were collected separately and dried at room temperature.

2.6.2 Instrumentation

HPLC analyses were performed using on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) with solvent delivery system of two pumps (Model LC–20AT, Prominence Liquid Chromatograph, Shimadzu), column oven (Model CTO-20A), UV detector (Model SPD–20A, Prominence Diode Array Detector, Shimadzu) and degasifier (DGU-20A5). Data collection and integration were accomplished using LC Solutions, 1.25 version software. Separation was achieved with on a C18 reverse phase column ZORBAX Eclipse XDB®, Agilent (4.6 mm x 250 mm, 5µm) equipped with a guard column of same packing material.

2.6.3 HPLC conditions

The methanol solutions of ethyl acetate and dichloromethane fractions were used to detect flavonoids and tannins, respectively. HPLC assay for flavonoids detection was carried out using an isocratic elution with a flow rate of 1 mL/min, the column temperature was set to 40ºC, a mobile phase of methanol and 2% phosphoric acid (pH 2.60) (40% : 60% v/v) and a detection wavelength of 350 nm. The injection volume was 20 µL of solution. The total run time was 20 minutes for each injection. Identification of flavonoids was performed by comparing their retention times (RT) and spectra with those of known standards. The standards used were chlorogenic acid, rutin, quercetin and luteolin (Sigma-Aldrich, USA). Chromatographic peaks in the samples were identified on the basis of retention time of the standards. Amounts of flavonoids were calculated using a calibration curve equation for each standard. The data are presented as averages of triplicate measurements (ROSS; BETA; ARNTFIELD, 2009).

Tannin detection was performed using the mobile phase with 0.1% acid formic solution (Solvent A) and methanol (Solvent B), the flow rate was adjusted to 0.7 mL/min and the column was thermostatically controlled at 40°C. A gradient elution was carried by varying the proportion of solvent B to solvent A as follows: 0–15 min, 15% to 33% B; 15–17 min, 33% to 15% B; 17–22 min, 15% B to wash and equilibrate of the column. The injection volume was kept at 20 µl and a detection wavelength of 270 nm. The quantification of the sample was measured by comparing their retention times and spectra with the standard, gallic acid (Vetec, Brazil). The data were reported in triplicate.

2.7. Statistical analysis

Genetic variability analysis was performed with XLSTAT for Windows (Addinsoft, New York, USA) program. The reproducible and well-resolved fragments were scored as present (1) or absent (0) as binary data for each of the ISSR markers. From these data, the genetic proximity was estimated based on Jaccard similarity coefficient. Clustering analysis was conducted using the Unweighted Pair Group Method Arithmetic Mean (UPGMA) method. For phytochemical analysis and antioxidant assay were performed analysis of variance (ANOVA) followed *test t* and the differences between measurements were considered to be significant at $p < 0.05$, using

ASSISTAT version 7.7 beta software (Assistat Software, Campinas, São Paulo, Brazil.). Principal component analysis (PCA), Pearson linear correlation and hierarchical cluster analysis (HCA) were performed using XLSTAT for Windows (Addinsoft, New York, USA) in order to visualize relationships between the *V. curassavica* samples in each collection area.

3. Results and discussion

3.1. Genetic variability

Documenting the genetic diversity may provide important information for identification of useful genotypes that could be used as cultivar. In the present study it was evaluated the genetic structure of natural populations of *V. curassavica* and to compare them with their phytochemical variability. Therefore, the 20 ISSR primers tested in the 10 populations *V. curassavica* showed good amplification profiles. A total of 136 DNA fragments were produced by 20 ISSR primers with average number of fragments per primer of 6.8 with 79.89% polymorphism. Among the 20 ISSR primers, UBC840 produced highest (13) followed by UBC848 (12), while UBC854 amplified the lowest (1) number of fragments. Nine ISSR markers, UBC834, UBC840, UBC848, UBC850, UBC854, UBC855, UBC856, UBC865 and UBC888 yielded 100% polymorphism, while lowest polymorphism was observed with primer ISSRCR-8 (25%) (Suppl. Table S3). To establish the genetic relationship among studied plant populations from different locality based on obtained ISSR markers, a dendrogram was constructed according to UPGMA cluster analysis using Jaccard's coefficients (Figure 1). The UPGMA dendrogram based on Jaccard's coefficients indicated that 10 populations were divided into three major clusters. The first cluster was divided into two sub-clusters, the first one included DM, MF, ST and MG populations, and the second one contained two populations ARA and ANC. The second cluster included JAC, JAG and GUA populations and third cluster contained only one population VIA. Based on proximity matrix of Jaccard´s coefficients (Suppl. Table S4) the highest similarity genetic was observed between DM and MF populations, with an index of 0.720. On the other hand, the VIA and ST showed the lowest genetic similarity with an index of 0.402.

Levels of similarity between samples were relatively high suggesting close genetic relations. Genetic diversity is crucial for effective maintaining biological diversity wich allows species to adapt to environmental changes (Frankham, 2003). Brito et. al, 2016 observed relatively high genetic diversity by Jaccard similarity coefficient for many pairs of accessions in germoplasm bank of *V. curassavica*. E qual a conclusão para fechar esta discussão?

3.2. Phytochemical and antioxidant analysis

Phenolic compounds have redox properties, which allow them to act as antioxidants (Rice-Evans et al., 1997) therefore the total phenolic, taninn and flavonoid content of all crude HAE was mensured (Table 1). The antioxidant activity of phenolic compounds is mostly due to possessing one or more aromatic rings bearing one or more hydroxyl substituents (Huang et al., 2009). *V. curassavica* extracts have shown a great variability contents of polyphenolic groups and consequently in antioxidant activity (Santi et al., 2014). The total phenolic content (TPC) (Table 1) of all 10 plant extracts under study was found in ranging from 25.22 to 218.40 mg $GAE.g^{-1}$. The maximum amount of TPC was recorded with the extract of the plants grown at locality ST and JAG had the lowest TPC. The total phenolic content found in present study was greater than found in methanol extrat the *Cordia evolutior*, specie of the same family, for Arunachalam & Parimelazhagan (2013). Michielin et al. (2011) found highest values of the TPC in *Cordia verbenacea*, however other extraction method was used since a was carried fractionation of the crude extract of the plant out by the method COSE (classical organic solvent extraction) and the highest TPC value in this research was obtained by COSE when using the solvent ethyl acetate. The total flavonoid content (TFC) (Table 1) ranges from 121.67 to 353.33 mg $RE.g^{-1}$ and the highest TFC was found in sample MF and ANC had the lowest TFC. Total tannin content (TTC) (Table 1) was higher in VIA (194.59 mg $TA.g^{-1}$) and MG had the lowest TTC (30.99 mg $TA.g^{-1}$) among the 10 extracts estimated.

The antioxidant ability of the *V. curassavica* HAE has been determined by using ABTS assay. ABTS radical scavenging assay involves a method that generates a blue/green $ABTS^{\bullet+}$ chromophore via the reaction of ABTS and potassium persulfate. The ABTS radical cation is reduced in the presence of hydrogen-donating antioxidants wich can be measured spectrophotometrically (Re et al., 1999). The results of ABTS assay was expressed as IC50 value ($\mu g.mL^{-1}$) (Table 1). IC50 value indicates ability of the extract to achieve 50% scavenging reaction, therefore, the lower the IC50 value of plant extracts used the higher was their free radical scavenging activity. ST sample showed a radical scavenging activity significantly higher than the other locations (107.57 $\mu g.mL^{-1}$).

Pearson correlation analysis demonstrated high significant correlation between ABTS with TPC (Table 5). Our results corroborate this since the extract samples ST and VIA present higher a radical scavenging activity mensured by ABTS and are among the samples with the highest values of TPC. Therefore, the antioxidant activity of these populations *V. curassavica* is determined by the total phenolic content.

Environmental factors, such as water availability, light intensity, carbon dioxide levels, temperature and nutrient supply influence in the secondary metabolites biosynthesis, which perform biological roles as an adaptive response to the change in environment conditions, can affect the concentrations of phenolic compounds (Borges et al., 2017; Borges et al., 2013). Thus, the amount of micronutrients available to the plant in the soil and leaves directly influences the production of phenolic compounds and consequently their antioxidant activity.

Our results demonstrated by Pearson's correlation that manganese (Mn), present in the leaves (Table 5), has a high correlation with TPC (Table 6), being that as samples ST and VIA presented a greater amount of manganese in the leaves and consisted of the samples with the highest content of TPC and with the best values of ABTS. Plants with manganese deficiency are impaired in the accumulation of phenolic compounds in their tissues. This occurs due to the influence of the Mn in the schikimic acid pathway, resulting in the biosynthesis of many phenolic compounds, such as flavonoids, tannins and lignin. Manganese activates several enzymes of the shikimic acid pathway including 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP), which condenses the phosphoenol pyruvate and erythrose-4-phosphate to a seven-carbon heterocyclic compound in the first step this biochemical pathway (Borges et al., 2017; Broadley et al., 2012; Herman, 1995).

3.3. HPLC analysis

Plants are rich source of effective and safe medicines due to presence of different bioactive molecules among other compounds as flavonoids, tannins and others phenolic compounds due to the fact they exhibit strong antioxidant activities (Meena et al. 2009; Minh et al., 2016). Therefore, free phenolic acids were identified and quantified in more details by HPLC (Table 2) that is a common technique employed for separation and quantification of compounds present in complex samples as plant extracts. The ethyl acetate fraction of *V. curassavica* was used to detect flavonoids simultaneously and was obtained good separation for most peaks. HPLC analysis demonstrated that the chlorogenic acid occurs in all populations and the content was higher in sample JAC (3.98 µg/mL). The content of total rutin ranged from 5.73 µg/mL to 71.63 µg/mL and higher levels were observed in samples of the ST (54.88 µg/mL) and VIA (71.63 µg/mL). In the present study quercetin was detected only in two populations GUA (4.26 µg/mL) and JAC (3.68 µg/mL). The luteolin was detected in five populations and their concentrations ranged from 2.01 µg/mL to 3.20 µg/mL. The highest luteolin level was found in ANC sample. The content of tannins was evaluated using gallic acid as standard. Gallic acid levels ranged from 2.41 µg/mL for sample JAC to 6.55 µg/mL for population VIA.

Phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl groups. Among these, the rutin, a phenolic compound with glycosidic linkage, that in the present study is related with total phenols content in *V. curassavica* extracts. Rutin it is reported to exhibit significant pharmacological activities, including antioxidant activity (De Araújo et al., 2013). These results suggest that the antioxidant property of *V. curassavica* may be partly attributed to the presence of rutin, corroborating with Alam et al.

(2017), wich demonstrated that the presence of rutin strongly supported the high antioxidant capacity of the tested *Acacia* species.

3.4. Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA)

Principal Component Analysis (PCA) was performed to determine differences among? samples based on their metabolite profiles and antioxidant activity (Figure 2). The PCA is probably the most common calculation used in processing of multivariate data. The objective of PCA is reduction of dimensionality of a data set with a large number of correlated variables or traits and it has proven to be a valuable tool to understand relationships between characteristics as well as between populations (Jolliffe, 2002; Khadivi-Khub et al., 2014).

In this study, two principal component (PC) of the score plot explained 61.31% of the total variance. Results of PCA revealed that the first componente (PC1) accounted for 40.67% of variance and the second componente (PC2) for 20.65% variance. Analysis of the data indicated that rutin, gallic acid, chlorogenic acid, quercetin, luteolin, TTC and TPC contributed most and positively to the PC1, with a satisfactory degree of correlation, while the correlation with the TFC and ABTS were inverse. The PC2 was mainly related to ABTS and luteolin. From this biplot, it was observed that VIA sample showed positive correlation with the amount rutin, gallic acid, TTC and negative correlation with the ABTS which indicated a good antioxidant activity of this population.

To evaluate the likely similarities and relationships among *V. curassavica* populations studied, the Hierarchical Cluster Analysis (HCA) was performed based on antioxidant activity and phytochemical content of the *V. curassavica*. The HCA results are presented in the form of a dendrogram (Figure 3). Cluster analysis confirmed the results of the PCA classification. Based on this analysis, the V. curassavica populations were classified into two major groups. First cluster is divided into two sub-clusters, the first one included GUA, ANC and MG, and the second one included JAG, ARA, MF, DM and JAC. The second cluster contained 2 populations ST and VIA. Similarly PCA analyss, the ST and VIA sanples formed a single group characterized by higher quantities of rutin and TPC. Since the TPC value is directly related to such phenolic structures as rutin, these samples might bestructurally different from others and were located differently from the others in the PCA. Results clearly showed that the *V. curassavica* collected from different regions were successfully distinguished by their antioxidant activity assay, total phenolic and rutin contents.

Comparison of clusters generated through phytochemical and genetic diversity, showed different pattern of grouping among the *V. curassavica* polutations. Therefore, chemical differences could not be correlated to the genetic profiles of these populations. It could be attributed to either environmental influence on phytochemical composition or the genes involved in phenolics compounds biosynthesis may not have been completely covered by the sequences of the markers used. These results are in accordance with data for six populations of *Teucrium arduini* L. from sub-Mediterranean part of Croatia (Samec et al., 2015) which did not show positive correlation between the molecular (RAPD) and phytochemical markers.

4. Conclusion

This work represent first comparative molecular and phytochemical investigation of ten *V. curassavica* wild populations and proposes a comprehensive study of factors that influence in its quality *as well as e*stimating genetic structure of their populations growing in the Southeastern Region Brazil. The results from the comparative study of the chemical and genetic analysis in ten populations of *V. curassavica* showed that the phytochemical variation is caused by environmental factors rather than genetic variability. Levels of ISSR based similarity between samples were relatively high suggesting that analyzed populations are in close genetic relations. The phytochemical levels demostrated a great variation among the populations, but there was no correlation with the genetic diversity. Results suggest that growing location is important factors influencing the overall chemical accumulation in *V. curassavica.* This study provides a solid basis for the combined use of chemical and genetic fingerprints in efficiently evaluating quality that will allow the production of plant material with homogeneous chemical profiles with appropriate pharmacological properties of *V. curassavica.*

Acknowledgements

The authors thank Fundação de Amparo a Pesquisa do Estado da Espírito Santo (FAPES), for their financial support to research. We acknowledge the Laboratory of Biomolecular Analysis (LABIOM) at Health Sciences Center from Federal University of Espírito Santo (UFES), Vitória-ES, Brazil for their support with the use of HPLC and the Nucleus of Genetics Applied to the Biodiversity Conservation UFES for support genetic analisys.

References

Alam, P., Alajmi, M. F., Arbab, A. H., Parvez, M. K., Siddiqui, N. A., Alqasoumi, S. I., . . . Basudan, O. A. (2017). Comparative study of antioxidant activity and validated RP-HPTLC analysis of rutin in the leaves of different *Acacia* species grown in Saudi Arabia. *Saudi Pharmaceutical Journal*, *25*(5), 715-723.

Alansi, S., Tarroum, M., Al-Qurainy, F., Khan, S., & Nadeem, M. (2016). Use of ISSR markers to assess the genetic diversity in wild medicinal *Ziziphus spina-christi* (L.) Willd. collected from different regions of Saudi Arabia. *Biotechnology & Biotechnological Equipment*, *30*(5), 942-947.

Arunachalam, K., & Thangaraj, P. (2013). *Evaluation of Phenolic Content, Antioxidant Activity, and Nutritional Composition of Cordia evolutior (Clarke) Gamble* (Vol. 17).

Borges, C. V., Minatel, I. O., Gomez-Gomez, H. A., & Lima, G. P. P. (2017). Medicinal Plants: Influence of Environmental Factors on the Content of Secondary Metabolites. In M. Ghorbanpour & A. Varma (Eds.), *Medicinal Plants and Environmental Challenges* (pp. 259-277). Cham: Springer International Publishing.

Borges, L. L., Alves, S. F., Sampaio, B. L., Conceição, E. C., Bara, M. T. F., & Paula, J. R. (2013). Environmental factors affecting the concentration of phenolic compounds in *Myrcia tomentosa* leaves. *Revista Brasileira de Farmacognosia, 23,* 230-238.

Brito, F. A., Aparecida, D., Silva, A. V., Diniz, L. E. C., Rabbani, A. R., Arrigoni-Blank, M. F., Blank, A. (2016). Genetic diversity analysis of *Varronia curassavica* Jacq. Accessions using ISSR markers. *Genet Mol Res,* 15(3), 1-10.

Broadley, M., Brown, P., Cakmak, I., Rengel, Z., & Zhao, F. (2012). Chapter 7 - Function of Nutrients: Micronutrients A2 - Marschner, Petra. In *Marschner's Mineral Nutrition of Higher Plants (Third Edition)* (pp. 191-248). San Diego: Academic Press.

Chen, D. X., Li, L. Y., Zhang, X., Wang, Y., & Zhang, Z. (2013). Genetic diversity in wild *Dipsacus chinensis* populations from China based on ISSR markers. *Genet Mol Res, 12*(2), 1205-1213.

De Oliveira, D. M. C., Luchini, A. C., Seito, L. N., Gomes, J. C., Crespo-López, M. E., & Di Stasi, L. C. (2011). *Cordia verbenacea* and secretion of mast cells in different animal species. *Journal of Ethnopharmacology, 135*(2), 463-468.

De Araújo, M. E. M. B., Moreira Franco, Y. E., Alberto, T. G., Sobreiro, M. A., Conrado, M. A., Priolli, D. G., . . . de Oliveira Carvalho, P. (2013). Enzymatic de-glycosylation of rutin improves its antioxidant and antiproliferative activities. *Food Chemistry, 141*(1), 266-273. doi:https://doi.org/10.1016/j.foodchem.2013.02.127

De Carvalho, P. M., Rodrigues, R. F. O., Sawaya, A. C. H. F., Marques, M. O. M., & Shimizu, M. T. (2004). Chemical composition and antimicrobial activity of the essential oil of *Cordia verbenacea* D.C. *Journal of Ethnopharmacology, 95*(2), 297-301.

Doyle, J. J. and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19 (1), 11-15.

Facanali, R., Colombo, C. A., Teixeira, J. P. F., Ming, L. C., Zucchi, M. I., & Marques, M. O. M. (2015). Genetic and chemical diversity of native populations of *Ocimum selloi* Benth. *Industrial Crops and Products, 76,* 249-257.

Feijó, E. V. R. d. S., Oliveira, R. A. d., & Costa, L. C. d. B. (2014). Light affects *Varronia curassavica essential* oil yield by increasing trichomes frequency. *Revista Brasileira de Farmacognosia, 24*, 516-523.

Frankham, R. (2003). Genetics and conservation biology. *Comptes Rendus Biologies*, 326 (1), 22-29.

Gasparino, E. C., & Barros, M. A. V. d. C. (2009). Palinotaxonomia das espécies de Cordiaceae (Boraginales) ocorrentes no Estado de São Paulo. *Brazilian Journal of Botany, 32*, 33-55.

Godwin, I. D., Aitken, E. A. B., & Smith, L. W. (1997). Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis*, *18*(9), 1524-1528.

Herrmann, K. M. (1995). The Shikimate Pathway: Early Steps in the Biosynthesis of Aromatic Compounds. *The Plant Cell, 7*(7), 907-919.

Honório, I. C. G., Bertoni, B. W., Telles, M. P. d. C., Braga, R. d. S., França, S. d. C., Coppede, J. d. S., . . . Pereira, A. M. S. (2017). Genetic and chemical diversity of *Uncaria tomentosa* (Willd. ex. Schult.) DC. in the Brazilian Amazon. *PLoS ONE, 12*(5), e0177103.

Huang, W.-Y., Cai, Y.-Z., & Zhang, Y. (2009). Natural Phenolic Compounds From Medicinal Herbs and Dietary Plants: Potential Use for Cancer Prevention. *Nutrition and Cancer, 62*(1), 1-20.

Khadivi-Khub, A., Karimi, E., & Hadian, J. (2014). Population genetic structure and trait associations in forest savory using molecular, morphological and phytochemical markers. *Gene*, *546*(2), 297-308.

Kumar, S., Paul, S., Walia, Y., Kumar, A., & Singhal, P. (2015). Therapeutic Potential of Medicinal Plants: A Review. *Journal of Biological and Chemical Chronicles*, 1, 46-54.

Li, L., Zheng, S., Brinckmann, J. A., Fu, J., Zeng, R., Huang, L., & Chen, S. (2017). Chemical and genetic diversity of *Astragalus mongholicus* grown in different eco-climatic regions. *PLoS ONE, 12*(9), e0184791.

Lipina, C., & Hundal, H. S. (2016). Modulation of cellular redox homeostasis by the endocannabinoid system. *Open Biology, 6*(4), 150276.

Liu, W., Yin, D., Li, N., Hou, X., Wang, D., Li, D., & Liu, J. (2016). Influence of Environmental Factors on the Active Substance Production and Antioxidant Activity in *Potentilla fruticosa* L. and Its Quality Assessment. *Scientific Reports*. 6:28591.

Liu, W., Liu, J., Yin, D., & Zhao, X. (2015). Influence of Ecological Factors on the Production of Active Substances in the Anti-Cancer Plant *Sinopodophyllum hexandrum* (Royle) T.S. Ying. PLoS ONE, 10(4), e0122981.

Matias, E. F. F., Alves, E. F.,Santos, B. S., De Souza, C. E. S., . . . Da Costa, J. G. M. (2013). Biological Activities and Chemical Characterization of *Cordia verbenacea* DC. as Tool to Validate the Ethnobiological Usage. *Evidence-Based Complementary and Alternative Medicine, 2013*, 7.

Matias, E. F. F., Alves, E. F., Silva, M. K. N., Carvalho, V. R. A., Medeiros, C. R., Santos, F. A. V., . . . Coutinho, H. D. M. (2016). Potentiation of antibiotic activity of aminoglycosides by natural products from *Cordia verbenacea* DC. *Microbial Pathogenesis, 95*, 111-116.

Meena, A., Bansal, P., & Kumar, S. (2009). Plants–herbs wealth as a potential source of ayurvedic drugs. *Asian Journal of Traditional Medicines*, 4, 152-170.

Michielin, E. M. Z., de Lemos Wiese, L. P., Ferreira, E. A., Pedrosa, R. C., & Ferreira, S. R. S. (2011). Radical-scavenging activity of extracts from *Cordia verbenacea* DC obtained by different methods. *The Journal of Supercritical Fluids, 56*(1), 89-96.

Minh, N. T., Khang, T. D., Tuyen, T. P., Minh, T. L., Anh, H. L., Quan, V. N., . . . Xuan, D. T. (2016). Phenolic Compounds and Antioxidant Activity of *Phalaenopsis* Orchid Hybrids. *Antioxidants, 5*(3), 31.

Moustafa, M. F., Hesham, A. E. L., Quraishi, M. S., & Alrumman, S. A. (2016). Variations in genetic and chemical constituents of *Ziziphus spina-christi* L. populations grown at various altitudinal zonation up to 2227m height. *Journal of Genetic Engineering and Biotechnology, 14*(2), 349-362.

Mraihi, F., Journi, M., Chérif, J. K., Sokmen, M., . . . Trabelsi-Ayadi, M. (2013). Phenolic Contents and Antioxidant Potential of *Crataegus* Fruits Grown in Tunisia as Determined by DPPH, FRAP, and β-Carotene/Linoleic Acid Assay. *Journal of Chemistry, 2013*, 6.

Nizio, D. A. d. C., Brito, F. d. A., Sampaio, T. S., Melo, J. d. O., Silva, F. L. S. d., Gagliardi, P. R., . . . Blank, A. F. (2015). Chemical diversity of native populations of *Varronia curassavica* Jacq. and antifungal activity against *Lasiodoplodia theobromae*. *Industrial Crops and Products, 76*, 437-448.

Pansera, M. R., Santos, A. C. A., Paese, K., Wasum, R., Rossato, M., Rota, L. D., . . . Serafini, L. A. (2003). Análise de taninos totais em plantas aromáticas e medicinais cultivadas no Nordeste do Rio Grande do Sul. *Revista Brasileira de Farmacognosia, 13*, 1, 17-22.

Parisotto, E. B., Michielin, E. M. Z., Biscaro, F., Ferreira, S. R. S., Filho, D. W., & Pedrosa, R. C. (2012). The antitumor activity of extracts from *Cordia verbenacea* D.C. obtained by supercritical fluid extraction. *The Journal of Supercritical Fluids, 61*, 101-107.

Passos, G. F., Fernandes, E. S., da Cunha, F. M., Ferreira, J., Pianowski, L. F., Campos, M. M., & Calixto, J. B. (2007). Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*. *Journal of Ethnopharmacology, 110*(2), 323-333.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9), 1231-1237.

Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, *2*(4), 152-159.

ROSS, K. A.; BETA, T.; ARNTFIELD, S. D. A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. *Food Chemistry*, v. 113, n. 1, p. 336–344, 2009.

Sampaio, B. L., Edrada-Ebel, R., & Da Costa, F. B. (2016). Effect of the environment on the secondary metabolic profile of *Tithonia diversifolia*: a model for environmental metabolomics of plants. *Scientific Reports*, *6*, 29265.

Santi, M. M., Sanches, F. S., Silva, J. F. M., Santos, P. M. L. (2014). Determinação do perfil fitoquímico de extrato com atividade antioxidante da espécie medicinal Cordia verbenacea DC. por HPLC-DAD. Revista Brasileira de Plantas Medicinais, 16(2), 256-261.

Shahraki, S., Khajavirad, A., Shafei, M. N., Mahmoudi, M., & Tabasi, N. S. (2016). Effect of total hydroalcholic extract of *Nigella sativa* and its n-hexane and ethyl acetate fractions on ACHN and GP-293 cell lines. *Journal of Traditional and Complementary Medicine, 6*(1), 89-96.

Simplicio, R. R., Waldschmidt, A. M., Amorim, M. B., Almeida, B. S., & Pereira, D. G. (2015). Genetic diversity and structure in natural populations of *Maytenus truncata* Reiss, 1861, a medicinal plant vulnerable to extractivism in Bahia State, Brazil. *Genet Mol Res, 14*(4), 18241-18248.

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (2005). Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 579*(1), 200-213.

Zhang, Q., Zhang, J., Shen, J., Silva, A., Dennis, D. A., & Barrow, C. J. (2006). A Simple 96-Well Microplate Method for Estimation of Total Polyphenol Content in Seaweeds. *Journal of Applied Phycology, 18*(3), 445-450.

Šamec, D., Durgo, K., Grúz, J., Kremer, D., Kosalec, I., Piljac-Žegarac, J., & Salopek-Sondi, B. (2015). Genetic and phytochemical variability of six *Teucrium arduini* L. populations and their antioxidant/prooxidant behaviour examined by biochemical, macromolecule- and cell-based approaches. *Food Chemistry, 186*, 298-305.

Table 1: ISSR primers, sequence, total number of locus and polymorphic loci used in molecular characterization of *V. curassavica* populations.

S. nº	Primer	Sequence	Total number of loci	Polymorphic loci	%
1	UBC809	AGAGAGAGAGAGAGAGG	6	4	66.67
2	UBC816	CACACACACACAGG	8	7	87.50
3	UBC825	ACACACACACACACACT	6	4	66.67
4	UBC826	ACACACACACACACACC	7	6	85.71
5	UBC834	AGAGAGAGAGAGAGAGCTT	7	7	100.00
6	UBC835	AGAGAGAGAGAGAGAGCTC	6	4	66.67
7	UBC840	GAGAGAGAGAGAGAGACTT	13	13	100.00
8	UBC848	CACACACACACACACAAGG	12	12	100.00
9	UBC850	GTGTGTGTGTGTGTGTCTC	7	7	100.00
10	UBC854	TCTCTCTCTCTCTCTCAGG	1	1	100.00
11	UBC855	ACACACACACACACACCTT	7	7	100.00
12	UBC856	ACACACACACACACACCTA	5	5	100.00
13	UBC861	ACCACCACCACCACCACC	7	3	42.86
14	UBC864	ATGATGATGATGATGATG	10	7	70.00
15	UBC865	CCGCCGCCGCCGCCGCCG	2	2	100.00
16	UBC866	CTCCTCCTCCTCCTCCTC	10	8	80.00
17	UBC880	GGAGAGGAGAGGAGA	9	6	66.67
18	UBC888	CGTAGTCGTCACACACACACACA	4	4	100.00
19	ISSRCR-8	GTGGTGGTGGTGGTGGC	4	1	25.00
20	ISSRCR-2	CACACACACACACACAAG	5	2	40.00
Total			109	110	79.89

Table 2: Similarity matrix using Jaccard´s coefficient among *Varronia curassavica* populations.

	MG	ST	DM	MF	ANC	ARA	JAG	JAC	GUA	VIA
MG	1									
ST	0.600	1								
DM	0.617	0.697	1							
MF	0.537	0.622	0.720	1						
ANC	0.548	0.613	0.611	0.667	1					
ARA	0.459	0.455	0.527	0.614	0.663	1				
JAG	0.433	0.529	0.556	0.552	0.530	0.531	1			
JAC	0.414	0.455	0.535	0.515	0.467	0.451	0.646	1		
GUA	0.441	0.491	0.486	0.467	0.491	0.422	0.589	0.613	1	
VIA	0.493	0.402	0.459	0.469	0.538	0.481	0.467	0.478	0.505	1

ANC: Anchieta; ARA: Aracruz; DM: Domingos Martins; GUA: Guarapari; JAC: Jacaraípe; JAG: Jaguaré; MF: Marechal Floriano; MG: Manguinhos; ST: Santa Teresa; VIA: Viana.

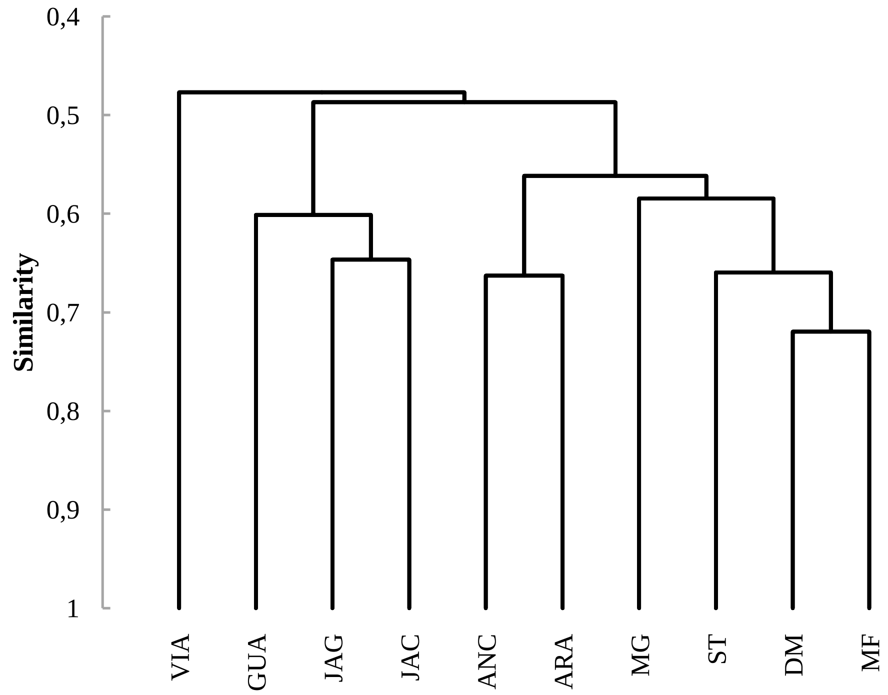


Figure 1: Dendrogram based on UPGMA of Jaccard's similarity matrix represents the genetic relationship among ten *V. curassavica* populations. ANC: Anchieta; ARA: Aracruz; DM: Domingos Martins; GUA: Guarapari; JAG: Jaguaré; JAC: Jacaraípe; MF: Marechal Floriano; MG: Manguinhos; ST: Santa Teresa; VIA: Viana.

Table 3: Content of phenolic compounds and antioxidant activity of ten *Varronia curassavica* populations.

	ANC	ARA	DM	GUA	JAC	JAG	MG	MF	ST	VIA	TROLOX
TPC (mg GAE.g^{-1}d.w.)	71.41±0.59^{ef}	93.00±3.93^{cde}	156.49±3.93^{b}	137.60±3.65^{bc}	78.56±0.40^{def}	25.22±0.96^{f}	78.87±0.99^{cdef}	86.65±1.32^{cde}	218.40±10.25^{a}	133.95±0.23^{bcd}	
TFC (mg RE.g^{-1}d.w.)	121.67±1.33^{e}	250.00±1.84^{b}	174.44±1.89^{cd}	159.44±2.30^{cde}	131.67±2.25^{e}	263.61±2.71^{b}	138.61±2.30^{de}	353.33±4.78^{a}	183.61±2.15^{c}	143.06±2.07^{de}	
TTC (mg TA.g^{-1}d.w.)	56.46±0.35^{ef}	104.71±0.84^{c}	114.43±2.25^{c}	134.98±1.02^{b}	132.68±0.95^{b}	79.79±0.57^{d}	30.99±0.40^{g}	53.50±0.74^{f}	71.15±0.42^{de}	194.59±1.05^{a}	
ABTS IC50 (mg.mL^{-1})	424,72±7.19^{a}	294.73±6.66^{b}	243.44±2.78^{b}	415.05±1.84^{a}	274.37±4.97^{b}	408.41±1.74^{a}	483.00±4.61^{a}	308.07±1.19^{b}	107.57±2.16^{cd}	164.03±1.63^{c}	78.54±2.90^{d}

ANC: Anchieta; ARA: Aracruz; DM: Domingos Martins; GUA: Guarapari; JAC: Jacaraípe; JAG: Jaguaré; MF: Marechal Floriano; MG: Manguinhos; ST: Santa Teresa; VIA: Viana; TROLOX: antioxidant standard. All the values are expressed as mean ± SE (n=3). SE: standard error; Total phenols content (TPC); Total tannins content (TTC), Total flavonoids content (TFC). Means in the rows with different letters are significantly different, ANOVA, test-Tukey (p< 0.05).

Table 4: HPLC analysis data of the *Varronia curassavica* populations.

	ANC	ARA	DM	GUA	JAC	JAG	MF	MG	ST	VIA
Chlorogenic acid (µg/mL)	3.05±0.001 ^{de}	3.29±0.007 ^{c}	2.84±0.002 ^{f}	3.13±0.016 ^{cd}	3.98±0.001^{a}	3.06±0.001 ^{de}	2.88±0.003 ^{ef}	3.77±0.036^{b}	3.27±0.004 ^{c}	3.75±0.011^{b}
Rutin (µg/mL)	15.01±0.02^{f}	24.72±0.10^{e}	16.25±0.53^{f}	48.14±0.46^{c}	35.42±0.05^{d}	5.73±0.01^{g}	7.73±0.03^{g}	ND	54.88±0.27^{b}	71.63±0.52^{a}
Quercetin (µg/mL)	ND	ND	ND	4.26±0.06^{a}	3.68±0.23^{a}	ND	ND	ND	ND	ND
Luteolin (µg/mL)	3.20±0.01^{a}	ND	2.50±0.15^{ab}	2.48±0.01^{ab}	ND	ND	ND	2.01±0.01^{b}	ND	2.63±0.02^{ab}
Galic acid (µg/mL)	2.88±0.014^{cde}	2.93±0.009^{cd}	2.91±0.003^{cde}	5.17±0.061^{b}	2.41±0.005^{e}	2.89±0.012 ^{cde}	2.47±0.012^{de}	3.09±0.002^{c}	2.71±0.018^{cde}	6.55±0.073^{a}

ANC: Anchieta; ARA: Aracruz; DM: Domingos Martins; GUA: Guarapari; JAC: Jacaraípe; JAG: Jaguaré; MF: Marechal Floriano; MG: Manguinhos; ST: Santa Teresa; VIA: Viana. ND: not detect. All the values are expressed as mean ± SE (n=3). SE: standard error. Means in the rows with different letters are significantly different, ANOVA, test-Tukey (p< 0.05).

Table 5: Chemical analysis data (A) soil and (B) leaves of *Varronia curassavica* populations.

A

Location	Manganese [(1)] ($g.kg^{-1}$)	Potassium [(1)] ($g.kg^{-1}$)	Phosphorus [(1)] ($g.kg^{-1}$)	Magnesium [(2)] ($g.dm^{-3}$)	Iron [(1)] ($g.kg^{-1}$)	Zinc [(1)] ($g.kg^{-1}$)	Organic matter [(3)] ($dag.kg^{-1}$)	pH
ANC	0.022	0.097	0.11	0.00385	0.402	0.0057	1.7	6.8
ARA	0.076	0.052	0.124	0.005	0.23	0.0171	1.6	6.6
DM	0.018	0.02	0.007	0.002	0.1	0.0042	2.5	5.8
GUA	0.01	0.027	0.012	0.002	0.064	0.008	2.4	6.5
JAC	0.009	0.04	0.018	0.01	0.02	0.0004	3.7	7.4
JAG	0.118	0.08	0.055	0.004	0.076	0.0135	2.2	6.2
MF	0.008	0.051	0.004	0.0005	0.034	0.0012	1.2	5.2
MG	0.014	0.043	0.008	0.0055	0.02	0.0005	3.3	8.2
ST	0.198	0.25	0.002	0.023	0.103	0.0004	2.1	8.6
VIA	0.186	0.22	0.163	0.0065	0.172	0.012	2.1	7.2

ANC: Anchieta; ARA: Aracruz; DM: Domingos Martins; GUA: Guarapari; JAC: Jacaraípe; JAG: Jaguaré; MF: Marechal Floriano; MG: Manguinhos; ST: Santa Teresa; VIA: Viana. (1)Extracted with HCI 0.05 mol.L-1 and H2SO4 0.025 mol.L-1, (3)extracted with KCI 1mol.L-1, (4)Oxidation with: Na2Cr2O72H2O 4 mol.L-1 and H2SO4 10 mol.L-1.

B

Location	Nitrogen ($g.kg^{-1}$)	Phosphorus ($g.kg^{-1}$)	Potassium ($g.kg^{-1}$)	Calcium ($g.kg^{-1}$)	Magnesium ($g.kg^{-1}$)	Sulfur ($g.kg^{-1}$)	Iron ($g.kg^{-1}$)	Zinc ($g.kg^{-1}$)	Copper ($g.kg^{-1}$)	Manganese ($g.kg^{-1}$)	Boron ($mg.kg^{-1}$)
ANC	32.62	1.80	25.00	41.49	3.75	2.32	0.417	0.05	0.023	0.09	27
ARA	31.57	2.11	9.38	26.08	5.44	3.54	0.537	0.055	0.016	0.064	57
DM	30.31	2.06	16.25	25.64	4.38	3.54	0.667	0.086	0.02	0.124	33
GUA	31.64	1.93	18.75	35.57	3.13	2.66	0.247	0.087	0.02	0.052	19
JAC	30.52	2.57	15.63	48.67	6.25	2.31	0.001259	0.017	0.008	0.04	31
JAG	40.88	2.29	18.75	29.76	4.38	3.13	0.305	0.031	0.006	0.078	49
MF	30.73	2.23	5.25	42.62	6.25	3.16	1.196	0.03	0.006	0.071	55
MG	30.45	2.36	15.63	32.40	4.00	3.44	1.157	0.106	0.015	0.109	31
ST	27.65	3.92	9.38	33.38	4.31	4.45	0.564	0.023	0.014	0.365	37
VIA	39.83	3.13	18.13	26.69	4.13	4.33	0.577	0.045	0.02	0.154	39

ANC: Anchieta; ARA: Aracruz; DM: Domingos Martins; GUA: Guarapari; JAC: Jacaraípe; JAG: Jaguaré; MF: Marechal Floriano; MG: Manguinhos; ST: Santa Teresa; VIA: Viana. (1) Hot acid digestion, (2) Nitro-perchloric digestion, (3) Dry digestion.

Tabela 6: Pearson correlation analysis between phenolic compounds content, antioxidant activity and leaves nutrients.

	TPC	TFC	TTC	ABTS	N	P	K	Ca	Mg	S	Fe	Zn	Cu	Mn	B
TPC	1														
TFC	-0.220	1													
TTC	0.241	-0.285	1												
ABTS	-0.634	-0.008	-0.468	1											
N	-0.457	0.058	0.396	0.131	1										
P	0.585	-0.040	0.181	-0.751	-0.065	1									
K	-0.239	-0.109	0.291	0.130	0.435	-0.350	1								
Ca	-0.265	-0.458	-0.304	0.329	-0.338	-0.109	0.008	1							
Mg	-0.271	-0.162	-0.168	0.026	-0.198	0.049	-0.650	0.413	1						
S	0.586	0.234	0.193	-0.651	0.076	0.732	-0.425	-0.684	-0.116	1					
Fe	-0.147	0.071	-0.253	-0.049	-0.407	0.085	-0.459	0.496	0.688	-0.102	1				
Zn	0.101	0.468	0.023	0.125	-0.169	-0.453	0.269	-0.419	-0.577	-0.016	-0.093	1			
Cu	0.409	-0.173	0.353	-0.195	-0.079	-0.156	0.527	-0.361	-0.697	0.099	-0.482	0.561	1		
Mn	0.731	-0.001	-0.075	-0.656	-0.252	0.854	-0.252	-0.256	-0.233	0.745	-0.147	-0.212	0.128	1	
B	-0.286	0.204	-0.247	0.094	0.233	0.084	-0.653	-0.250	0.624	0.339	0.138	-0.434	-0.555	-0.032	1

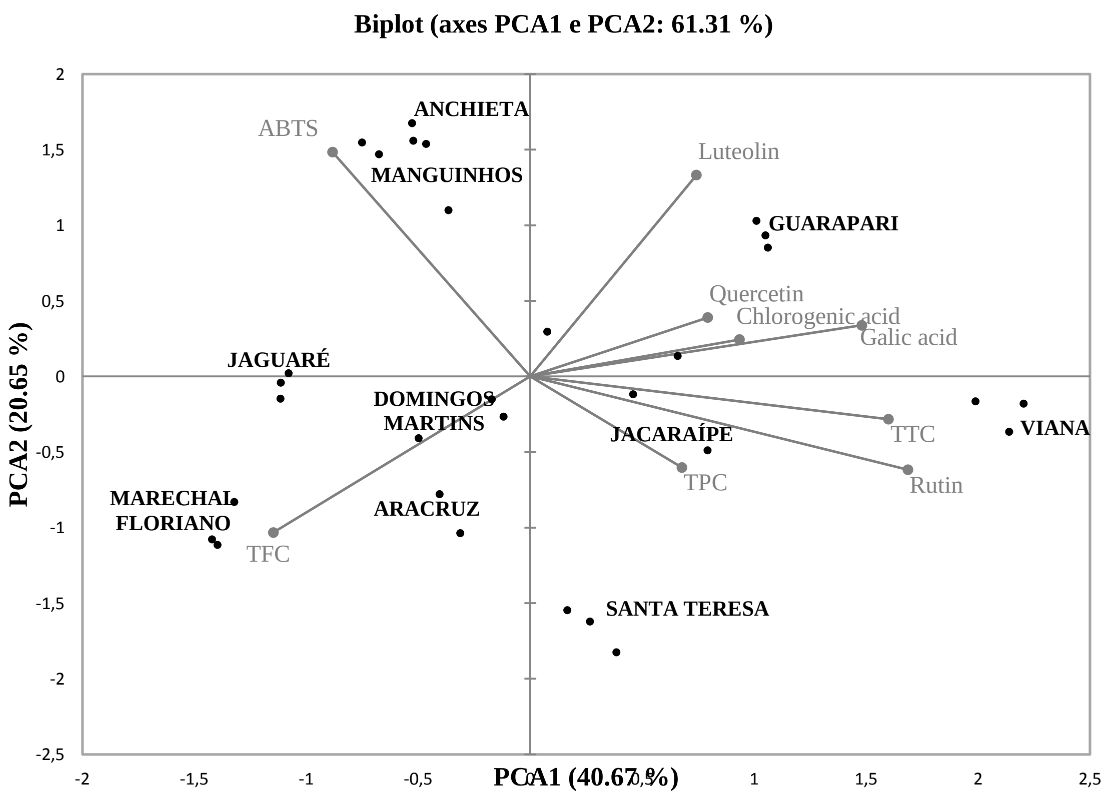


Figure 2: Principal components analysis (PCA) of the phytochemical profiles of *V. curassavica* populations and their antioxidant activity.

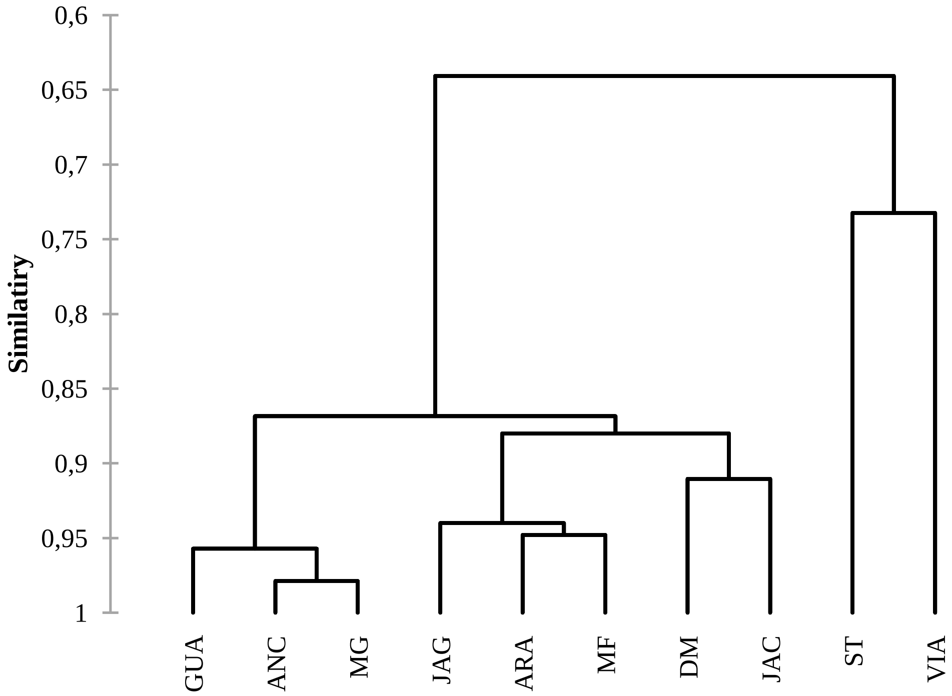


Figure 3: Hierarchical clusters analysis based on phytochemical content and antioxidant activity of HAE from ten *V. curassavica* populations. ANC: Anchieta; ARA: Aracruz; DM: Domingos Martins; GUA: Guarapari; JAG: Jaguaré; JAC: Jacaraípe; MF: Marechal Floriano; MG: Manguinhos; ST: Santa Teresa; VIA: Viana.

CAPÍTULO 2 – ASSESSMENT OF GENETIC AND PHYTOCHEMICAL VARIATIONS AMONG *Momordica charantia* L. NATURAL POPULATIONS

Após revisão da língua inglesa, e considerações da banca examinadora, este artigo será submetido à revista: Arabian Journal of Chemistry (ISSN: 1878-5352).

Assessment of genetic and phytochemical variations among M*omordica charantia* L. natural populations.

Mainã Mantovanelli da Mota*, Anny Carolyne da Luz[a], Irany Rodrigues Pretti[a], Jean Carlos Vencioneck Dutra[a], Maria do Carmo Pimentel Batitucci[a].

[a]Departamento de Ciências Biológicas, Centro de Ciências Humanas e Naturais, Universidade Federal do Espírito Santo, Vitória, Brazil.

*Corresponding author: Mainã Mantovanelli da Mota

Departamento de Ciências Biológicas

Laboratório de Genética Vegetal e Toxicológica

Universidade Federal do Espírito Santo

Av. Fernando Ferrari 514, Goiabeiras, 29075-910, Vitória, ES, Brazil

Phone: 55 27 998311735

Email address: maina.mantovanelli@gmail.com

Abstract

Momordica charantia L. belongs to the family Curcubitaceae and is commonly known as bitter melon or bitter gourd, has several medicinal properties such as: antidiabetic, carminative, antihelmintic, antimicrobial, antiviral, anticancerigenous and antioxidant. The objective of this study was determine the genetic and chemical variability between seven populations of *M. charantia* from Espírito Santo, Brazil, to investigate possible correlation between genetic and enviromental factors in the accumulation of bioactive compounds. Genetic diversity among *M. charantia* populations were assessed by ISSR markers and analysis of chemical components of extracts from these populations as well as its antioxidant actions were performed in order to determine its phytochemical variability. Were generated 119 amplicons from 18 inter simple sequence repeats (ISSR) primers producing an average of 56.35% polymorphism. The UPGMA cluster analysis based on the ISSR data resulted in the aggregation of *M. charantia* populations into two main clusters and levels of similarity among samples were relatively high. Cluster analysis and principal component analysis were used to characterize the samples according to phenolic compounds content and their antioxidant activity. The results showed low phytochemical variability among populations, then that the variation of the chemical composition the samples was influenced both for enviromental and genetic factors.

Keywords: Bitter gourd, ISSR, phenolics compounds, antioxidant activity

1. Introduction

In developing countries the majority of the population continues to use traditional medicine. For many of people this practice is the main source of health care, and sometimes the only one source of care (WHO, 2013). The presence of bioactive compounds in some plants is mainly related to the content of secondary metabolites, these are organic compounds the great majority of which do not to participate directly in growth and development. Among these secondary metabolites, include the phenolics compounds that acts as effective antioxidant and their beneficial effect is attributed to their donating electrons able to scavenging and neutralize reactive oxygen species (ROS) (Kumar et al., 2015; Rice-Evans et al., 1997). Cellular metabolism produced ROS that have crucial roles in maintaining of their redox homeostasis by modulating a numerous process in the cell. However, when at high concentrations, oxidative stress is generated and this consists of imbalance between the formation of reactive oxygen species and antioxidant defense mechanisms leading to damage all cell structures, which is associated with cancer, autoimmune disorders, cardiovascular and neurodegenerative diseases (Lipina and Hundal, 2016; Pham-Huy et al., 2008).

In the last years, researches has focused on scientific evaluation of natural products of plants origin and *Momordica charantia* L. has been frequently studied to evaluate the effect of its use in traditional medicine.

*M. charantia (*Cucurbitaceae) is a species herbaceous popularly known as bitter gourd or bitter melon. The plant grows in tropical areas including East Africa, South America, Asia, the Caribbean, and India (Dinakaran et al., 2010; Dandawate et al., 2016). Some of its common uses in most countries are for the treatment of diabetes and colics (Anilakumar et al., 2015). Furthermore, pharmacological studies have demonstrated several properties of *M. charantia* include antioxidant, antitumor, anti inflammatory, antibacterial, antifungal, hepatoprotective, and neuroprotective effects (Wei et al., 2013; Kwatra et a., 2013; Chao et al., 2014; Costa et al., 2010; Santos et al., 2010; Lu et al., 2014; Malik et al., 2011).

Researches have demonstrated that the total content and biological activities of secondary metabolites in plants are frequently affected by abiotic factors, such as light intensity, photoperiod, temperature, water availability, carbon dioxide levels, soil composition (Borges et al., 2013; Liu et al., 2015; Sampaio et al., 2016; Liu et al., 2016) and also by genetic factors (Sampaio et al., 2015; Moustafa et al., 2016). Changes in secondary metabolities amounts influence directly the quality of the plant for medicinal application (Borges et al., 2013). Then, the present study evaluated genetic and phytochemical variability of *M. charantia* populations in order to determine factors that influence an accumulation of polyphenolic compounds and their antioxidant activity. To assess the genetic diversity among and within seven wild populations of *M. charantia* collected from diferents locations in the southeastern Brazil, we used ISSR markers. For determine the phytochemical diversity it was mensured the total phenol, total flavonoid, total tannins and individual phenolic acids contents of differents extracts of *M. charantia* and it was also evaluated their antioxidant activitie by ABTS assay.

2. Material and methods

2.1. Plant material

The aerial parts of *Momordica charantia* L. were arbitrarily selected and collected at seven localities of Southeastern Brazil region: Anchieta (ANC) (20°48'25.4"S; 40°38'48.3"W), Cariacica (CAR) (20°19'20.3"S; 40°22'20.6"W), Domingos Martins (DM) (20°22'34.6"S; 40°34'56.8"W), Fundão (FUN) (19°56'09.5"S; 40°25'23.2"W), Jaguaré (JAG) (18°57'19.6"S; 40°08'43.5"W), Praia Grande (PG) (20°02'18.4"S; 40°11'22.9W), Santa Teresa (ST) (19°56'28.0"S, 40°35'07.0"W), during June to September of 2015. Voucher specimen (41241) was deposited in the Central Herbarium of Universidade Federal do Espírito Santo/VIES, Brazil. For each of seven populations, the soil and leaf samples were collected and their chemichal compositions were analized, as presented in Table 1A and 1B respectively. Leaves were also collected and immediately cooled for DNA extraction from leaf tissues.

2.2 DNA extraction

The genomic DNA was extracted from leaf samples, based on the protocol described by Doyle (1987) with modifications.

Young leaves were ground to a fine powder in liquid nitrogen in presence of polyvinylpolypyrrolidone (PVPP). The powder (50 mg) was placed in 1.5 mL microtubes containing 1 mL of CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH 8, 20 mM EDTA pH 8), 20 µL of β-mercaptoethanol and 10 µL of proteinase-K, microtubes were then vortexed for 10 s and incubated at 65ºC for 30 minutes. After, 500 µL chloroform:isoamyl alcohol (24:1 v/v) was added to the solution and gently mixed for 1 min and centrifuged for 5 min at 12000 rpm, the supernatant was transferred to a new tube following the addition of 500 mL chloroform-isoamylalcohol (24:1) and this stage was repeated once. The supernatant was treated with RNAse A (10 µg/µL), to 37 ºC for 30 min. Cold isopropanol was added to the supernatant (0.7 of the total volume of supernatant collected), samples were gently mixed by inversion and centrifuged at 12,000 rpm for 5 min. The liquid solution was then released and DNA washed with 70% etanol, the pellet was set to dry at room temperature and was ressuspended in 100 µL Tris–EDTA pH 8.0 (TE Buffer). This solution was then stored at –20ºC. The quantity and purity of the extracted DNA were evaluated spectrophotometrically by NanoDrop 3300 (Termo Scientific). The purity of the DNA was also confirmed by gel electrophoresis system using 1.0% agarose stained with GelRedTM (BIOTIUM^{TM}).

2.3 ISSR analysis

Eighteen ISSR primers (Eurofins, Operon Technologies, Louisville, KY, USA) (Table 2) were selected for this study based on the presence of clear, reproducible and polymorphic amplified bands. The reactions were carried out in a Veriti® 96-Well Thermal Cycler (Apllied Biosystems^{TM}). Reactions without DNA were used as negative controls. Each 25 µL reaction volume contained 25 ng DNA template , 5 µL de 5x reaction buffer, 2.5 µL 25 mM $MgCl_2$, 0.5µL 10mM dNTP, Taq DNA polymerase (1.25 U) and 0.5 µL (10 µM) of specific primer. The thermocycler was programmed for an initial denaturation step of 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, annealing at 55 ºC for 2 min and elongation at 72°C for 2 min and a final extension at 72°C for 5 min and a hold temperature of 4°C at the end. PCR products were electrophoresed on 2% agarose gel in 1X TAE buffer at 200 V for 60 min and were stained with GelRedTM (BIOTIUM^{TM}). Gel with amplification fragments were visualized and photographed by gel documentation system (Loccus Biotecnologia, Brazil). 100 pb molecular ladder was used as molecular marker to know the size of the fragments.

2.3. *Extract preparation*

The powder of dried aerial parts of *M. charantia* were submitted to exhaustive extraction with ethanol-water solution (95%) in a ratio 5:1 (v/w) by 72h, protected from light, at room temperature. This process was repeated twice with same power to extract the maximum of constituents. After the maceration, resulting solutions were filtered and the solvent recovered in a rotatory evaporator under vacuum to obtain the crude hydroalcoholic extracts (HAE) from each population of *M. charantia*.

2.4. Polyphenols analysis

2.4.1. Total phenolic content (TPC)

Folin–Ciocalteu method (Zhang et al. 2006) was used to determine the total phenolic content of the extract. 20 µL ethanol solution of HAE (500µg.mL^{-1}) was mixed to 100 µL of Folin-Ciocalteu reagent diluted in distilled water (1:10). The mixture was incubated for 5 min at room temperature, followed by the addition of 80 µL of $NaCO_3$ (7,5%) solution and incubated in the dark at 25 °C for 2 h. The reaction mixture was measured by absorbance at 750 nm in spectrophotometric microplate reader (Epoch Microplate Spectrophotometer – BioTek). TPC was expressed as milligrams gallic acid equivalent per gram of dry weight of the extract (mg GAE.g^{-1} d.w.) from a calibration curve with gallic acid. The concentrations of gallic acid were 12.5, 25, 50, 100, 250, 500 and 1000 µg.mL^{-1}. Ethanol was used as a blank. The analysis was run in triplicate and conducted for the crude HAE extracts from all locations.

2.4.2. Total tannins content (TTC)

The total tannin content (TTC) was determined by Folin-Denis method which is based on the reducing power of the phenolic hydroxyl group of tannins (Pansera et al., 2003) with a few modifications. A volume of 500 µL of ethanol solution of HAE (500 µg.mL^{-1}) was added to 500 µL of of Folin-Denis reagent. The solution was mixed gently, fter 3 min, 500 µL of Na_2CO_3 solution (8%) were added. The preparation was mixed and allowed to stand for 2 hours. Subsequently, the material was centrifuged at 2000 rpm for 5 min and the absorbance measured at 725 nm. To construct the standard curve, different concentrations of tannic acid (7.8, 15.62, 31.25, 62.5, 125, 250 and 500 µg.mL^{-1}). were used and the result was expressed as tannic acid equivalents per gram of dry weight (mg TA.g^{-1} d.w.). Ethanol was used as blank. For each sample, three replications were performed.

2.4.2. Total flavonoid content (TFC)

Total flavonoid content (TFC) was estimated according to the spectrophotometric method with aluminum chloride (Mraihi et al., 2013). In sealed tubes, 1.5 mL of a 2% methanol solution of $AlCl_3{\cdot}6H_2O$ was added to 0.5 mL of methanol solution of HAE 500 µg.mL^{-1} and then kept in dark for 10 min at room temperature. After 10 min, the absorbance of the solutions was read at λ = 430 nm using the rutin solution for comparison, methanolic $AlCl_3$ was used as blank and samples were analyzed in 3 replicates. The TFC in extract was expressed as rutin equivalent per gram of dry weight (mg RE.g^{-1} DW).

2.5. Free radical scavenging activity by ABTS assay

ABTS (2,2'-azinobis-3- ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay was carried out according to the method reported by Re et al. (1999), with modifications. The ABTS radical cation ($ABTS^{\bullet+}$) was generated by mixing the following solutions; 5 mL of an ABTS aqueous solution (7 mM) with 88 µL potassium persulfate (2.5 mM). The reagent was kept in darkness at room temperature for 16 h to complete the reaction after which this solution was diluted with ethanol to get the ABTS working solution having absorbance of 0.70. Then, 200 µL of $ABTS^{\bullet+}$ solution were mixed with 40 µL of the test solution (15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg.mL^{-1}), samples of the HAE and standard. Trolox was used as an antioxidant standard. The absorbance was measured in 734 nm, after 6 min incubation in the dark. The control was prepared using 40 µL mL of ethanol and 200 µL of ABTS radical solution. The test was performed in triplicate and the scavenging activity was determined by the following formula: % scavenging of $ABTS^{\bullet+}$ = [(Abs0 – Abs1) / Abs0] x 100, where Abs0 = absorbance of control and Abs1 = absorbance of the sample. Results were expressed as IC50 value ($\mu g.mL^{-1}$).

2.6 HAE *Extract fractionation*

In order to prepare the fractions were used different solvents with increasing polarity (Shahraki et. al, 2016). The obtained HAE from *M. charantia* (0.5 g) was suspended in 50 mL of water and transferred to a decanter funnel. The n-hexane (50 mL) solvent was added to the funnel, and the hexanic fraction was then extracted. In the next step, the remainder of the solvent in the decanter funnel was combined with dichloromethane solvent, and this fraction was then extracted. Subsequently, the remaining solvent from the previous steps was mixed with ethyl acetate, and the ethyl acetate fraction was extracted. At the end, n-butanol solvent was added to the funnel for obtain butanolic fractions. All fractionates were collected separately and dried at room temperature.

2.7 High-performance liquid chromatography (HPLC) analysis

High performance liquid chromatography (HPLC) analyses were performed using on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) with solvent delivery system of two pumps (Model LC–20AT, Prominence Liquid Chromatograph, Shimadzu), column oven (Model CTO-20A), UV detector (Model SPD–20A, Prominence Diode Array Detector, Shimadzu) and degasifier (DGU-20A5). Data collection and integration were accomplished using LC Solutions, 1.25 version software. Separation was achieved with on a C18 reverse phase column ZORBAX Eclipse XDB®, Agilent (4.6 mm x 250 mm, 5µm) equipped with a guard column of same packing material.

HPLC assay for flavonoids detection was used the methanol solutions of ethyl acetate and was carried out using an isocratic elution with a flow rate of 1 mL/min, the column temperature was set to 40ºC, a mobile phase of methanol and 2% phosphoric acid (pH 2.60) (40% : 60% v/v) and a detection wavelength of 350 nm. The injection volume was 20 µL of solution. The total run time was 20 minutes for each injection. Identification of flavonoids was performed by comparing their retention times (RT) and spectra with those of known standards. The standards used were chlorogenic acid, rutin, quercetin and luteolin (Sigma-Aldrich, USA). Chromatographic peaks in the samples were identified on the basis of retention time of the standards. Amounts of flavonoids were calculated using a calibration curve equation for each standard. The data are presented as averages of triplicate measurements.

The methanol solution of dichloromethane fraction was used to detect tannin. Tannin detection was performed using the mobile phase with 0.1% acid formic solution (Solvent A) and methanol (Solvent B), the flow rate was adjusted to 0.7 mL/min and the column was thermostatically controlled at 40°C. A gradient elution was carried by varying the proportion of solvent B to solvent A as follows: 0–15 min, 15% to 33% B; 15–17 min, 33% to 15% B; 17–22 min, 15% B to wash and equilibrate of the column. The injection volume was kept at 20 µl and a detection wavelength of 270 nm. The quantification of the sample was measured by comparing their retention times and spectra with the standard, gallic acid (Vetec, Brazil). The data were reported in triplicate.

2.7. Statistical analysis

Genetic variability analysis was performed with XLSTAT for Windows (Addinsoft, New York, USA) program. The reproducible and well-resolved fragments were scored as present (1) or absent (0) as binary data for each of the ISSR markers. From these data, the genetic proximity was estimated based on Jaccard similarity coefficient. Clustering analysis was conducted using the Unweighted Pair Group Method Arithmetic Average (UPGMA) method. For phytochemical analysis and antioxidant assay were performed ANOVA followed *test t* and the differences between measurements were considered to be significant at $p < 0.05$, using ASSISTAT version

7.7 beta software (Assistat Software, Campinas, São Paulo, Brazil.). Principal component analysis (PCA), Pearson Linear Correlation and Hierarchical cluster analysis (HCA) were performed using XLSTAT for Windows (Addinsoft, New York, USA) in order to visualize relationships between the *M. charantia* samples in each collection area.

3. Results

3.1. Polymorphism revealed by ISSR markers

The DNA based markers provide an important tool for estimation of genetic struture of populations and ISSR markers have been shown to be efficient in several studies of the analysis of genetic variability in medicinal plants (Liu et al 2011; Alansi et al., 2015; Kumar et al., 2016; Natarajan et al., 2018). In the present study it was evaluated the genetic structure of natural populations of *M. charantia* to compare with their chemical variability. Genetic diversity analysis tested in the seven populations *M. charantia* using 18 polymorphic ISSR primers generated 119 bands. Of these, 67 bands (56.35%) were found to be polymorphic (Table 1). Maximum number of fragments (10 bands) was found with the primers UBC840, UBC864 and UBC880, whereas the smallest number (2 bands) was generated by primers UBC888 and UBC889. Among here used primers, ISSRCR-2 appeared as the most informative one resulting in 100% of polymorphic fragments, followed by UBC848 resulting in 87.50% of polymorphic fragments. The highest number of ISSR fragments was obtained for DM (95), whereas the lowest (71) was for ANC. At the populations level, the highest percentage of polymorphism loci (21.00%) was observed in ANC while the lowest (5.88%) was observed in PG (data not shown). The similarity coefficients based on ISSR marker scoring data calculated using Jaccard's coefficient ranged from 0.651 to 0.851 with an average of 0.77 (Table 2). Based on proximity matrix of Jaccard´s coefficients the highest similarity genetic was observed between PG and JAG populations. On the other hand, the FUN and ANC showed the lowest genetic similarity. Levels of similarity between samples were relatively high suggesting close genetic relations.

The dendrogram obtained for *M. charantia* samples constructed according to UPGMA cluster analysis using Jaccard's coefficients shows two main clusters, first with sample from ANC and the second cluster was divided into two sub-clusters, the first one included FUN, CAR and DM and the second one contained three populations ST, PG and JAG.

3.2. Phytochemical content and antioxidant analysis

M. charantia ethanol extracts have shown a great variability in contents polyphenolic groups. The total phenolic content (TPC) of seven plant extracts is shown in Table 3. The TPC ranging from 18.87 to 44.27 mg GAE.g^{-1} and the maximum amount of TPC was recorded with the extract of the samples JAG, PG and FUN. The total flavonoid content (TFC) (Table 3) was higher in JAG (575.28 mg RE.g^{-1}) and ANC had the lowest TFC (46.67 mg RE.g^{-1}) among the seven extracts estimated. Total tannin content (TTC) ranges from 12.36 to 30.39 mg TA.g^{-1} and the highest TTC was found in samples JAG, FUN and CAR. Considering populations, the highest amount of phenolic compounds has been found in hydroalcoholic extract of plants grown at locality Jaguaré and it was observed a high variation among the localities.

In the present investigation, the commonly accepted assay ABTS was used for the evaluation of antioxidant activity of *M. charantia* extracts of the differents populations. Percentage scavenging effect of ABTS radicals versus concentration of extracts and standards were plotted (Figure 2). In table 4, scavenging effect of ABTS radicals are shown as IC50 values (μg.mL^{-1}). A lower IC50 value indicates a higher ability of the extract to act as a scavenger, while higher IC50 value indicates lower ability of the extract to achieve 50% scavenging activity. For the ABTS assay, the scavenging activity of samples JAG and FUN was significantly higher among the populations (Table 3).

3.3. HPLC analysis

Phenolic compounds (chlorogenic acid, rutin, quercetin, luteolin and gallic acid) were identified and quantified in more details by HPLC-DAD in samples of *Momordica charantia* (Table 5). The compounds were identified by comparison of the retention time and UV spectra of the peaks with those of the available reference standards, chlorogenic acid (RT = 2.954 min), rutin (RT = 5.502 min), quercetin (RT = 14.111 min) and luteolin (RT = 17.200 min). For tannins quantification, were used the spectra and retention time of gallic acid (RT = 7.558 min).

The ethyl acetate fraction of *M. charantia* was used to quantification of chlorogenic acid and flavonoids (Table 4). HPLC analysis demonstrated that the chlorogenic acid occurs in all populations and the content was higher in samples JAG (3.07 $\mu g.mL^{-1}$) and CAR (3.00 $\mu g.mL^{-1}$). The content of total rutin ranged from 5.10 $\mu g.mL^{-1}$ to 29.72 $\mu g.mL^{-1}$ and higher levels was observed in sample FUN. In this study quercetin and luteolin was detected only in one population CAR, 4.00 $\mu g.mL^{-1}$ and 3.47 $\mu g.mL^{-1}$, respectively. The quantification of tannins was performed from the dichloromethane fraction of HAE of the *M. charantia* and the highest galic acid level was found in sample CAR.

3.4 Multivariate analysis

The correlation of pearson was performed to verify the relation of the phenolic compounds with the antioxidant activity, as well as with the chemical components present in the leaves of the studied plants (Table 5). This analysis demonstrated high significant correlation between TPC and TFC; TPC and ABTS; ABTS and Boron; TPC and Boron; TFC and Zinc (Table 6). It was observed that the phenolic contents correlates well with ABTS assay. The correlation coefficients of TPC with ABTS is -0.891 confirming that phenolic compounds are likely to contribute to radical scavenging activity of these plant extracts. Our results corroborate this since the extract samples JAG and FUN present higher a radical scavenging activity mensured by ABTS assays and are among the samples with the highest values of TPC and TTC. Therefore, the antioxidant activity of these populations *M. charantia* is determined by the total phenolic content.

In the present study was demonstrated by Pearson's correlation that boron (B), present in the leaves, has a high correlation with TPC and ABTS and the zinc (Zn) present in the leaves is correlated with TFC.

To evaluate the likely similarities and relationships among *M. charantia* populations studied, the Hierarchical Cluster Analysis (HCA) was performed based on antioxidant activity and phytochemical content. The HCA results are presented in the form of a dendrogram (Fig. 4). The *M. charantia* populations were classified into two main groups. First cluster divided into two sub-clusters, the first sub-cluster included ST, DM and ANC and the second subcluster included PG, CAR, and FUN. The second cluster contained only 1 population JAG. Similarly, PCA analysis, the ST and VIA samples formed a single group characterized by higher contents of rutin and TPC. Comparison of clusters generated through phytochemical and genetic diversity, showed different pattern of grouping among the *M. charantia* polutations. However genetic and chemical dissimilarity is very low, indicates that phytochemical and antioxidant variations among populations are influenced by genetics factors as well as environmental conditions.

PCA was performed to determine differences among samples based on their metabolite profiles and antioxidant activity (Fig. 5). The PCA is probably the most common calculation used in processing of multivariate data. The objective of PCA is reduction of dimensionality of a data set with a large number of correlated variables or traits and it has proven to be a valuable tool to understand relationships between characteristics as well as between populations (Jolliffe, 2002; Khadivi-Khub et al., 2014).

In this study, two principal component (PC) of the score plot explained 72.26% of the total variance. Results of PCA revealed that the first componente (PC1) accounted for 48.05% of variance and the second componente (PC2) for 24.22% variance. Analysis of the data indicated that gallic acid, chlorogenic acid, quercetin, luteolin, TTC, TPC and TFC contributed most and positively to the PC1, with a satisfactory degree of correlation, while the correlation with the ABTS were inverse. The PC2 was mainly related to luteolin, quercetin and ABTS. From this biplot, it was observed that JAG sample showed positive correlation with the amount TPC and TTC and negative correlation with the ABTS which indicated a good antioxidant activity of this population.

4. Discussion

Different environmental factors, such as light incidence/intensity, temperature, water availability, soil composition (Borges et al., 2013; Liu et al., 2015; Sampaio et al., 2016; Liu et al., 2016) and genetics (Sampaio et al., 2015 et al., 2016; Moustafa et al., 2016) may contribute to the different quality levels of medicinal plants. In this study, the *M. charantia* samples were collected from in the Southeast Region of Brazil. The samples were analyzed in terms of their chemical and genetic, which were the main factors of influence in the total content and biological activities of secondary metabolites in plants.

To explore the genetic variation between different locations, we used ISSR markers. DNA-based molecular markers could be utilized for the comparison of genetic architecture of different plant population and inter-simple sequence repeats (ISSR) are one of the used markers due to their simple and high reproducible, furthemore, target many sequences throughout the genome as their application does not need any previous information about the sequence (Mishra et al. 2015).

Levels of similarity between samples were relatively high (between 65% and 85%) (Table 3) suggesting close genetic relations. The UPGMA dendrogram also revealed that geographical and genetic cluster among the seven *M. charantia* populations were not correlated, thus the genetic structure of populations is not reflected in the geographical proximity of the individuals. *M. charantia* is a monoecious specie, in which a plant carries both unisexual flowers (male and female in a single plant), fruit formation occurred through crossed-pollination and self-pollination, however, in both cases this plants need transporters pollen for pollination to occur (Balina et al., 2015; Lenzi et al., 2005). The mechanisms of seed dispersal model the genetic structure of plant populations. In general, species with efficient and diverse mechanisms of pollen and seed dispersal exhibit genetic heterogeneity within populations than among populations, while species with wide seed dispersion should have a lower genetic heterogeneity due to the gene flow of seeds (Loveless and Hamrick 1984; Hamrick et al., 1993). Therefore, the zoochory dispersion has a great influence on the patterns of gene flow and the intra and interpopulation genetic structure (Jordano et al., 2014). Thus, the efficiency of *M. charantia* in dispersing seeds and pollen through insect pollinators (Balina et al., 2015; Lenzi et al., 2005) certainly contributed to the observed results.

Phenolic compounds are widely distributed in plants and representes most pronounced secondary metabolites found in these, have been associated with human health and beneficial implications derived from their antioxidant activities and free radical-scavenging abilities (Balasundram et al., 2006; Lin et al., 2016). Therefore, the content of phenolic compounds was measured in samples from different populations. *M. charantia* HAE have shown a significant differences in contents of total and individual phenolics compounds and consequently in antioxidant activity.

ABTS is rapid assay reflecting the free radical scavenging potential of compounds with ability to donate either hydrogen or electron (Floegel et al., 2011). For the ABTS assay, the scavenging activity of JAG was higher among the localities. The antioxidant activity of *M. charantia* has been confirmed in many studies (Kubola et al., 2008; Thenmozhi and Subramanian, 2011; Divya et al., 2013; Svobodova et al., 2016).

In general, the phenolic contents were positively correlated with antioxidant activities being indicative that phenolic compounds greatly contributed to antioxidant capacity of *Momordica charantia*. Several studies have reported these strongly positive linear correlations between free radical scavenging activities and phenolic acids concentrations in medicinal plants. These works suggest that the ability to radical eliminate of medicinal plants are strongly influenced by the presence and position of the phenolic hydroxyl groups. Their antioxidant activity seems to be related to their molecular structure and the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation (Horax et al., 2005; Kubola et al., 2008; Chauke et al., 2012; Gawron-Gzella et al., 2013; Blum-Silva et al., 2015).

HPLC analyzes presented low levels of rutin, chlorogenic acid, quecertin, luteolin and gallic acid were found. Horax et al (2006) observed that majoritary phenolic acids in leaves were gallic acid, gentisic acid, catechin, chlorogenic acid, and epicatechin. The results in the present study suggest that phenolic compounds mensured by HPLC are not the determining factors affecting antioxidant activity.

Secondary metabolites represent a chemical interface between plants and environment, therefore their accumulation can be influenced by environmental factors (Gobbo-Neto & Lopes, 2007). Pearson correlation analysis (Table 6) suggest that there the main nutrients capable to influence the levels of the phenolics compounds were: N, K, Zn, Cu, B. In the present study, the sample with the highest amount of nitrogen in the leaves had the highest contents of phenols, falvonoids and tannins. Palumbo et al. (2007) discuss that discusses that the production of phenolic compounds is influenced by the rate between photosynthetic products and nitrogen reports that nitrogen supplied in excess of growth requirements is allocated to nitrogen-based secondary metabolism.

Data showed potassium levels found leaves presented significant negative correlation with total phenols and flavonoids. Potassium exhibits well-defined action in disease resistance, possibly to compensate for potassium deficiency occurs increased synthesis of secondary metabolites such as phenolic compounds (Yamada, 2004).

Micronutrients, especially Cu, Zn and B, are essential for the smooth functioning of the shikimic acid pathway. Data showed cupper, zinc and boron levels found leaves presented significant negative, negative and positive correlation, respectively. Under copper deficiency, there is an increase in the phenol content due to the decrease in polyphenol oxidase activity enzime which catalyzes the oxidation of phenolic compounds (Marchner, 1997).

Results of the Hierarchical Cluster Analysis (HCA) performed on the content and antioxidant activity not resembles the grouping obtained by the ISSR analysis. However, a high similarity was observed among populations when comparing their chemical content. This analysis indicates that chemical and antioxidant variations among populations are influenced for genetics fator as well as enviromental conditions.

5. Conclusion

This work represent the comparative molecular and phytochemical investigation of *M. charantia* wild populations growing in the Espírito Santo-Brazil to understand what factors that influence in the production and accumulation of bioactive metabolites. The results from the comparative study of the chemical and genetic analyses in seven populations of *M. charantia* showed that the phytochemical variation is caused both by environmental and genetic factors. Levels of ISSR based similarity between samples were relatively high suggesting that analyzed populations are in close genetic relations. The chemical levels demostrated a low variation among the populations, but there was no correlation with the genetic struture.The present study assumes significance as it provides valuable information about the pattern of genetic variation and environmental conditions that seem to exert an important influence on the production of phenolic compounds from this considerable medicinal plant. This study provides a solid basis for the combined use of chemical and genetic fingerprints in efficiently evaluating quality that will allow the production of plant material with homogeneous chemical profiles with appropriate pharmacological properties of *M. charantia.*

6. Acknowledgements

The authors thank Fundação de Amparo a Pesquisa do Estado da Espírito Santo (FAPES), for their financial support to research. We acknowledge the Laboratory of Biomolecular Analysis (LABIOM) at Health Sciences Center from Federal University of Espírito Santo (UFES), Vitória-ES, Brazil for their support with the use of HPLC and the Nucleus of Genetics Applied to the Biodiversity Conservation UFES for support genetic analisys.

7. References

Alansi S, Tarroum M, Al-Qurainy F, Khan S, Nadeem M. 2016. Use of ISSR markers to assess the genetic diversity in wild medicinal *Ziziphus spina-christi* (L.) Willd. collected from different regions of Saudi Arabia. Biotechnology & Biotechnological Equipment 30(5):942-947.

Balasundram N, Sundram K, Samman S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chemistry 99(1):191-203.

Balina PK, Sharma SK, Rana MK. 2012. Diversity, abundance and pollination efficiency of native bee pollinators of bitter gourd (*Momordica charantia* L.) in India. Journal of Apicultural Research 51(3):227-231.

Blum-Silva CH, Chaves VC, Schenkel EP, Coelho GC, Reginatto FH. 2015. The influence of leaf age on methylxanthines, total phenolic content, and free radical scavenging capacity of *Ilex paraguariensis* aqueous extracts. Revista Brasileira de Farmacognosia 25:1-6.

Borges CV, Minatel IO, Gomez-Gomez HA, Lima GPP. 2017. Medicinal Plants: Influence of Environmental Factors on the Content of Secondary Metabolites. In: Ghorbanpour M, Varma A, editors. Medicinal Plants and Environmental Challenges. Cham: Springer International Publishing. p. 259-277.

Chao CY, Sung PJ, Wang WH, Kuo YH. Anti-inflammatory effect of *Momordica charantia* in sepsis mice. (1420-3049 (Electronic)).

Chauke AM, Shai LJ, Mphahlele PM, Mogale MA. 2012. Radical Scavenging Activity of Selected Medicinal Plants From Limpopo Province of South Africa. African Journal of Traditional, Complementary, and Alternative Medicines 9(3):426-430.

Costa JGM, Nascimento EMM, Campos AR, Rodrigues FFG. 2010. Antibacterial activity of *Momordica charantia* (Curcubitaceae) extracts and fractions. Journal of Basic and Clinical Pharmacy 2(1):45-51.

Dandawate PR, Subramaniam D, Padhye SB, Anant S. 2016. Bitter melon: a panacea for inflammation and cancer. Chinese Journal of Natural Medicines 14(2):81-100.

DINAKARAN, S. et al. A medicinal potency of *Momordica charantia*. International Journal of Pharmaceutical Sciences Review and Research, p. 95-100, 2010.

Divya D, Hettiarachchy NS, Ganesh V, Kannan A, Rayaprolu S. 2013. Phenolic extracts from leaves of bitter melon (*Momordica charantia*) with antioxidant properties. Journal of Agricultural Science and Applications 2(1):28-34.

Floegel A, Kim D-O, Chung S-J, Koo SI, Chun OK. 2011. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. Journal of Food Composition and Analysis 24(7):1043-1048.

Hamrick JL, Murawski DA, Nason JD. 1993. The influence of seed dispersal mechanisms on the genetic structure of tropical tree populations. Vegetatio 107(1):281-297.

Horax R, Hettiarachchy N, Islam S. 2006. Total Phenolic Contents and Phenolic Acid Constituents in 4 Varieties of Bitter Melons (*Momordica charantia*) and Antioxidant Activities of their Extracts. Journal of Food Science 70(4):C275-C280.

Jordano P, Galetti M, Pizo M, Silva W. 2006. Ligando Frugivoria e Dispersão de Sementes à Biologia da Conservação.

Kubola J, Siriamornpun S. 2008. Phenolic contents and antioxidant activities of bitter gourd (*Momordica charantia* L.) leaf, stem and fruit fraction extracts in vitro. Food Chemistry 110(4):881-890.

Kumar A, Mishra P, Baskaran K, Shukla AK, Shasany AK, Sundaresan V. 2016. Higher efficiency of ISSR markers over plastid psbA-trnH region in resolving taxonomical status of genus *Ocimum* L. Ecol Evol 6(21):7671-7682.

Kumar S, Paul S, Walia Y, Kumar A, Singhal P. 2015. Therapeutic Potential of Medicinal Plants: A Review.

Kwatra D, Venugopal A Fau - Standing D, Standing D Fau - Ponnurangam S, Ponnurangam S Fau - Dhar A, Dhar A Fau - Mitra A, Mitra A Fau - Anant S, Anant S. Bitter melon extracts enhance the activity of chemotherapeutic agents through the modulation of multiple drug resistance. (1520-6017 (Electronic)).

Lenzi M, Orth AI, Guerra TM. 2005. Ecologia da polinização de Momordica charantia L. (Cucurbitaceae), em Florianópolis, SC, Brasil. Brazilian Journal of Botany 28:505-513.

Lin D, Xiao M, Zhao J, Li Z, Xing B, Li X, Kong M, Li L, Zhang Q, Liu Y et al. . 2016. An Overview of Plant Phenolic Compounds and Their Importance in Human Nutrition and Management of Type 2 Diabetes. Molecules 21(10).

Liu D, He X, Liu G, Huang B. 2011. Genetic diversity and phylogenetic relationship of Tadehagi in southwest China evaluated by inter-simple sequence repeat (ISSR). Genetic Resources and Crop Evolution 58(5):679-688.

Liu W, Liu J, Yin D, Zhao X. 2015. Influence of Ecological Factors on the Production of Active Substances in the Anti-Cancer Plant *Sinopodophyllum hexandrum* (Royle) T.S. Ying. PLOS ONE 10(4):e0122981.

Liu W, Yin D, Li N, Hou X, Wang D, Li D, Liu J. Influence of Environmental Factors on the Active Substance Production and Antioxidant Activity in *Potentilla fruticosa* L. and Its Quality Assessment. (2045-2322 (Electronic)).

Lu, K. H. et a.Wild bitter gourd protects against alcoholic fatty liver in mice by attenuating oxidative stress and inflammatory responses. Food & Function, 5(5), 1027-37, 2014.

Mishra P, Kumar LD, Kumar A, Gokul S, Ravikumar K, Shukla AK, Sundaresan V. 2015. Population Dynamics and Conservation Implications of *Decalepis arayalpathra* (J. Joseph and V. Chandras.) Venter., a Steno Endemic Species of Western Ghats, India. Appl Biochem Biotechnol 176(5):1413-30.

Moustafa MF, Hesham AE-L, Quraishi MS, Alrumman SA. 2016. Variations in genetic and chemical constituents of *Ziziphus spina-christi* L. populations grown at various altitudinal zonation up to 2227m height. Journal of Genetic Engineering and Biotechnology 14(2):349-362.

Natarajan S, Mishra P, Vadivel M, Basha MG, Kumar A, Velusamy S. 2018. ISSR Characterization and Quantification of Purpurin and Alizarin in *Rubia cordifolia* L. Populations from India. Biochem Genet.

Palumbo MJ, Putz FE, Talcott ST. 2007. Nitrogen Fertilizer and Gender Effects on the Secondary Metabolism of Yaupon, a Caffeine-Containing North American Holly. Oecologia 151(1):1-9.

Rajendran, K.; Vikram Reddy, E.; Khanna, A. Anticancer effect of *Mesua ferrea* extracts on Human Pancreatic Cancer Cell line. International Journal of Life-Sciences Scientific Research, 198-205, 2016.

Sampaio BL, Edrada-Ebel R, Da Costa FB. 2016. Effect of the environment on the secondary metabolic profile of *Tithonia diversifolia*: a model for environmental metabolomics of plants. Scientific Reports 6:29265.

Santos, K. K., et al. Trypanocide, cytotoxic, and antifungal activities of *Momordica charantia*. Pharmaceutical Biology, 50(2), 162-166, 2012.

Svobodova B, Barros L, Calhelha RC, Heleno S, Alves MJ, Walcott S, Bittova M, Kuban V, Ferreira ICFR. 2017. Bioactive properties and phenolic profile of *Momordica charantia* L. medicinal plant growing wild in Trinidad and Tobago. Industrial Crops and Products 95:365-373.

Thenmozhi AJ, Subramanian P. Antioxidant Potential of *Momordica Charantia* in Ammonium Chloride-Induced Hyperammonemic Rats. (1741-4288 (Electronic)).

Wei L, Shaoyun W Fau - Shutao L, Shutao L Fau - Jianwu Z, Jianwu Z Fau - Lijing K, Lijing K Fau - Pingfan R, Pingfan R. Increase in the free radical scavenging capability of bitter gourd by a heat-drying process. (2042-650X (Electronic)).

Šamec D, Durgo K, Grúz J, Kremer D, Kosalec I, Piljac-Žegarac J, Salopek-Sondi B. 2015. Genetic and phytochemical variability of six Teucrium arduini L. populations and their antioxidant/prooxidant behaviour examined by biochemical, macromolecule- and cell-based approaches. Food Chemistry 186:298-305.

Table 1: ISSR primes, sequence, total number of locus and polymorphic loci used in molecular characterization of *Momordica charantia* populations.

S. nº	Primer	Sequence	Total number of loci	Polymorphic loci	%
1	UBC808	AGAGAGAGAGAGAGAGC	9	4	44.44
2	UBC816	CACACACACACAGG	8	4	50.00
3	UBC826	ACACACACACACACACC	7	1	14.28
4	UBC834	AGAGAGAGAGAGAGAGCTT	7	3	42.85
5	UBC835	AGAGAGAGAGAGAGAGCTC	4	2	50.00
6	UBC840	GAGAGAGAGAGAGAGACTT	10	7	70.00
7	UBC848	CACACACACACACACAAGG	8	7	87.50
8	UBC850	GTGTGTGTGTGTGTGTCTC	8	4	50.00
9	UBC855	ACACACACACACACACCTT	4	2	50.00
10	UBC856	ACACACACACACACACCTA	7	6	85.71
11	UBC861	ACCACCACCACCACCACC	7	2	28.57
12	UBC864	ATGATGATGATGATGATG	10	4	40.00
13	UBC866	CTCCTCCTCCTCCTCCTC	4	1	25.00
14	UBC880	GGAGAGGAGAGGAGA	10	6	60.00
15	UBC888	CGTAGTCGTCACACACACACACA	3	1	33.33
16	UBC889	AGTCGTAGTACACACACACACAC	3	2	66.67
17	ISSRCR-8	GTGGTGGTGGTGGTGGC	4	2	50.00
18	ISSRCR-2	CACACACACACACACAAG	6	6	100.00
Total			119	67	56.35

Tabela 2: Similarity matrix using Jaccard´s coefficient among *Momordica charantia* populations.

	ST	FUN	CAR	DM	ANC	PG	JAG
ST	1						
FUN	0,757	1					
CAR	0,717	0,811	1				
DM	0,681	0,802	0,813	1			
ANC	0,667	0,651	0,724	0,670	1		
PG	0,757	0,785	0,762	0,723	0,714	1	
JAG	0,769	0,764	0,679	0,704	0,660	0,851	1

ANC: Anchieta; CAR: Cariacia; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa.

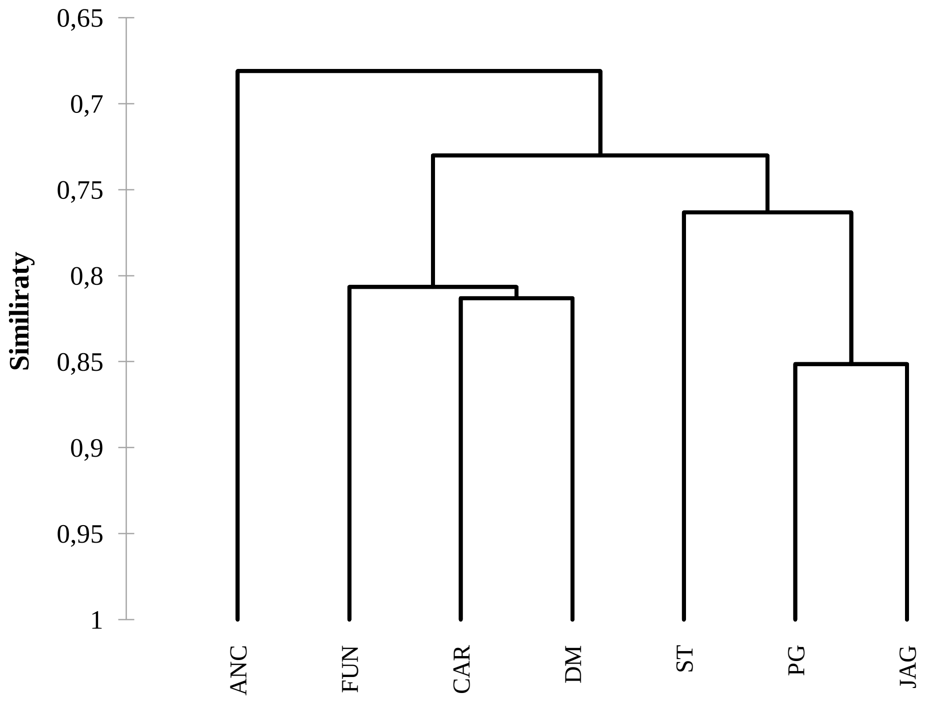


Figure 1: Dendrogram based on UPGMA of Jaccard’s similarity matrix represents the genetic relationship among ten *V. curassavica* populations. ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa.

Table 3: Content of phenolic compounds and antioxidant activity of *Momordica charantia* populations.

	ANC	CAR	DM	FUN	JAG	PG	ST	TROLOX
TPC (mg GAE.g^{-1}d.w.)	18.87±1.20^{c}	33.79±1.51^{ab}	23.00±1.23^{bc}	34.43±0.73^{ab}	44.27±0.39^{a}	29.98±1.12^{bc}	26.65±0.59^{bc}	
TFC (mg RE.g^{-1}d.w.)	46.67±0.98^{f}	457.50±5,3^{b}	190.28±2.37^{e}	311.94±3.65^{d}	575.28±5.64^{a}	391.11±3.33^{c}	70.00±0.83^{f}	
TTC (mg TA.g^{-1}d.w.)	17.00±0.16^{cd}	22.96±0.47^{b}	13.45±0.06^{de}	25.47±0.12^{b}	30.39±0.27^{a}	17.71±0.20^{c}	12.36±0.18^{e}	
ABTS IC50 (mg.mL^{-1})	778.56±21.0^{ab}	598.04±5.9^{c}	865.37±10.5^{a}	513.85±6.76^{cd}	390.64±2.58^{d}	613.20±6.93^{cd}	569.74±0.86^{cd}	78.54±2.90^{e}

ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa; VIA: Viana; TROLOX: antioxidant standard. All the values are expressed as mean $\pm$ SE (n=3). SE: standard error; Total phenols content (TPC); Total tannins content (TTC), Total flavonoids content (TFC). Means in the rows with different letters are significantly different, ANOVA, test-Tukey (p< 0.05).

Table 4: HPLC analysis data of the *Momordica charantia* populations.

	ANC	CAR	DM	FUN	JAG	PG	ST
Chlorogenic acid (µg/mL)	2.69±0.005^{c}	3.00±0.202^{ab}	2.70±0.004^{c}	2.77±0.011^{bc}	3.07±0.117^{a}	2.69±0.003^{c}	2.69±0.002^{c}
Rutin (µg/mL)	10.1±0.13^{c}	5.42±0.13^{e}	8.86±0.42^{c}	29.72±1.29^{a}	7.23±0.38^{d}	5.10±0.08^{e}	13.97±0.07^{b}
Quercetin (µg/mL)	ND	4.00±0.43^{a}	ND	ND	ND	ND	ND
Luteolin (µg/mL)	ND	3.47±0.26^{a}	ND	ND	ND	ND	ND
Galic acid (µg/mL)	ND	2.47±0.006^{a}	2.29±0.015^{b}	2.28±0.022^{b}	2.26±0.00^{b}	2.29±0.012^{b}	2.26±0.00^{b}

ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa; VIA: Viana; TROLOX: antioxidant standard. All the values are expressed as mean $\pm$ SE (n=3). SE: standard error. Means in the rows with different letters are significantly different, ANOVA, test-Tukey (p< 0.05). ND: not detect. All the values are expressed as mean $\pm$ SE (n=3). SE: standard error. Means in the rows with different letters are significantly different, ANOVA, test-Tukey (p< 0.05).

Table 5: Chemical analysis data (A) soil and (B) leaves of *Momordica charantia* populations.

A

Location	Manganese ($mg.dm^{-3}$)	Potassium ($mg.dm^{-3}$)	Phosphorus ($mg.dm^{-3}$)	Magnesium ($cmolc.dm^{-3}$)	Iron ($mg.dm^{-3}$)	Zinc ($mg.dm^{-3}$)	Organic matter ($Dag.kg^{-1}$)	pH
ANC	8	21	2	0.3	28	0.1	1.5	7.2
CAR	120	180	98	0.7	37	12.8	1.2	7.6
DM	123	160	27	0.9	89	11.6	2.4	6.4
FUN	168	140	11	0.6	446	8.1	1.2	7.2
JAG	118	80	55	0.8	76	13.5	2.2	6.2
PG	37	86	16	0.4	106	1.3	2.4	7.7
ST	190	220	32	0.8	64	12.9	2	6.5

B

Location	Nitrogen ($g.kg^{-1}$)	Phosphorus ($g.kg^{-1}$)	Potassium ($g.kg^{-1}$)	Calcium ($g.kg^{-1}$)	Magnesium ($g.kg^{-1}$)	Sulfur ($g.kg^{-1}$)	Iron ($mg.kg^{-1}$)	Zinc ($mg.kg^{-1}$)	Copper ($mg.kg^{-1}$)	Manganese ($mg.kg^{-1}$)	Boron ($mg.kg^{-1}$)
ANC	37.66	4.14	40.63	62.4	8.44	3.37	602	55	15	67	24
CAR	43.26	2.71	20	64.92	7.54	3.48	1.133	35	13	113	59
DM	40.6	2.49	17.5	48.97	10.57	3.37	589	49	14	147	28
FUN	39.97	2.17	18.75	32.4	11.48	2.46	770	52	11	153	42
JAG	53.62	3.33	16.88	28.33	9.79	3.33	294	32	8	40	57
PG	37.87	2.97	25	95.78	15.63	3.01	992	32	8	67	31
ST	45.78	3.04	28.75	48.42	8.63	3.85	692	70	13	127	46

ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa.

Tabela 6: Pearson correlation analysis between phenolic compounds content, antioxidant activity and leaves nutrients.

	TPC	TFC	TTC	ABTS	N	P	K	Ca	Mg	S	Fe	Zn	Cu	Mn	B
TPC	**1**														
TFC	0,817	**1**													
TTC	0,823	0,796	**1**												
ABTS	-0,773	-0,632	-0,707	**1**											
DPPH	-0,601	-0,512	-0,729	0,744											
N	0,665	0,507	0,515	-0,655	**1**										
P	-0,264	-0,276	-0,090	0,114	0,060	**1**									
K	-0,628	-0,700	-0,468	0,362	-0,438	0,784	**1**								
Ca	-0,367	-0,076	-0,433	0,354	-0,606	0,195	0,378	**1**							
Mg	0,079	0,231	-0,024	-0,048	-0,355	-0,273	-0,193	0,466	**1**						
S	-0,237	-0,296	-0,458	0,187	0,363	0,437	0,293	0,076	-0,579	**1**					
Fe	-0,129	0,073	-0,191	0,116	-0,544	-0,357	0,011	0,674	0,182	-0,126	**1**				
Zn	-0,531	-0,874	-0,599	0,274	-0,178	0,069	0,471	-0,283	-0,372	0,291	-0,179	**1**			
Cu	-0,697	-0,756	-0,573	0,694	-0,394	0,161	0,467	-0,056	-0,655	0,396	0,074	0,637	**1**		
Mn	-0,257	-0,373	-0,357	0,297	-0,324	-0,751	-0,293	-0,251	-0,125	-0,158	0,294	0,522	0,481	**1**	
B	0,734	0,654	0,617	-0,739	0,764	-0,244	-0,527	-0,398	-0,399	0,177	0,066	-0,293	-0,336	-0,070	**1**

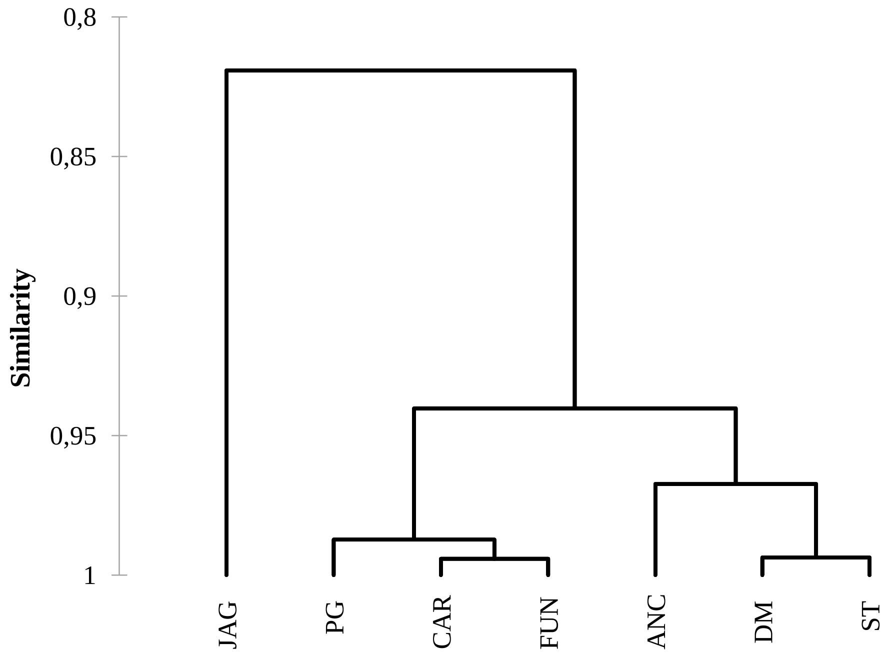


Figure 4: Hierarchical cluster analysis based on phytochemical content and antioxidant activity of HAE from seven *M. charantia* populations of different locations. ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa.

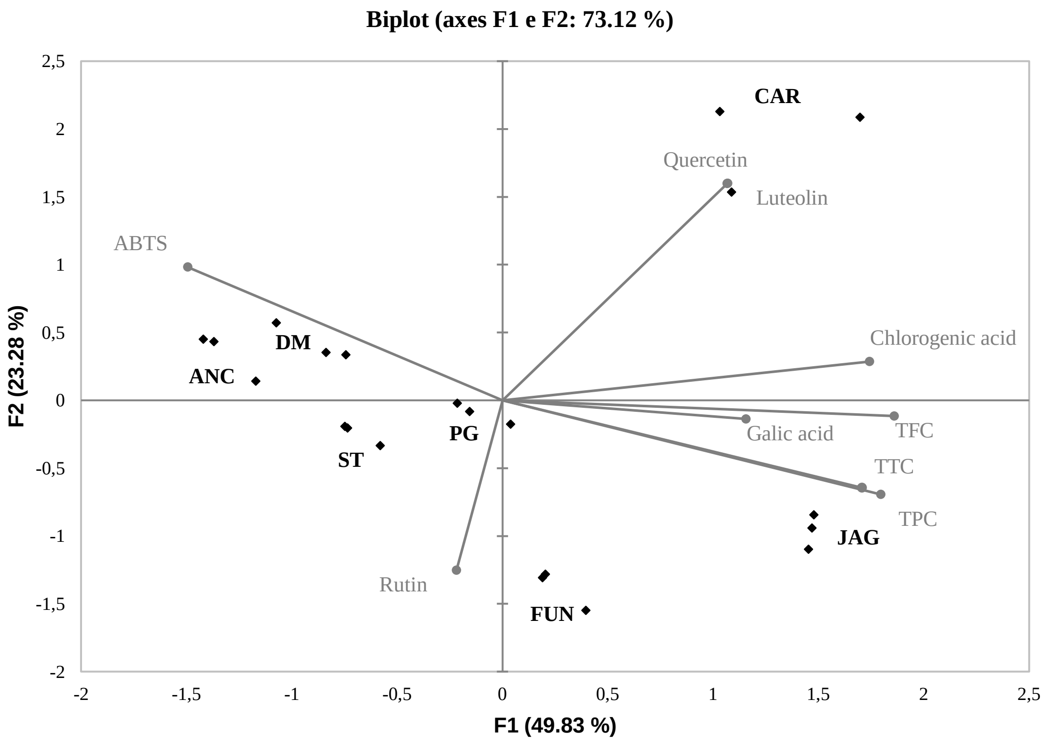


Figure 5: Principal components analysis (PCA) of the phytochemical profiles of *M. charantia* populations and their antioxidant activity. ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa.

CAPÍTULO 3 – *Varronia curassavica* Jacq. INDUCES ANTIPROLIFERATIVE EFFECTS IN SARCOMA 180 CELLS IN VITRO

Após revisão da língua inglesa, este artigo será submetido à revista: Asian Pacific Journal of Tropical Biomedicine (Print Edition ISSN: 2231-2560).

Varronia curassavica Jacq. induces antiproliferative effects in sarcoma 180 cells in vitro

Mainã Mantovanelli da Mota[a*], Anny Carolyne da Luz[a], Jean Carlos Vencioneck Dutra[a], Suiany Vitorino Gervásio, Maria do Carmo Pimentel Batitucci[a].

[a]Departamento de Ciências Biológicas, Centro de Ciências Humanas e Naturais, Universidade Federal do Espírito Santo, Vitória, Brazil.

*Corresponding author: Mainã Mantovanelli da Mota
Departamento de Ciências Biológicas
Laboratório de Genética Vegetal e Toxicológica
Universidade Federal do Espírito Santo
Av. Fernando Ferrari 514, Goiabeiras, 29075-910, Vitória, ES, Brazil
Phone: 55 27 998311735
Email address: maina.mantovanelli@gmail.com

Abstract

Cancer is the second major cause of deaths after cardiovascular diseases. Natural products have been shown to be useful sources of chemoprotective agents against cancer and medicinal plants are an important area of research for drug discovery for cancer therapy. Several plants have been described with potential antitumor activity, such as *Varronia curassavica* Jacq. (*Cordia verbenacea* DC. synonymy). *V. curassavica* is a medicinal plant, widely distributed along the Brazilian coast and commonly used to treat inflammation. However, the qualities of medicinal plants are influenced by environmental conditions. Thus, the objective of this study was to analyze the differences in antioxidant, cytotoxic, and antitumor effects of the hydroalcoholic extract of *V. curassavica in vitro*, as well as to analyze the anticancer effect of these compounds *in vivo*. *V. curassavica* showed greater antioxidant activity in the DPPH assay than FRAP and Fe^{2+} chelating ions assays. In addition, *V. curassavica* induced a significant decrease of sarcoma 180 cell viability at 24 h of treatment and did not induce cytotoxicity in human lymphocytes. Our findings suggest that the antioxidant and antitumor activity are influenced by factors of the environmental growing. However, it was not able to reduce tumor growth *in vivo*. These results will contribute to determining ideal condition for plant growth and extract production aimed at improving anticancer effects *in vivo*.

Keywords: *Cordia verbenacea*, antitumor activity, MTT, antioxidant activity

INTRODUCTION

Cancer is most devastating disease worldwide and the number of cases and deaths are increasing annually[1,2]. Natural products have been shown to be useful as sources of chemoprotective agents against cancer[3]. Several plants have been described with potential antitumor activity, including *Polygala campestris*[4], *Mesua ferrea*[5], *Artemisia capillaris*[6], and *Varronia curassavica*[7].

Varronia curassavica Jacq. (*Cordia verbenacea* DC. synonymy), popularly known as "erva-baleeira", belongs to the family *Cordiaceae* and is a medicinal species native to Central and South traditionally used to treat inflammation[8,9]. Pharmacological studies have demonstrated several properties of *V. curassavica*, including antibacterial, antifungal, anti-allergic, antitumor, and antioxidant activities[10-13].

The therapeutic use of plants to prevent and/or cure diseases is mainly related to the content of secondary metabolites, such as phenolic compounds that act as effective antioxidants; their beneficial effects have been attributed to the donation of electrons able to scavenge and neutralize reactive oxygen species (ROS)[14,15]. When present in excess, ROS can cause damage that is frequently associated with cardiovascular and neurodegeneration disorders[16]. Studies have demonstrated that the total content and biological activities of secondary metabolites in plants can be affected by environmental factors, such as light incidence/intensity, temperature, water availability, soil composition[17,18] and genetics[19].

Thus, the objective of this work was to study the differences in antioxidant, cytotoxic, and antitumor effects of the hydroalcoholic extracts (HAE) of *V. curassavica in vitro* from 10 different localities, as well as to analyze the anticancer effect *in vivo* from a selected sample.

MATERIALS AND METHODS

Plant material and preparation of extract

The fresh aerial part of *V. curassavica* was collected from June to September in 2015 at ten locations in Brazil: Anchieta (ANC; 20°47'30.6"S; 40°34'58.0"W), Aracruz (ARA; 19°57'32.3"S; 40°08'15.6"W), Domingos Martins (DM; 20°23'01.3"S; 40°38'27.5"W), Guarapari (GUA; 20°36'08.6"S; 40°25'35.7"W), Jaguaré (JAG; 18°57'19.6"S; 40°08'43.5"W), Marechal Floriano (MF; 20°24'37.0"S; 40°40'32.8"W), Serra (MG; 20°12'04.3"S; 40°11'42.9W), Serra (JAC; 20°10'24.4"S; 40°11'11.3"W), and Viana (VIA; 20°23'05.7"S; 40°29'52.9"W). The plant material was identified by Dra. Luciana Dias Thomaz and one voucher specimen (41243) was deposited in the Central Herbarium of Universidade Federal do Espírito Santo/VIES, Brazil. Plant leaves and soil from each population were collected for chemical analysis of nutrients.

The dry aerial parts of *V. curassavica* plants from each population were triturated and macerated with 70% ethanol (v/v), using a proportion of 1:5 of powder to solvent (w/v) for 72 h at room temperature. The process was repeated twice to extract the maximum constituents. The resultant extract was filtered and concentrated under a vacuum rotary evaporator TE-210 (TECNAL, Brazil) to remove the solvent and obtain the HAE of *V. curassavica* for each of the ten populations.

DPPH assay

To measure the radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH)[20], HAE samples and the standard (Trolox) were diluted in methanol (15.62, 31.25, 62.5, 125, 250, 500, and 1000 μg.mL^{-1}). Then, 200 μL methanolic DPPH solution (0.3 mM) were added to 100 μL of the each sample. The mixtures were kept in the dark for 30 min and the absorbance was measured at 517 nm using a spectrophotometric microplate reader (Epoch Microplate

Spectrophotometer, BioTek). The test was conducted in triplicate and the percentage inhibition of DPPH was calculated as follows: % inhibition of DPPH = [(Abs0 – Abs1) / Abs0] × 100, where Abs0 = absorbance of control and Abs1 = absorbance of the sample. The control solution was prepared by mixing ethanol and DPPH radical solution and ethanol was used as the blank. Results were expressed as the IC_{50} value ($\mu g.mL^{-1}$); i.e., the effective concentration of antioxidant agent required to scavenge 50% of DPPH.

Chelating activity of Fe^{+2} ions

Iron (II) binding ability of chelators was determined according to the procedure of Tang et al.[21]. Briefly, 1 mL of methanolic solutions of the samples and the EDTA standard (15.62, 31.25, 62.5, 125, 250, 500, and 1000 $\mu g.mL^{-1}$) were each mixed with 50 μL of $FeCl_2$ solution (2 mM) and 200 μL of ferrozine (5 mM) to start the reaction. The resulting mixture was mixed thoroughly and left to stand for 10 min at room temperature. The absorbance was measured spectrophotometrically at 562 nm with a microplate reader (Epoch Microplate Spectrophotometer, BioTek). The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated from the formula $[(Abs_0 – Abs_1) / Abs_0] \times 100$, where Abs0 is the absorbance of the ferrozine-Fe^{2+} complex and Abs_1 is the absorbance of the sample. The control contains $FeCl_2$, ferrozine and methanol (solvent). The assay was carried out in triplicate.

FRAP assay

Measurement of ferric reducing antioxidant power (FRAP) of the HAE was carried out based on the procedure of Rufino et al.[22]. First, 25 mL of sodium acetate buffer (0.3 M), 2.5 mL of 10 mM 2,4,6-tri(2-196 pyridyl)-s-triazine (TPTZ) and 2.5 mL of 20 mM iron (III) chloride solution were mixed to generate the FRAP reaction solution immediately prior to use. Then, 30 μL of HAE ethanolic solution (15.62, 31.25, 62.5, 125, 250, 500, and 1000 $\mu g.mL^{-1}$) plus

90 μL of distilled water were incubated with 900 μL of the working FRAP solution at 37°C in the dark. After 30 min, the absorbance of the sample was recorded at 593 nm. The standard curve was constructed using Trolox solution. The results are expressed as IC_{50} values ($mg.mL^{-1}$). Each assay was carried out in triplicate.

Cell culture

Human lymphocytes were obtained from a peripheral blood sample from a healthy donor aged between 20 to 30 years. The lymphocytes were isolated using Ficoll® Paque Plus (Sigma-Aldrich) density gradients and cultured as described previously[23]. Briefly, blood was diluted 1:1 in phosphate-buffered saline (PBS) and Ficoll-Paque. The blood was centrifuged at 200 × *g* for 10 min at room temperature. The lymphocyte layer was removed and washed in PBS at 200 × *g* for 10 min. Cell density was determined with a hemocytometer. Typically, each culture consisted of an initial density of 1×10^6 cells in 300 μL of culture medium. Lymphocyte cultures were grown in a humidified incubator with 5% CO_2 at 37°C in a 96-well microplate. The experimental protocol was approved by the Research Ethical Committee of UFES (certificate 2.333.879).

Mouse sarcoma 180 ascites (S180) cells were acquired from Banco de Células do Rio de Janeiro and incubated in *Mus musculus Swiss* mice. Human lymphocytes and S180 cells were cultured in RPMI 1640 (Cultilab) supplemented with the antibiotic gentamicin (50 $mg.L^{-1}$), the antifungal compound amphotericin B (2 $mg.L^{-1}$), and 20% fetal bovine serum (Gibco) at 37°C and 5% CO_2.

MTT assay

Cytotoxicity of the HAEs of the different populations of *V. curassavica* was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is based on the reduction of the yellow tetrazolium salt, which forms a soluble blue formazan product via mitochondrial enzymes. The amount of formazan produced is directly proportional to the number of living cells. Human lymphocytes and S180 cells were plated in 96-well plates with 2×10^5 cells in each well and treated with different concentrations of HAE of *V. curassavica* (62.5, 125, 250, and 500 μg.mL^{-1}), in RPMI 1640 medium for 24 h at 37°C and 5% CO_2. After exposure, the medium was removed, MTT (5 mg.mL^{-1} of stock in PBS) was added (20 μL/well), and cells were incubated for an additional 3 h with MTT. Then, 100 μL of dimethyl sulfoxide was added and mixed thoroughly to dissolve the dark blue formazan crystals [24]. The formation of formazan was quantified in a microplate reader at 595 nm. The experiment was performed in triplicate and the results are expressed as a relative percentage of cell viability compared to the control, calculated with the following equation: % cell viability = $(Abs_{sample} / Abs_{control}) \times 100$.

Preparation of tumor cells

In vivo antitumor activity was tested against S180 cells, which were obtained from Banco de Células do Rio de Janeiro. S180 cells were maintained in the ascitic form *in vivo* in *Mus musculus Swiss* mice by means of serial intraperitoneal transplantation. Ascitic fluid was drawn from mice 7 days after transplantation. The freshly drawn fluid was diluted with sterile isotonic saline and the tumor cell count was adjusted to 5×10^7 cells/mL by sterile isotonic saline.

***In vivo* antitumor activity**

The animals were divided into five groups ($n = 6$ per group). With the exception of the first group, all groups received 0.1 mL of S180 cell suspension (5×10^7 cells/mouse) by intraperitoneal injection in the back region. The second group served as the tumor control. Three days after inoculation, the mice received HAEs of *V. curassavica* from the ST population diluted in saline solution at doses of 5, 10, and 20 mg.kg^{-1} body weight, administered by intraperitoneal injection every day for 15 days. The dosages chosen for the experiments were based on the LD_{50} of the crude extract of the leaves of the *V. curassavica*[25]. The healthy and tumor control groups were treated with 0.9% (w/v) NaCl. After 15 days, all mice were sacrificed and the tumors were immediately dissected. This experiment was performed according to the guidelines of Animal Experimentation of the Universidade Federal do Espírito Santo and approved by the Ethics Committee for the Use of Animals (CEUA/UFES) under process number 89/2015.

Tumor inhibition

At the end of the experimental period, the tumors of the mice of each experimental group were removed. The tumors were weighed and the mean tumor weight of each experimental group was used to calculate the percentage of tumor inhibition as follows:

$$\frac{\text{(\%) Tumor inhibition} = (\text{Tumor weight in A} - \text{Tumor weight in B})}{(\text{Tumor weight in A})} \times 100$$

where "A" is the mean of the tumor weight of sarcoma group; and "B" is the mean of the tumor weight of the *V. curassavica* treatment groups.

Statistical analysis

Data are expressed as mean ± standard deviation. Differences between groups were assessed for statistical significance by the Tukey test. p values < 0.05 were considered statistically significant. Principal component analysis (PCA) was performed using XLSTAT for Windows to visualize relationships between the *V. curassavica* samples in each collection area. To analyze the antitumor effect *in vivo*, the Mann–Whitney test ($p < 0.05$) was performed to compare differences between the sarcoma and experimental groups.

RESULTS AND DISCUSSION

ROS, which be scavenged by the antioxidant system of the body, are formed during metabolic processes and contact with the external environment. Any imbalance in the levels of oxidants and antioxidants induces excessive production of free radicals. This oxidative stress in the human body is associated with some diseases, such as Alzheimer's disease, cardiovascular disorders, and cancer. Therefore, natural antioxidants found in medicinal plants may play an important role in the prevention and treatment of chronic diseases[17,26].

Antioxidant compounds are products of secondary metabolism of medicinal plants. Polyphenols, such as flavonoids and phenolic acids, are the most significant compounds that contribute to the antioxidant properties of plants[27]. In the present study, we evaluated the antioxidant capacity of *V. curassavica* collected in different regions using DPPH free radical scavenging, FRAP, and iron chelating activity assays.

The DPPH assay is a widely used method for screening the antioxidant activity of medicinal plants[28]. The table 1, shows the antioxidant activity of the ethanol extracts of the selected plants. The results of the DPPH assay are expressed as IC_{50} values ($\mu g.mL^{-1}$), which indicates the ability of the extract to achieve 50% scavenging; therefore, a high IC_{50} value indicates a weak ability of the extract to act as a scavenger, while a low IC_{50} value indicates that less extract is necessary to achieve 50% scavenging. Samples from ST, VIA, DM, GUA, and JAC showed radical scavenging activities of 64.80, 84.98, 91.34, 126.37, and 138.05 $\mu g.mL^{-1}$, respectively, which were significantly higher than the other locations (Table 1). The standard Trolox exhibited an IC_{50} value of 73.536 $\mu g.mL^{-1}$.

The FRAP assay measures the ability of antioxidants to reduce the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and produce the intense blue ferrous compound tripyridyltriazine (Fe^{2+}-TPTZ)[28]. The abilities of the extracts to chelate Fe^{2+} ions were expressed as IC_{50} values (μg.mL^{-1}; Table 3). The IC_{50} values of the studied extracts varied from 413.94 μg.mL^{-1} (GUA) to 2092.2 μg.mL^{-1} (MG).

Excess free irons have been implicated in the induction and formation of free radicals in biological systems, since iron is an important catalyst for the generation of highly reactive hydroxyl radicals via the Fenton reaction both *in vivo* and *in vitro*[28]. IC_{50} values representing chelating activities of the studied plant samples varied from 876.84 μg.mL^{-1} (MG) to 1645.33 μg.mL^{-1} (JAC). Overall, *V. curassavica* demonstrated low chelating activities and the reducing ability was highest in the DPPH assay. The difference in the antioxidant capacity of the extracts of *V. curassavica* is related to the principle of the assays employed; i.e., the DPPH assay is based on the ability of antioxidants to neutralize radicals, while the FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. Chelating activity verifies the ability of compounds to sequester free metal ions. Therefore, the extracts of *V. curassavica* tested in this study are more efficient in in the DPPH system.

Several studies have associated plant antioxidant activity with the content of phenolic compounds[29,30]. Similarly, it was observed that the antioxidant activity of *V. curassavica* is related to the phenolic content of the extracts (data not shown). The antioxidant activity of phenolic compounds is linked to their hydrogen donation, reducing power, and metal ion chelating ability, because phenolic compounds possessing one or more aromatic rings which have one or more substituent hydroxyls, structural characteristic responsible for the strong antioxidant activity attributed to this secondary metabolite. Phenolic compounds represent the most important group of natural antioxidants[15]. Free radicals play a central role in various physiological conditions such as cell signaling pathways; they control cell viability, migration, and differentiation, protecting cells against and inactivating pathological and infectious agents[31]. But at higher concentrations, free radicals cause oxidative stress, which induces a number of diseases, including diabetes mellitus, neurodegenerative disorders, cardiovascular diseases, respiratory diseases, and certain types of cancers. It is thus important for the body to receive exogenous natural antioxidants such as phenolic compounds[32]. Therefore, studies that elucidate the action of antioxidants from natural products are important.

Table 1. Content of phenolic compounds and antioxidant activity of the different *V. curassavica* populations.

	ANC	ARA	DM	GUA	JAC	JAG	MG	MF	ST	VIA	TROLOX	EDTA
DPPH IC_{50} ($\mu g.mL^{-1}$)	223.85 ± 2.59^{bc}	176.29 ± 0.38^{cd}	91.34 ± 0.37^{def}	126.37 ± 0.31^{def}	138.05 ± 0.11^{cdef}	268.04 ± 0.28^{ab}	335.37 ± 17.35^{a}	153.69 ± 0.63^{cde}	64.80 ± 0.58^{f}	84.98 ± 0.08^{ef}	73.536 ± 0.79^{ef}	
FRAP IC_{50} ($\mu g.mL^{-1}$)	876.07 ± 2.02^{c}	735.47 ± 4.64^{d}	347.86 ± 0.50^{g}	413.94 ± 2.35^{fg}	466.93 ± 1.20^{f}	1027.77 ± 1.83^{b}	2092.20 ± 6.52^{a}	877.04 ± 1.86^{c}	413.94 ± 2.35^{fg}	567.64 ± 10.85^{e}	137.52 ± 1.62^{h}	
Chelating activity IC_{50} ($\mu g.mL^{-1}$)	1111.47 ± 5.47^{de}	1258.69 ± 1.35^{bc}	1023.28 ± 5.09^{de}	1824.23 ± 5.19^{a}	1645.33 ± 9.56^{ab}	846.42 ± 4.95^{e}	876.84 ± 3.83^{de}	1216.81 ± 14.26^{bcd}	1115.12 ± 7.31^{cd}	1142.63 ± 2.84^{cd}		49.86 ± 0.44^{f}

ANC, Anchieta; ARA, Aracruz; DM, Domingos Martins; GUA, Guarapari; JAC, Jacaraípe; JAG, Jaguaré; MF, Marechal Floriano; MG, Manguinhos; ST, Santa Teresa; and VIA, Viana. The values are presented as means ±standard error.

Cytotoxicity and antitumoral activity of *V. curassavica* extracts *in vitro* were determined by MTT assay. The MTT assay is a cell viability assay that was used to determine the cytotoxicity of human lymphocytes[33] and mouse S180 cells following exposure to natural products[34,35]. The MTT assay is based on reduction of the yellow tetrazolium salt and the formation of the soluble blue product formazan. MTT is cleaved by mitochondrial succinate dehydrogenase, yielding the measurable purple product formazan. Only viable cells with active metabolism can convert MTT to formazan; therefore, formazan production is directly proportional to viable cell number and inversely proportional to the degree of cytotoxicity[25]. *V. curassavica* extracts of all ten populations were tested for cytotoxic effects *in vitro* in human lymphocytes and S180 cells using the MTT assay. Results of the different concentrations (62.5, 125, 250, and 500 $\mu g.mL^{-1}$) of *V. curassavica* extract against S180 cells (Fig. 1A) and lymphocytes human (Fig. 1B) are shown.

Our results demonstrated that HAE of *V. curassavica* reduced the viability of S180 cells. The percentage of growth inhibition increased with increasing concentration of the test compounds. Thus, the highest rate of cell death was observed at 500 $\mu g.mL^{-1}$. Only the MF sample did not show effective antitumoral effects, with 70.71% viable cells. On the other hand, in human lymphocytes, HAE of *V. curassavica* was not cytotoxic, presenting viabilities of approximately 90%.

In agreement with the present study, Parisoto et al.[7] also observed antitumor activity of the extract of *Cordia verbenacea*, which corresponds to a synonym of the species studied in the present study, in the human cancer breast cell line MCF-7. The possible mechanisms involved in the antitumor effects of *V. curassavica* include the downregulation of pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α and cyclooxygenase (COX)-2 [7,36,6]. TNF-α is a transmembrane protein that plays an important role in initiating the inflammatory reactions of the immune system. TNF-α initiates a highly complex biological cascade, primarily due to activation of NF-κB that leads to the expression of inflammatory genes[37,38]. However, TNF-α can stimulate tumorigenesis when produced in the tumor microenvironment. Kulbe et al.[39] demonstrated that tumor growth and propagation *in vivo* were significantly decreased with stable knockout of TNF-α, which was explained by the fact that tumors derived from TNF-α knockout cells showed high levels of apoptosis and were noninvasive, resulting in reduced vascularization of TNF-α knockout tumors.

A pharmacological study performed by Parisoto et al.[7] observed that the extract of *C. verbenacea* was able to reduce COX-2 expression in the tumoral lineage of breast cancer, denominated MCF-7. The COX isoenzyme converts arachidonic acid to prostaglandins. Three isoforms have been identified: COX-1, COX-2, and COX-3; COX-2 has been associated with inflammatory diseases and carcinogenesis[17]. Maeng et al.[40] observed upregulation of COX-2 in non-small-cell lung cancer cell lines and mouse lung cancer models. Using publicly available datasets, Kochel et al.[41] showed high expression of COX-2 in breast cancer. Similarly, Parisoto et al.[7] attributed the antitumor capacity of the extract of *C. verbenacea* to the inhibition of COX-2, which blocked cell survival by inducing apoptosis.

PCA was performed to analyze differences between samples based on their metabolite profiles and antioxidant activity to verify the determinant factor for antitumor activity *in vitro* (Fig. 2). PCA is a multivariate technique that exploits data which are described by diverse intercorrelated quantitative dependent variables. PCA is likely the most common calculation used in processing multivariate data. The objective of PCA is the reduction of dimensionality of a data set with a large number of correlated variables or traits. It has been proven to be a valuable tool for understanding relationships between characteristics as well as between populations[42].

In this study, two principal components (PCs) of the score plot explained 56.93% of the total variance. PCA results revealed that the first component (PC1) accounted for 37.77% of the variance and the second component (PC2) for 19.16% of the variance. Analysis of the data indicated that rutin, gallic acid, chlorogenic acid, quercetin, luteolin, total phenol content (TPC), and Fe^{2+} chelation contributed the most and positively to PC1, with a satisfactory degree of correlation, while the correlation with ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid), DPPH and FRAP assays were inverse. PC2 was mainly related to TFC and antitumoral activity (S180 cells). From this biplot, it was observed that the ST sample showed a positive correlation with the amount of TPC and a negative correlation with S180 cells, which indicated a good *in vitro* antitumoral activity of this population.

This multivariate analysis showed that antitumoral activity is inversely related to TPC (data not shown), indicating that the higher the TPC values, the lower the survival percentage of S180 tumor cells. As discussed earlier, phenolic compounds are good antioxidants; therefore, their antioxidant properties could inhibit the progression of tumor cells. Chen et al.[43] observed significantly increased antitumor activities of phenolic compounds isolated from sugar beet molasses.

In addition, antitumor activity is positively related to total flavonoid content (TFC). In accordance with this analysis, the MF sample presented the highest TFC and highest antitumor activity, such that S180 cells had the highest percentages of survival; therefore, this sample was shown to be inefficient as an antitumor agent. Flavonoids may act as antioxidants or prooxidants depending on the conditions[44]. Eren-Guzelgun et al.[45] emphasized the importance of identifying the conditions under which they have carcinogenic or anticarcinogenic effects.

The present study investigated the antitumor actions of the *V. curassavica* HAE in S180 tumor cells. Table 4 shows the antitumor activities induced by different doses of the *V. curassavica* extract in mice with sarcoma. In comparison to the sarcoma group, animals receiving the *V. curassavica* extract at the treatment doses of 20.0, 10.0, or 5.0 mg/kg body weight did not show tumor reduction. There was no significant difference in the tumor weight following treatment with 20.0, 10.0, or 5.0 mg/kg body weight of *V. curassavica* extracts compared with the sarcoma group. This occurred because the tumors evaluated were not homogenous in size. In studies conducted by Dutra et al.[46] were also found a large variation in tumor weight. According to Chignola et al.[47], this growth variability is an intrinsic property of each tumor, such that each individual tumor develops differently. Although there was no significant difference among the weights of the tumors, due to the heterogeneity in tumor size, the extracts were shown to induce tumor reduction against sarcomas *in vivo*, whereas our results indicated that the extract doses of 10.0 and 20.0 mg/kg body weight resulted in 18.95% and 78.31%, respectively, of tumor inhibition growth.

Table 4. Tumor weight of *Swiss* albino mice treated with *V. curassavica* extract (5.0, 10.0, or 20.0 mg/kg body weight).

Treatment	**Tumor weight (g)**	***p***	**% Tumor inhibition**
Sarcoma + NaCl (0.9%)	0.438 ± 0.127	–	–
Sarcoma + *V. curassavica* 5.0 mg/kg b.w.	1.182 ± 0.129	0.17349	–
Sarcoma + *V. curassavica* 10.0 mg/kg b.w.	0.130 ± 0.131	0.45496	18.95
Sarcoma + *V. curassavica* 20.0 mg/kg b.w.	0.095 ± 0.037	0.11471	78.31

The values are presented as means ±standard error. Tumor weight of *V. curassavica* treatment groups were compared to the sarcoma group by Mann–Whitney test ($p < 0.05$).

CONCLUSIONS

This comparative study of ten *V. curassavica* populations from Brazil showed that environmental conditions contribute to the plant’s biological properties, and that antioxidant and antitumor activity can change according to growing conditions. In addition, our results indicate that the *V. curassavica* extract exhibits selective cytotoxicity against tumor cells *in vitro*, likely due to the inhibition of pro-inflammatory factors and antioxidant action, leading to reduced tumor growth. Future studies aimed at standardizing the ideal growing environment and the production of extracts should be performed to improve anticancer effects *in vivo.*

Acknowledgements

The authors thank Fundação de Amparo a Pesquisa do Estado da Espírito Santo (FAPES), for their financial support to research.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] IQBAL, J. et al. Plant-derived anticancer agents: A green anticancer approach. **Asian Pacific Journal of Tropical Biomedicine,** v. 7, n. 12, p. 1129-1150, 2017.

[2] RAYAN, A.; RAIYN, J.; FALAH, M. Nature is the best source of anticancer drugs: Indexing natural products for their anticancer bioactivity. **PLOS ONE,** v. 12, n. 11, p. e0187925, 2017.

[3] SOLOWEY, E. et al. Evaluating Medicinal Plants for Anticancer Activity. **The Scientific World Journal,** v. 2014, p. 12, 2014.

[4] TIZZIANI, T. et al. Antitumor screening of crude extracts of ten medicinal plants of *Polygala* genus from Southern Brazil. **Journal of Applied Pharmaceutical Science**, p. 79-83, 2017.

[5] RAJENDRAN, K.; VIKRAM REDDY, E.; KHANNA, A. Anticancer effect of *Mesua ferrea* extracts on Human Pancreatic Cancer Cell line. **Int. J. Life. Sci. Scienti. Res.,** v. 2, .n. 2, p: 198-205, 2016.

[6] JANG, E. et al. Evaluation of antitumor activity of *Artemisia capillaris* extract against hepatocellular carcinoma through the inhibition of IL-6/STAT3 signaling axis. **Oncol. Rep.** 37 (1), 526–532, 2017.

[7] PARISOTTO, E. B. et al. The antitumor activity of extracts from *Cordia verbenacea* D.C. obtained by supercritical fluid extraction. **The Journal of Supercritical Fluids,** v. 61, p. 101-107, 2012.

[8] Nizio, D. A. C. et al. Chemical diversity of native populations of *Varronia curassavica* Jacq. and antifungal activity against Lasiodoplodia theobromae. Ind. Crops Prod. 76: 437-448, 2015.

[9] MATIAS, E. F. F. et al. Biological Activities and Chemical Characterization of *Cordia verbenacea* DC. as Tool to Validate the Ethnobiological Usage. **Evidence-Based Complementary and Alternative Medicine,** v. 2013, p. 7, 2013.

[10] PASSOS, G. F. et al. Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*. **J Ethnopharmacol,** v. 110, n. 2, p. 323-33, Mar 21 2007.

[11] Carvalho, P. M., Rodrigues, R. F. O., Sawaya, A. C. H. F., Marques, M. O. M., & Shimizu, M. T. Chemical composition and antimicrobial activity of the essential oil of Cordia verbenacea D.C. Journal of Ethnopharmacology, 95(2), 297-301, 2004.

[12] MICHIELIN, E. M. Z. et al. Radical-scavenging activity of extracts from *Cordia verbenacea* DC obtained by different methods. **The Journal of Supercritical Fluids,** v. 56, n. 1, p. 89-96, 2011.

[13] KUMAR, S. et al. Therapeutic Potential of Medicinal Plants: A Review. 2015. 46-54. **J. Biol. Chem. Chron.**, 1(1):46-54, 2015.

[14] RICE-EVANS, C.; MILLER, N.; PAGANGA, G. Antioxidant properties of phenolic compounds. **Trends in Plant Science,** v. 2, n. 4, p. 152-159, 1997.

[15] RAY, P. D.; HUANG, B.-W.; TSUJI, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. **Cellular signalling,** v. 24, n. 5, p. 981-990, 2012.

[16] LIPINA, C.; HUNDAL, H. S. Modulation of cellular redox homeostasis by the endocannabinoid system. **Open Biology,** v. 6, n. 4, p. 150276, 2016.

[17] LIU, B.; QU, L.; YAN, S. Cyclooxygenase-2 promotes tumor growth and suppresses tumor immunity. **Cancer Cell International,** v. 15, n. 1, p. 106, 2015.

[18] Liu, W., Yin, D., Li, N., Hou, X., Wang, D., Li, D., & Liu, J. Influence of Environmental Factors on the Active Substance Production and Antioxidant Activity in *Potentilla fruticosa* L. and Its Quality Assessment. **Scientific Reports.** 6:28591, 2016.

[19] ŠAMEC, D. et al. Genetic and phytochemical variability of six *Teucrium arduini* L. populations and their antioxidant/prooxidant behaviour examined by biochemical, macromolecule- and cell-based approaches. *Food Chemistry*, v. 186, p. 298–305, 2015.

[20] Rufino, M.S.M.; Alves, R.E.; Brito, E.S.; Morais, S.M.; Sampaio, C.G.; Pérez-Jiménez, J.; Saura-Calixto, F. Comunicado técnico-metodologia científica: Determinação da atividade antioxidante total em frutas pela captura do radical livre DPPH. **Fortaleza Embrapa,** 1, 1–4, 2007.

[21] Tang, S.Z., Kerry, J.P., Sheehan, D., and Buckley, D.J. Antioxidative mechanisms of tea catechins in chicken meat system. **Food Chem.,** 76: 45–51, 2002.

[22] Rufino, M. D. S. M.; Alves, R. E.; Brito, E. S. De; Morais, S. M. De; Sampaio, C. D. G.; Pérez-Jiménez, J.; Saura-Calixto, F. D. Metodologia científica: determinação da atividade antioxidante total em frutas pelo método de redução do ferro (FRAP). Comun. técnico, 125, 1–4. 528, 2006.

[23] Yager, J. W.; Eastmond, D. A.; Robertson, M. L.; Paradisin,W. M.; Smith, M. T. Characterization of micronuclei induced in human lymphocytes by benzene metabolites. Cancer Res., 90, 393-399, 1990.

[24] Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol.Methods 65: 55-63, 1983.

[25] SERTIE, J. A. A. et al. Pharmacological Assay of *Cordia verbenacea* ; Part 1. Anti-Inflammatory Activity and Toxicity of the Crude Extract of the Leaves. **Planta Médica**, 54: 7-10. 1988.

[26] BOULILA, A. et al. Antioxidant properties and phenolic variation in wild populations of *Marrubium vulgare* L. (Lamiaceae). **Industrial Crops and Products,** v. 76, p. 616-622, 2015.

[27] Najafabad, A.; Jamei R. Free radical scavenging capacity and antioxidant activity of methanolic and ethanolic extracts of plum (Prunus domestica L.) in both fresh and dried samples. **Avicenna J Phytomed.,** 4(5):343-53, 2014.

[28] Wong, F.C.; Yong, A. L.; Ting, E. P.; Khoo, S. C.; Ong, H. C.; Chai, T. T. Antioxidant, Metal Chelating, Anti-glucosidase Activities and Phytochemical Analysis of Selected Tropical Medicinal Plants. Iran J Pharm Res.,13(4),1409-15, 2014.

[29] Tohma, H. et al., Antioxidant activity and phenolic compounds of ginger (*Zingiber officinale* Rosc.) determined by HPLC-MS/MS, J. Food Meas. Charact., 556-566, 2017.

[30] MOO-HUCHIN, V. M. et al. Antioxidant compounds, antioxidant activity and phenolic content in peel from three tropical fruits from Yucatan, Mexico. **Food Chemistry,** v. 166, p. 17-22, 2015.

[31] Phaniendra, A.; Jestadi, D. B.; Periyasamy, L. Free radicals: properties, sources, targets, and their implication in various diseases. **Indian J Clin Biochem.** 30(1):11-26, 2014.

[32] Huyut Z, Beydemir Ş, Gülçin İ. Antioxidant and Antiradical Properties of Selected Flavonoids and Phenolic Compounds. **Biochem Res Int.** 2017:7616791, 2017.

[33] SAGRILLO, M. R. et al. Tucumã fruit extracts (*Astrocaryum aculeatum* Meyer) decrease cytotoxic effects of hydrogen peroxide on human lymphocytes. **Food Chemistry,** v. 173, p. 741-748, 2015.

[34] LIMA, A. P. et al. Cytoxicity and Apoptotic Mechanism of Ruthenium(II) Amino Acid Complexes in Sarcoma-180 Tumor Cells. **PLOS ONE,** v. 9, n. 10, p. e105865, 2014.

[35] FERREIRA, P. M. P. et al. Preclinical anticancer effectiveness of a fraction from *Casearia sylvestris* and its component Casearin X: in vivo and ex vivo methods and microscopy examinations. **Journal of Ethnopharmacology,** v. 186, p. 270-279, 2016.

[36] FERNANDES, E. S. et al. Anti-inflammatory effects of compounds alpha-humulene and (-)-trans-caryophyllene isolated from the essential oil of *Cordia verbenacea*. **Eur J Pharmacol,** v. 569, n. 3, p. 228-36, 2007.

[37] SETHI, G.; SUNG, B.; AGGARWAL, B. B. TNF: a master switch for inflammation to cancer. **Front Biosci,** v. 13, p. 5094-107, 2008.

[38] BALKWILL, F. TNF-alpha in promotion and progression of cancer. **Cancer Metastasis Rev,** v. 25, n. 3, p. 409-16, Sep 2006.

[39] KULBE, H. et al. The inflammatory cytokine tumor necrosis factor-alpha generates an autocrine tumor-promoting network in epithelial ovarian cancer cells. **Cancer Res,** v. 67, n. 2, p. 585-92, Jan 15 2007.

[40] MAENG, H.-J. et al. Upregulation of COX-2 in the lung cancer promotes overexpression of multidrug resistance protein 4 (MRP4) via PGE2-dependent pathway. **European Journal of Pharmaceutical Sciences,** v. 62, p. 189-196, 2014.

[41] KOCHEL, T. J.; GOLOUBEVA, O. G.; FULTON, A. M. Upregulation of Cyclooxygenase-2/Prostaglandin E2 (COX-2/PGE2) Pathway Member Multiple Drug Resistance-Associated Protein 4 (MRP4) and Downregulation of Prostaglandin Transporter (PGT) and 15-Prostaglandin Dehydrogenase (15-PGDH) in Triple-Negative Breast Cancer. **Breast cancer : basic and clinical research,** v. 10, p. 61-70, 2016.

[42] JOLLIFFE, I. Principal Component Analysis. In: LOVRIC, M. (Ed.). **International Encyclopedia of Statistical Science**. Berlin, Heidelberg: Springer Berlin Heidelberg, p.1094-1096, 2011.

[43] Chen, M., Meng, H., Zhao, Y., Chen, F., & Yu, S. Antioxidant and in vitro anticancer activities of phenolics isolated from sugar beet molasses. **BMC complementary and alternative medicine,** 15, 313, 2015.

[44] Prochazkova, D.; Boušova, I.; Wilhelmova, N. Antioxidant and prooxidant properties of flavonoids. **Fitoterapia,** 82(4), 513–523, 2011.

[45] Eren-Guzelgun, B.; Ince, E.; Gurer-Orhan, H. In vitro antioxidant/prooxidant effects of combined use of flavonoids, **Natural Product Research,** 32:12, 1446-1450, 2017.

[46] DUTRA, et al. *Cereus jamacaru* D.C. Hydroalcoholic Extract Promotes Anti-Cytotoxic and Antitumor Activity. **Pharmaceuticals**, 11(4),130, 2018.

[47] CHIGNOLA, R.; SCHENETTI, A.; ANDRIGHETTO, G.; CHIESA, E.; FORONI, R.; SARTORIS, S.; TRIDENTE, G.; LIBERATI, D.: Forecasting the growth of multicell tumour spheroids: implication for the dynamic growth of solid tumours. **Cell Prolif.**, 33:219–229, 2000.

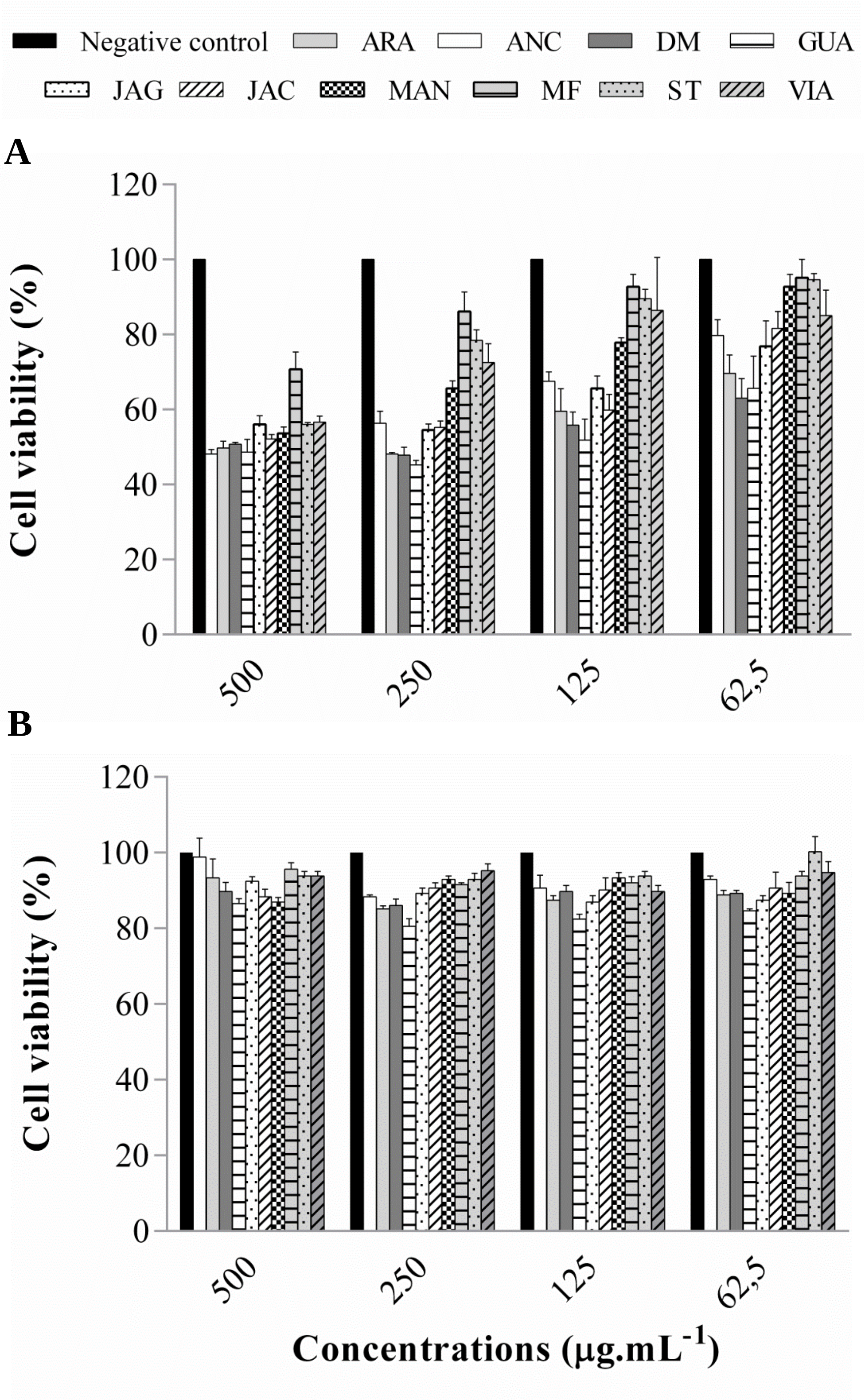


Figure 1. *In vitro* cytotoxicity in S180 cells (A) and human lymphocytes (B) determined by the MTT assay following exposure to hydroalcoholic extracts (HAEs) of *V. curassavica* all ten populations at 500, 250, 125, and 62.5 μg.mL^{-1} for 24 h. Data are presented as percentages (n = 3).

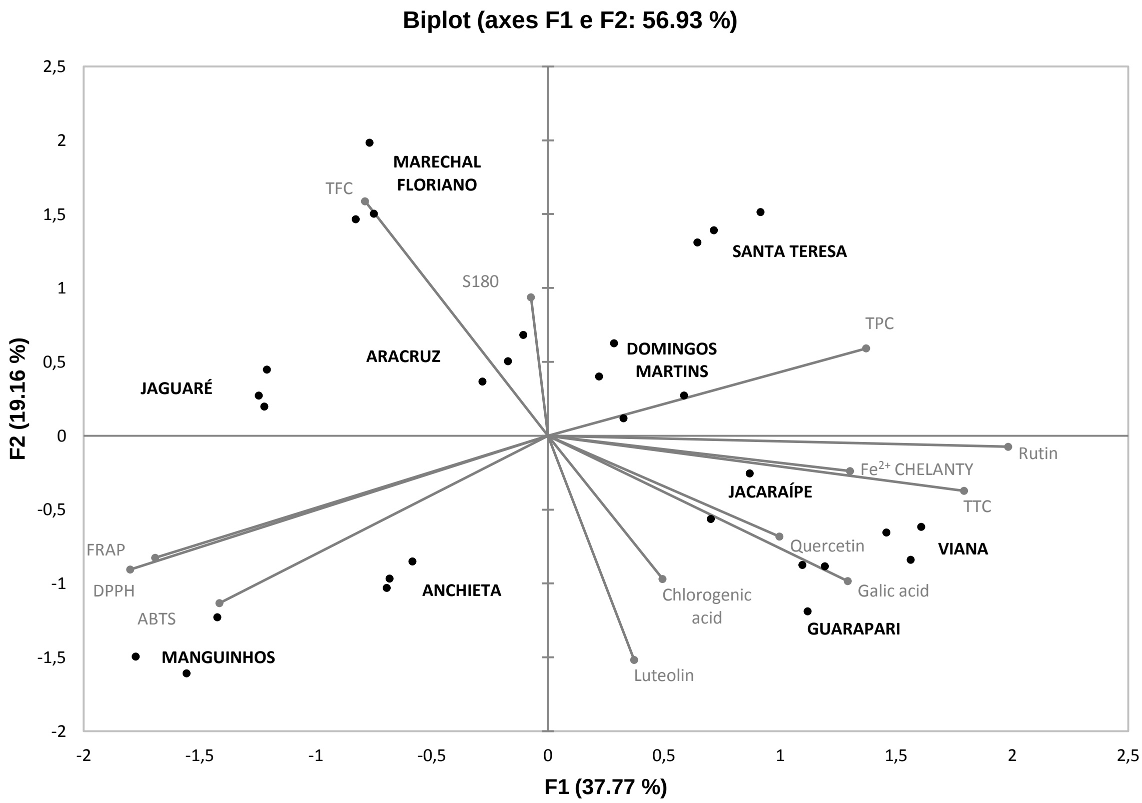


Figure 2. Principal components analysis (PCA) of the phytochemical profiles of *V. curassavica* populations as well as their antioxidant and antitumoral activity *in vitro*.

CAPÍTULO 4 – ANTIPROLIFERATIVE ACTIVITY OF *Momordica charantia* L. HYDROALCOHOLIC EXTRACTS AGAINST SARCOMA 180.

Após revisão da língua inglesa, este artigo será submetido à revista: Asian Journal of Biochemical and Pharmaceutical Research (Print Edition ISSN: 2231-2560).

Antiproliferative Activity of *Momordica charantia L.* hydroalcoholic extract against sarcoma 180

Mainã Mantovanelli da Mota[a*], Anny Carolyne da Luz[a], Suiany Vitorino Gervásio, Jean Carlos Vencioneck Dutra[a], Maria do Carmo Pimentel Batitucci[a].

[a]Departamento de Ciências Biológicas, Centro de Ciências Humanas e Naturais, Universidade Federal do Espírito Santo, Vitória, Brazil.

*Corresponding author: Mainã Mantovanelli da Mota
Departamento de Ciências Biológicas
Laboratório de Genética Vegetal e Toxicológica
Universidade Federal do Espírito Santo
Av. Fernando Ferrari 514, Goiabeiras, 29075-910, Vitória, ES, Brazil
Phone: 55 27 998311735
Email address: maina.mantovanelli@gmail.com

Abstract

Cancer causes mortality in millions of people worldwide and is one of the most devastating diseases. Several plants are described with potential antitumor activity such as *Momordica charantia* L. *M. charantia* is a species herbaceous commonly known as bitter gourd or bitter melon and is used as a vegetable, as well as medicinal plant in many countries. In the present study, hydroalcoholic extracts of *M. charantia* collected in different locations from state of southeastern Brazil were evaluated to determine differences in antioxidant activity *in vitro*, in the cytotoxic and antitumor effects in human lymphocytes and sarcoma 180 cells *in vitro* by MTT assay and antitumoral on mice sarcoma-induced *in vivo* of a selected sample. JAG sample of the *M. charantia* presented the greater antioxidant activity the DPPH (IC_{50} = 553.57µg.mL^{-1}) assay than and Fe^{2+} chelating ions (IC_{50} = 214,93 µg.mL^{-1}) assay. In addition, *M. charantia* induced significant decrease of sarcoma 180 viability at 24h of treatment and did not induces cytotoxicity in human lymphocytes. But it was not able to reduce tumor growth *in vivo*. These results contribute to the development of studies to standardize the ideal growing environment and the production of the extracts aimed at improve anticancer effects *in vivo.*

Keywords: Bitter gourd, Bitter melon, DPPH, FRAP, Sarcoma 180, MTT

Introduction

Cancer cells continually divide without any response of signaling apoptosis until form masses of cells wich grow in various tissues and interfere with the functions of many body systems (Torre et al., 2015; Sithisarn; Rojsanga, 2017). This disease is the second cause of global morbidity and is estimated to have occasioned the death approximately 9.6 million people in 2018 (WHO, 2018). Most existing cancer therapies wich often envolve surgical removal followed by radiation and/or chemotherapy. However, these treatments are not specific to only cancer cells and also cause damage to healthy cells resulting on severe side effects that can reduce patient's quality of life (Demain; Vaishnav, 2011; Rayan et al., 2017). Thus, due the high toxicity usually associated wich modern anticancer treatments is important to search for novel anti-tumour drugs active against tumours which reduces side effects and increases therapeutic efficacy (Deorukhkar et al., 2007; Shanmugam et al., 2011; Demain; Vaishnav, 2011). Therefore, has increasingly discovery and development of new drugs based on natural products and recentely many drugs used for the treatment of cancer present compounds isolated from natural products including paclitaxel, catharanthus alkaloids, and derivatives of podophyllotoxin (Rayan et al., 2017; Sithisarn; Rojsanga, 2017).

Researches have demonstred wich many plants present potential antitumor activity wich *Varronia curassavica* (Parisotto et. al, 2012), *Polygala campestris* and *Polygala densiracemosa* (Tizziani et. al, 2017), *Artemisia capillaris* (Jang et. al, 2017), *Mesua ferrea* (Rajendran et al., 2016), and also *Momordica charantia (*Jilka et al., 1983; Dia; Krishnan, 2016). *Momordica charantia L.* is a species herbaceous belonging to the family Cucurbitaceae commonly known as bitter gourd or bitter melon and can found in tropical and subtropical regions. This plant is cultivated in several regions around the world, including areas of East Africa, South America, Asia, the Caribbean, and India and it is used as a vegetable as well as medicinal plant (Dinakaran et al., 2010; Dandawate et al., 2016). In tradicional medicine, it is used to the treatment of diabetes and colics (Anilakumar et al., 2015). The pharmacological properties of *M. charantia* have been demonstrated by several studies, that in addition to its atitumoral activity also present antioxidant, anti inflammatory, antibacterial, antifungal, hepatoprotective and neuroprotective effects (Wei et al., 2013; Chao et al., 2014; Costa et al., 2010; Santos et al., 2012; Lu et al., 2014; Malik et al., 2011).

Most biologically active natural products used to prevent and/or to *cure diseases* are secondary metabolites with complex structures, such as the phenolics compounds that acts as effective antioxidant and their beneficial effect is attributed to their *ability* to *donate electrons* can to scavenging and neutralize reactive oxygen species (ROS) (Kumar et al., 2015; Rice-Evans et al., 1997).

Studies have showed that the there is a genetic control that regulates secondary metabolites, but their content and biological activities in plants can be affected by environmental factors, such as light incidence/intensity, temperature, water availability, soil composition (Borges et al., 2013; Liu et al., 2015; Sampaio et al., 2015; Liu et al., 2016). Therefore, the aim of this work was evaluates the differences in antioxidant, cytotoxic and antitumor effects *in vitro* of the *Momordica charantia* hydroalcoholic extract collected from diferents locations in the southeastern Brazil, as well as to analyze the anticancer effect *in vivo* from the selection of one of the samples studied.

Material and methods

Plant material

The aerial parts of ten *Momordica charantia* L. adult specimens were arbitrarily selected and collected at seven localities of southeastern Brazil region: Anchieta (ANC) (20°48'25.4"S; 40°38'48.3"W), Cariacica (CAR) (20°19'20.3"S; 40°22'20.6"W), Domingos Martins (DM) (20°22'34.6"S; 40°34'56.8"W), Fundão (FUN) (19°56'09.5"S; 40°25'23.2"W), Jaguaré (JAG) (18°57'19.6"S; 40°08'43.5"W), Praia Grande (PG) (20°02'18.4"S; 40°11'22.9W), Santa Teresa (ST) (19°56'28.0"S, 40°35'07.0"W), during June to September 2015. Voucher specimen (41243) was deposited in the Central Herbarium of the Universidade Federal do Espírito Santo/VIES, Brazil.

Hydroalcoholic extract

Dried plant material was powdered, macerated with aqueous etanol solution (70:30 v/v) using a solvent to powder ratio of ratio 5/1 (v/w) at room temperature (25–30 ◦C, protected from the light for 72h. Filtered and evaporated under reduced pressure at 60 ◦C to obtain the hydroalcoholic extracts (HAE) of the *M. charantia* for each of seven populations. The extract was stored at 6–10 ◦C and protected from the light until use.

DPPH assay

Free radical scavenging activities of *M. charantia* extracts were estimated according to the previously reported method (Rufino et al., 2007). DPPH• (2,2-diphenyl-1-picrylhydrazyl), which fixing an H• leads to a decrease in absorbance (Rufino et al., 2007). For each sample and standard (Trolox), eight concentrations (7.8, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 μg.mL^{-1}) were tested in order to obtain their calibration curves. Methanolic DPPH solution (0.3 mM, 200 μL) was added to 100 μL of the test solution. After 30 min at room temperature in the dark, the absorbance was taken by spectrophotometric microplate reader (Epoch Microplate Spectrophotometer – BioTek) at 517 nm and the test was performed in triplicate. The percentage inhibition of DPPH was calculated as follows: % inhibition of DPPH = [(Abs0 – Abs1) /Abs0] × 100, where Abs0 = absorbance of control and Abs1 = absorbance of the sample. The control solution was prepared by mixing methanol and DPPH radical solution and methanol was used as the blank. Results were expressed as the IC50 value (μg.mL^{-1}), that is the effective concentration of antioxidant agent required to scavenge 50% of DPPH.

Chelating activity on Fe^{+2} ions

The ferrous ion-chelating activity of *M. charantia* extracts was measured by the inhibition of ferrous–ferrozine complex formation according the procedure of Tang et al. (2002). Methanolic solutions of samples and EDTA standard (15.62, 31.25, 62.5, 125, 250, 500 and 1000 μg.mL^{-1}) were mixed with $FeCl_2$ solution (2 mM) and ferrozine (5 mM) to start the reaction. The resulting mixture was incubated at room temperature for 10 min. The absorbance was measured by the microplate reader at 562 nm. The percentage of ferrous ion chelating effect was calculated using the following equation: The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated from the formula [(Abs_0 – Abs_1) / Abs_0] × 100, where Abs0 is the absorbance of the ferrozine-Fe^{2+} complex and Abs_1 is the absorbance of the sample. The control contains $FeCl_2$ and ferrozine, complex formation molecules. The assay was carried out in triplicate.

Cytotoxicity in vitro

Human lymphocytes were obtained from peripheral blood sample from a healthy donor and aged between 20 to 30 years. The lymphocytes were isolated by the traditional method on Ficoll® Paque Plus gradient, as recommended by manufacturer, with minimal modifications. Briefly, blood was diluted 1:1 with phosphate-buffered saline PBS and Ficoll-Paque. The blood was centrifuged at 200 G for 10 min at room temperature. The lymphocyte layer was removed and washed in PBS at 200 G for 10 min. Cell density was counted with a hemocytometer and human lymphocytes were plated in 96-well plates with 2.10^5 cells in each well. The cells were grown with RPMI 1640 (Cultilab) supplemented with antibiotic gentamicin (50 $mg.L^{-1}$) and antifungal amphotericin B (2 mg.L-1), 20% fetal bovine serum (Gibco) at 37 °C with 5% CO at 37°C. The human lymphocytes cultured under these conditions for 24 h before starting the treatments. Treated cells received *M. charantia* extract diluted with water at 500, 250, 125 or 62.5 $\mu g.mL^{-1}$. After 24h the last treatment, cell viability was determined using MTT assay. Protocol involved was approved by the Research Ethical Committee of UFES (certificate 2.333.879).

In vitro Antitumor activity

Mouse sarcoma 180 ascites cells (S180) were acquired from Banco de Células do Rio de Janeiro. Sarcoma 180 cells were plated in 96-well plates with $2x10^5$ cells in each well and were cultured under the same conditions described for human lymphocytes. The cells were treated with *M. charantia* extract diluted with water at 500, 250, 125 or 62.5 $\mu g.mL^{-1}$. After 24h the last treatment, cell viability was evaluated using MTT assay to determine its antiproliferative effect.

MTT assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate cell viability and determine cytotoxicity and antiproliferative activity of the samples HAE *M. charantia* of different populations. The MTT assay is based on reduction of the yellow tetrazolium salt (MTT), to form a soluble blue formazan product by mitochondrial enzymes. The amount of formazan produced is directly proportional to the number of living cells (Mosmann et al., 1983). After exposure of cells (human lymphocytes and sarcoma 180) to treatment the medium was removed and MTT (5 $mg.mL^{-1}$ of stock in PBS) was added (20 μL/well), and cells were incubated for an additional 3 h with MTT. Then 100 μL of DMSO was added and mixed thoroughly to dissolve the dark blue crystals of formazan (Mosmann, 1983). Formation of formazan was quantified in a microplate reader at 595 nm. The experiment was performed in triplicate and the results were expressed as relative percentage of cell viability in comparison to control.

In vivo ***antitumor activity***

The albino male mice Swiss strain (Mus musculus), aged 6 to 8 weeks and approximately 40 g of body weight (b.w.) were supplied by the biotery of Univerdidade Federal do Espírito Santo. The animals were placed in polypropylene cages with metal bars and wood shavings and they passed an acclimatization period of 7 days before the start of the experiments, with free access to standard commercial feed and water and they were kept under light/dark cycles of 12 h.

The Sarcoma 180 cells were maintained in the ascitic form *in vivo* in Swiss mice by means of serial intraperitoneal transplantation. Ascitic fluid was drawn off 7 days after transplantation. The freshly drawn fluid was diluted with sterile isotonic saline and the tumor cell count was adjusted to 5 × 107 cells/mL by sterile isotonic saline.

The animals were divided into five groups (n = 5 per group). Except the first group, all groups received 0.1 mL of SARCOMA 180 cell suspension (5×10^7 cells/mouse, i.p.) cells by subcutaneous injection in the back region. The second group served as tumor control. Three day after inoculation, third, fourth and fifth, received HAE diluted in saline solution of *Momordica charantia* Jaguaré sample, at the doses of 5, 10 and 20 mg.kg^{-1} body weight, respectively and was administered by intraperitoneal injection every day for 15 days. The healthy group (healthy mice) and tumor control group were treated with 0.9% (w/v) NaCl. The weight of the tumor and organs of the heart, kidneys, spleen and liver were measured. After 15 days, all mice were sacrificed and the tumors dissected out immediately. The experiment was approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 89/2015).

Tumor Inhibition

The tumors were weighed and the mean tumor weight of each experimental group was used to calculate the percentage of tumor inhibition by the formula:

$$\frac{(\%)\ \text{Tumor inhibition} = (\text{Tumor weight in A} - \text{Tumor weight in B})}{(\text{Tumor weight in A})} \times 100$$

where “A” is the mean of the tumor weight of sarcoma group; “B” is the mean of the tumor weight of the *M. charantia* treatment groups.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were assessed for statistical significance by the Tukey test. P value < 0.05 denoted the presence of a statistically significant difference. Principal component analysis (PCA) was performed using XLSTAT for Windows (Addinsoft, New York, USA) in order to visualize relationships between the *Momordica charantia* samples in each collection area. To analyze the antitumor effect on *in vivo* mice treatments, it was performed a comparison between sarcoma group and experimental groups by Mann Whitney test (p<0.05).

Results and Discussion

Natural antioxidants found medicinal plants plays an important role in prevention and treatment chronic diseases, because the free radical in the human body is associated with some diseases, such as Alzheimer's disease, cardiovascular disorders, cancer, etc (Boulila et al., 2015; Lipina et al., 2016). Polyphenols, such as flavonoids and phenolic acids, are the most significant compounds that attribute antioxidant properties to plants, due to the ability of phenolic compounds to their donating electrons able to scavenging and neutralize reactive oxygen species (ROS) (Kumar et al., 2015; Rice-Evans et al., 1997).

In the previous studies, the measurement of phenolic compounds using colorimetric methods showed that *M. charantia* ethanol extracts have variability in contents polyphenolic groups and the population grown in Jaguaré was the sample with the highest quantities of phenolic compounds and presented the greater antioxidant activity measured by the ABTS method among the analyzed samples (data not showed). In the present investigation, the commonly accepted assays DPPH chelating activity on Fe^{+2} ions were used for the evaluation of antioxidant activity of plant extracts.

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay is relatively rapid, eficiente and is one of the most widely used method to evaluate free radical scavenging activity of plant extract (Najafabad and Jamei, 2014). The antioxidant activity of *M. charantia* extracts expressed in half maximal inhibitory concentration (IC_{50}) compared with Trolox (Table 1). IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity (color). Therefore, the high the IC_{50} value indicates a weak ability of the extract to act as a scavenger, while the low IC_{50} value indicates that less extract is necessary to achieve 50% scavenging reaction. The observed results show that JAG's extract exhibited lower IC_{50} value (553.57 $\mu g.mL^{-1}$) among the all samples. The standard Trolox exhibited IC_{50} value of the 28.86 $\mu g.mL^{-1}$. These results demonstred that most of the extracts were not effective in the DPPH system. However, several studies showed that *Momordica charantia* has a good antioxidant activity as measured by the DPPH method, such as Tan et al. (2014) that observed a good antioxidant activity of extracts of the fruits of this plant obtained from several solvents. Aljohi et al. (2016) also found that the extracts of the plant, both leaves and fruits, obtained from several solvents, are efficient scavenging of DPPH radical. In this case, the source of the plant material and the experimental conditions may be influenced by the antioxidant capacity of the extracts from the DPPH assay.

Excess free irons have been implicated in the induction and formation of free radicals in biological systems, the chelating activity has been used to determine the ability of components in plant extracts to sequester the free metal ions (Wong et al., 2015). Chelating activity IC_{50} value of the studied plant varied from 201.70 μg.mL^{-1} of sample of ST to 2818.08 μg.mL^{-1} of sample PG. The antioxidant activity by the Fe^{2+} chelating activity of HAE of ST and JAG have no significant difference when compared to the EDTA standard. The results observed in this study showed that the free radical scavenging action of extracts tested may depend to a large extent on iron chelation rather than the direct extinction of hydroxyl radicals.

Table 3: Antioxidant activity of *Momordica charantia* populations.

	ANC	CAR	DM	FUN	JAG	PG	ST	TROLOX	EDTA
DPPH IC50 (μg.mL^{-1})	901.75± 7.67^{bc}	1055± 5.53^{b}	1443.35± 5.53^{a}	781.19± 1.35^{c}	553.57± 4.52^{d}	830.82± 27.6^{c}	1062± 16.13^{b}	28.86± 0.16^{e}	
Chelating activity IC50 (μg.mL^{-1})	1187.92± 21,04^{c}	2338.09± 23,76^{b}	677.87± 3,11^{d}	1378.26± 45,82^{c}	214.93± 1,67^{e}	2818.08± 29,49^{a}	201.70± 4,80^{e}		49.86± 0,44^{e}

ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa; VIA: Viana; TROLOX: antioxidant standard. All the values are expressed as mean ± SE (n=3). SE: standard error; Total phenols content (TPC); Total tannins content (TTC), Total flavonoids content (TFC). Means in the rows with different letters are significantly different, ANOVA, test-Tukey (p< 0.05).

The cytotoxic activity of the extracts of *M. charantia* on human lymphocytes and sarcoma 180 cancer cell line from mouse was investigated in vitro 3-(4) 5-dimethyl-thiazol-zyl - 2,5 biphenyl tetrazolium bromide (MTT). Viable cells with active metabolism convert water-soluble yellow tetrazolium salt (MTT) into an insoluble purple colored formazan product. MTT is cleaved by mitochondrial enzyme dehydrogenase, the dead cells lose the ability to convert MTT into formazan. The absorbance of dissolved formazan, quantified spectrophotometrically, correlates with the number of intact viable cell (Mosmann et al., 1983). The extract *M. charantia* of the seven populations were tested *in vitro* for their cytotoxic and antiproliferative effects in both cell lines, human lymphocytes (Figure 1A) and sarcoma 180 (Figure 1B), using the MTT assay. The cell lines were exposed to the hydroalcoholic extract of *M. charantia* concentrations of the 62.5, 125, 250 and 500 $\mu g.mL^{-1}$, for 24h (Figure 1).

The data obtained from the cytotoxicity assay showed that *M. charantia* extracts not induced *in vitro* cytotoxicity for human lymphocytes, in these experimental conditions presenting the viability percentage in this cell line between 70% and 80% at 62.5 and 125 $\mu g.mL^{-1}$ and promoted lymphocytes proliferation at 500 and 250 $\mu g.mL^{-1}$, being that PG and FUN samples inducing a cell proliferation of around 80%. On the other hand, the HAE of the *M. charantia* reduced the viability of sarcoma 180. However, cell growth inhibition does not occur in a dose dependent manner. Thus, the highest rate of cell death was observed at the concentration of 250 and 125 $\mu g.mL^{-1}$ and only PG sample promotes the proliferation of the sarcoma at the concentration of 500 $\mu g.mL^{-1}$. The antiproliferative effect against tumor cells was more pronounced in all the samples (ANC, CAR, DM, FUN, JAG, ST) with an approximately 48% cell viability index, except the PG extract presenting the viability percentage in this cell line arond 70%. The data obtained in these studies corroborate with the results found by Shobha et al. (2015) in which the authors observed that the ethanol extract of *M. charantia* inhibits cervical and breast carcinoma cell growth. The antiproliferative capacity against tumor cells was also reported by Alshehri (2016), being that, and in his research showed that the methanolic extract of *M. charantia* promotes *in vitro* inhibition of the growth of against human colon, liver and breast cancer cell lines.

However, despite demonstrating that the tested samples of the *M. charantia* plant present, in the majority, a good *in vitro* antitumor activity, data showed that the growth location of this plant is not determinant for the antiproliferative activity, since the extracts of different localities presented similar results of antitumor activity *in vitro*. Studies by Lee et al. (2014) to characterize the differences between two species of the genus *Curcuma*, also demonstrated that the content of phenolic compounds and consequently their biological activity were not affected by the by the growth environment.

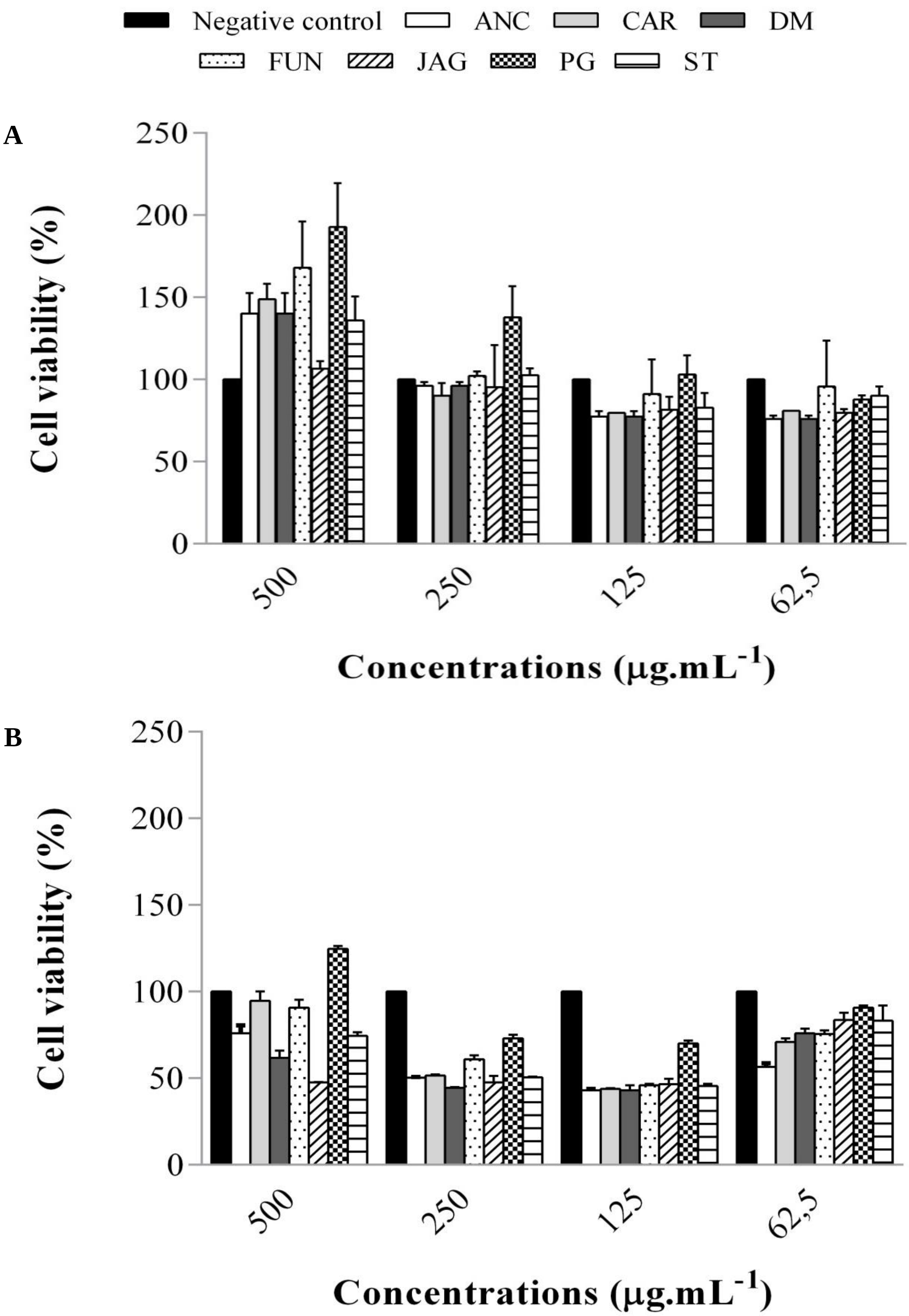


Figure 1: *In vitro* cytotoxicity in human lymphocytes (A) and cells sarcoma 180 cells (B) obtained by the methyl tetrazolium (MTT) assay after exposure to hydroalcoholic extracts of *M. charantia* from seven populations at dosage of 500, 250, 125 and 62,5 μg.mL^{-1}, for 24h. ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa. Data presented as percentage and all the values are expressed as mean ± SE (n=3); SE: standard error.

Principal Component Analysis (PCA) was performed to analyze differences between samples based on their metabolite profiles and antioxidant activity to verify the determinant factor for antitumor activity in vitro (Figure 2). The PCA is a multivariate technique that exploits data to identifying patterns in data, and expressing the data in such a way as to highlight their similarities and differences. The main advantage of PCA is reduction of dimensionality of a data, without much loss of information and it has proven to be a valuable tool to understand relationships between characteristics as well as between populations (Jollife, 2011; Everitt; Dunn, 2013). In the present study, two principal component (PC) of the score plot explained 61.87 % of the total variance. PCA results demonstred that the first componente (PC1) accounted for 38.11 % of variance and the second componente (PC2) for 23.77 % variance. The data indicate that content gallic acid, chlorogenic acid, quercetin, luteolin, TPC, TTC, TFC (data not show) and Fe^{2+} chelanty contributed most and positively to the PC1, with a satisfactory degree of correlation, while the correlation with the DPPH, ABTS (data not show) and antitumoral activity (S180) were inverse. The PC2 was mainly related to Fe^{2+} chelanty, quercetin and luteolin. From this biplot, it was observed that samples JAG and FUN they are positive correlation with the amount TPC and TTC and inversely correlated with the ABTS, DPPH and S180 which indicated a great antioxidant and antitumoral activity *in vitro* of these populations.

The DM and ANC were the samples with the highest values of DPPH and ABTS indicating a low atioxidant activity and are negatively correlated with the variables that measured the phenolic compounds (TPC, TFC and TTC) were the samples with the lowest amounts of compounds phenolic (data not show), however, such samples showed a great antiproliferative activity against sarcoma 180 cells, as well as the other extracts except the PG sample that did not present antitumoral activity in vitro. PG and CAR are grouped by the values of Fe^{2+} chelanty, gallic acid and TFC, but, different of the PG sample, CAR was cytotoxic against sarcoma 180. This multivariate analysis showed that it is not possible to establish the determinant variable for the antiproliferative activity of HAEs of *Momordica charantia*.

The anticancer activity *in vitro* of *Momordica charantia* extract has been enough reported and this effect is probably related to its ability to regulate the cell cycle and induce apoptosis in tumor cells. Weng et al. (2013), demonstred that the triterpene isolated from a crude extract of wild bitter gourd, induced apoptosis in breast cancer cells. And the author suggests that the basic mechanism for antitumor activity of wild bitter gourd is the activation of peroxisome proliferator-activated receptor γ, which acts in regulating lipid metabolism, insulin sensitivity, apoptosis, and cell differentiation is well recognized. Rajamoorthi et al. (2013), observed a reduced cell proliferation in Head and neck squamous cell carcinoma (HNSCC) after treatment of *M. charantia* extract suggesting that bitter melon inhibited c-Met signaling pathway in HNSCC cells. This pathway regulates tissue remodelling, which underlies developmental morphogenesis, wound repair, organ homeostasis and cancer metastasis, therefore, its activation can lead to tumor growth and metastatic progression of cancer cells.

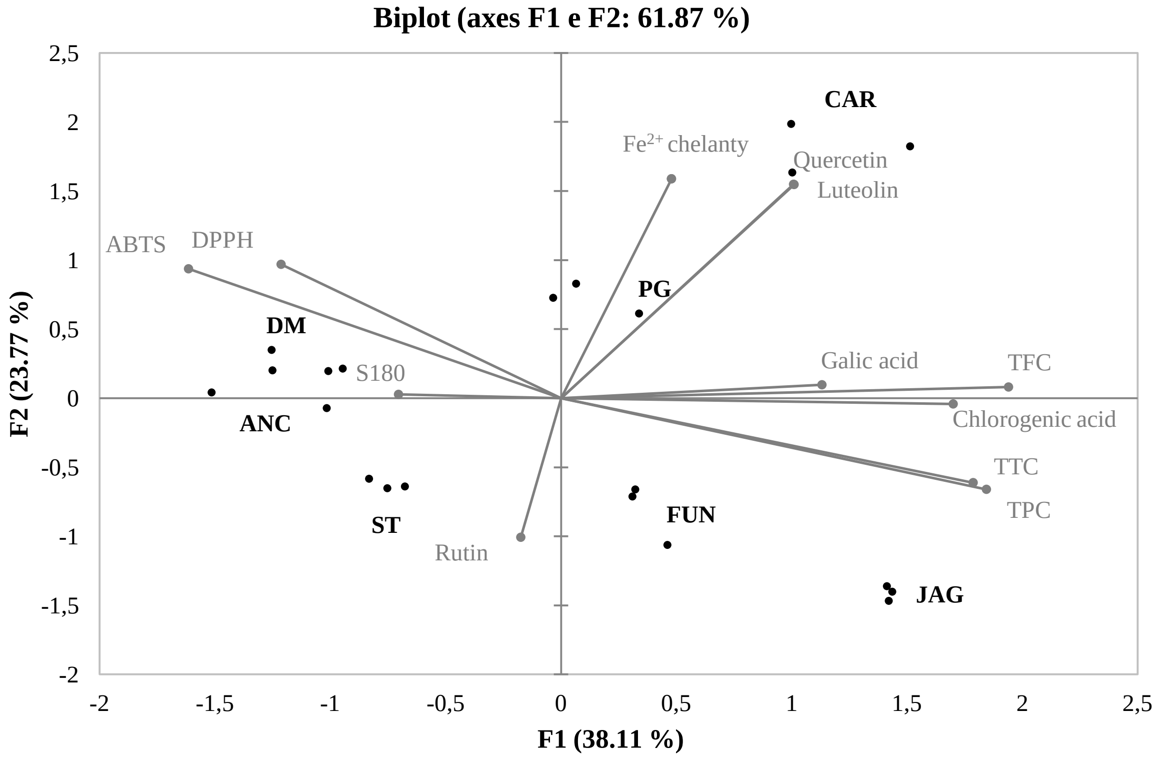


Figure 2: Principal components analysis (PCA) of the phytochemical profiles of *M. charantia* populations, their antioxidant and antitumoral *in vitro* activity. ANC: Anchieta; CAR: Cariacica; DM: Domingos Martins; FUN: Fundão; JAG: Jaguaré; PG: Praia Grande; ST: Santa Teresa.

Cancer is is the second cause of death globally and many drugs used in cancer prevention and therapy present compounds isolated from natural products obtained from various sources. Several plants are described with potential antitumor activity (Rayan et al., 2017; Demain and Vaishnav, 2011). Therefore, the presente study investigates the antitumour effects of the *Momordica charantia* hydroalcoholic extract of the JAG sample, in tumor cell line sarcoma 180. The results for the effects of *Momordica charantia* on sarcoma 180 ascites model in mice are shown in Table 2. In comparison to sarcoma group, animals receiving JAG HAE at the treatment dose of 20.0, 10.0 or 5.0 mg/kg b.w. showed that was no significant difference in the tumor weight any of the doses tested. These results can be due absence uniformity in the tumors size. In previous studies performed by Dutra et al. (2018) heterogeneity was also observed in tumor size of mice induced with sarcoma 180 tumors. However, although there is no significant difference between the weights of the tumors, due to the heterogeneity of tumor size, the extract induces tumor reduction against sarcoma *in vivo.* In previous studies by Dutra et al. (2018) also did not find uniformity in tumor sizes. According to the Chignola et al. (2011), that this growth variability is an intrinsic property of each tumor, so each individual develops the tumor differently. However, whereas the results show that the extract doses of 5.0 and 10.0 mg/kg b.w. present 59.55% and 90.55%, respectively, of the tumor inhibition growth.

Table 2: Tumor weight of Swiss albino mice sarcoma induced treated with *M .charantia* extract (5.0, 10.0 or 20.0 mg.kg^{-1} bw).

Treatment	**Tumor weight (g)**	***p***	**% Tumor Inhibition**
Sarcoma + NaCl (0.9%)	0.438±0.127	–	–
Sarcoma + *M. charantia* 5.0 mg/kg b.w.	0.177 ±0.047	0.7460	59.55
Sarcoma + *M. charantia* 10.0 mg/kg b.w.	0.041±0.020	0.4127	90.55
Sarcoma + *M. charantia* 20.0 mg/kg b.w.	0.826 ±0.222	0.5317	–

CONCLUSIONS

Comparative study of seven *Momordica charantia* populations from Brazil showed that geografical location not interfere in determining of their biological properties. The results of this study indicate that the antioxidant and antitumor activit no are influenced by factors of the environmental growing. We suggest that the anticancer activity of *M. charantia* extract is probably due to its ability to regulate the cell cycle and induce apoptosis in cells, leading to reduce tumour growth. The results suggest that *M. charantia* presents great therapeutic potential and can be used for the development of new drugs. Further studies are required to know the exact cause for the antitumor activity and to standardize the ideal growing environment and the production of the extract should be performed aimed at improve anticancer effects *in vivo.*

ACKNOWLEDGEMENTS

The authors thank Fundação de Amparo a Pesquisa do Estado da Espírito Santo (FAPES), for their financial support to research.

REFERENCES

1. Alshehri, M. A. Anticancer activity of methanolic extarct of *Momordica charantia* against human colon, liver and breast cancer cell lines- *In vitro*, Journal of Biology 2016, Agriculture and Healthcare, 6(6), 106-112 .

2. Anilakumar, K. R.; Kumar, G. P.; Ilaiyaraja, N. Nutritional, Pharmacological and Medicinal Properties of *Momordica charantia*. International Journal of Nutrition and Food Sciences 2015, 4(1), 75-83.

3. Boulila A, Sanaa A, Salem IB, Rokbeni N, M'Rabet Y, Hosni K, Fernandez X. Antioxidant properties and phenolic variation in wild populations of *Marrubium vulgare* L. (Lamiaceae). Industrial Crops and Products 2015;76:616-622.

4. Chao CY, Sung PJ, Wang WH, Kuo YH. Anti-inflammatory effect of *Momordica charantia* in sepsis mice. Molecules 2014 Aug 21;19(8):12777-88.

5. Costa JGM, Nascimento EMM, Campos AR, Rodrigues FFG. Antibacterial activity of *Momordica charantia* (Curcubitaceae) extracts and fractions. Journal of Basic and Clinical Pharmacy 2010;2(1):45-51.

6. Demain AL, Vaishnav P. Natural products for cancer chemotherapy. Microbial biotechnology 2011;4(6):687-699.

7. Deorukhkar A, Krishnan S, Sethi G, Aggarwal BB. Back to basics: how natural products can provide the basis for new therapeutics. Expert Opinion on Investigational Drugs 2007;16(11):1753-1773.

8. Dia VP, Krishnan HB. BG-4, a novel anticancer peptide from bitter gourd (*Momordica charantia*), promotes apoptosis in human colon cancer cells. Scientific Reports 2016;6:33532.

9. Dinakaran S, Vamshi Sharathnath K, Yogeswaran P, Avasarala H, Sudhakar K, Sudha P, Banji D. A medicinal potency of *Momordica charantia*. International Journal of Pharmaceutical Sciences Review and Research, 2010, p. 95-100.

10. Jang E, Kim SY, Lee NR, Yi CM, Hong DR, Lee WS, Kim JH, Lee KT, Kim BJ, Lee JH et al. Evaluation of antitumor activity of Artemisia capillaris extract against hepatocellular carcinoma through the inhibition of IL-6/STAT3 signaling axis. Oncol Rep. 2017 Jan;37(1):526-532.

11. Jilka, C. et al. *In vivo* antitumor activity of the bitter melon (*Momordica charantia*).Cancer research 1983, 43 (11), p. 5151-5.

12. Lee J, Jung Y, Shin J-H, Kim KH, Moon CB, Ryu HD, Hwang G-S. Secondary Metabolite Profiling of Curcuma Species Grown at Different Locations Using GC/TOF and UPLC/Q-TOF MS. Molecules 2014;19(7).

13. Lipina C, Hundal HS. Modulation of cellular redox homeostasis by the endocannabinoid system. Open Biology 2016;6(4):150276.

14. Lu, K. H. et a.Wild bitter gourd protects against alcoholic fatty liver in mice by attenuating oxidative stress and inflammatory responses. Food & Function 2014, 5(5), 1027-37.

15. Malik ZA, Singh M, Sharma PL. Neuroprotective effect of Momordica charantia in global cerebral ischemia and reperfusion induced neuronal damage in diabetic mice. J Ethnopharmacol 2011;133(2):729-34.

16. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol.Methods 1983, 65: 55-63.

17. Najafabad, A.; Jamei R. Free radical scavenging capacity and antioxidant activity of methanolic and ethanolic extracts of plum (*Prunus domestica* L.) in both fresh and dried samples. Avicenna J Phytomed. 2014, 4(5):343-53.

18. Parisotto EB, Michielin EMZ, Biscaro F, Ferreira SRS, Filho DW, Pedrosa RC. The antitumor activity of extracts from *Cordia verbenacea* D.C. obtained by supercritical fluid extraction. The Journal of Supercritical Fluids 2012;61:101-107.

19. Rajendran K, Vikram Reddy E, Khanna A. Anticancer effect of *Mesua ferrea* extracts on Human Pancreatic Cancer Cell line. 2016. 198-205 p.

20. Ramachandra S, Vishwanath P, Nataraj S, Akila P, Rangaswamy C, H B. In vitro anti-cancer activity of ethanolic extract of *Momordica charantia* on cervical and breast cancer cell lines. 2015.

21. Rayan A, Raiyn J, Falah M. Nature is the best source of anticancer drugs: Indexing natural products for their anticancer bioactivity. PLOS ONE 2017;12(11):e0187925.

22. Rice-Evans, C.; Miller, N.; Paganga, G. Antioxidant properties of phenolic compounds. Trends in Plant Science 1997, v. 2, n. 4, p. 152-159.

23. Rufino, M.S.M.; Alves, R.E.; Brito, E.S.; Morais, S.M.; Sampaio, C.G.; Pérez-Jiménez, J.; Saura-Calixto, F. Comunicado técnico-metodologia científica: Determinação da atividade antioxidante total em frutas pela captura do radical livre DPPH. Fortaleza Embrapa 2007, 1, 1–4.

24. Santos, K. K., et al. Trypanocide, cytotoxic, and antifungal activities of *Momordica charantia*. Pharmaceutical Biology 2012, 50(2), 162-166.

25. Shanmugam MK, Kannaiyan R, Sethi G. Targeting Cell Signaling and Apoptotic Pathways by Dietary Agents: Role in the Prevention and Treatment of Cancer. Nutrition and Cancer 2011;63(2):161-173.

26. Sithisarn, P.; Rojsanga, P. Anticancer Effects of Some Medicinal Thai Plants. In Badria, F. A. (Eds). Natural Products and Cancer Drug Discovery 2017, Mansoura University, Egypt, p. 25-41.

26. Tan SP, Stathopoulos C, Parks S, Roach P. An Optimised Aqueous Extract of Phenolic Compounds from Bitter Melon with High Antioxidant Capacity. Antioxidants (Basel) 2014;3(4):814-29.

27. TIZZIANI, T. et al. Antitumor screening of crude extracts of ten medicinal plants of Polygala genus from Southern Brazil. Journal of Applied Pharmaceutical Science 2017, p. 79-83.

28. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA: A Cancer Journal for Clinicians 2015;65(2):87-108.

29. Wei L, Shaoyun W Fau - Shutao L, Shutao L Fau - Jianwu Z, Jianwu Z Fau - Lijing K, Lijing K Fau - Pingfan R, Pingfan R. Increase in the free radical scavenging capability of bitter gourd by a heat-drying process. (2042-650X (Electronic)).

30. WHO. Cancer. World Health Organization, Geneva, 2018. Available from: ttps://www.who.int/news-room/fact-sheets/detail/cancer.

31. Wong, F.C.; Yong, A. L.; Ting, E. P.; Khoo, S. C.; Ong, H. C.; Chai, T. T. Antioxidant, Metal Chelating, Anti-glucosidase Activities and Phytochemical Analysis of Selected Tropical Medicinal Plants. Iran J Pharm Res. 2014,13(4),1409-15.

5. CONSIDERAÇÕES FINAIS

Nos estudos com a espécie *Varronia curassavica*

- A caracterização genética de diferentes populações do estado do Espírito Santo de *Varronia curassavica*, por meio da utilização dos marcadores moleculares ISSR, mostrou que os índices de similaridade entre essas populações variaram de 0,402 a 0,720, demonstrando uma similaridade interpopulacional relativamente alta. E a partir da análise desses resultados sugere-se que a similaridade genética entre as populações de *V. curassavica* pode estar relacionada com as estratégias de reprodução da espécie, tanto no que diz respeito à polinização quanto à dispersão de sementes;

- As quantificações de compostos fenólicos realizadas por espectrofotometria e HPLC mostraram a ocorrência de uma variação quantitativa nos teores dessas substâncias entre as diferentes populações;

- Os resultados obtidos a partir da avaliação da atividade antioxidante pelos ensaios ABTS, DPPH, FRAP e atividade quelante do Fe^{2+}, demonstraram que *V. curassavica* apresenta uma atividade antioxidante significativa, porém é mais eficiente quando utilizado o sistema ABTS. Além disso, esses resultados mostram que a atividade antioxidante varia de acordo com o local de desenvolvimento das populações, evidenciando que essa atividade biológica dos compostos químicos da espécie, nas populações estudadas, é influenciada por condições do meio;
- Os estudos comparativos baseados nas análises fitoquímicas, antioxidante e genética de populações de *V. curassica*, mostram que os fatores ambientais foram mais determinantes para a produção de compostos fenólicos, bem como para a atividade antioxidante, pois não houve correlação entre o agrupamento genético e agrupamento químico;
- Com as análises da atividade antitumoral, observou-se que os extratos de *V. curassica* apresentam uma citotoxicidade seletiva para células tumorais, sarcoma 180. A variação do efeito antiproliferativo entre as diferentes populações demonstram que essa propriedade é influenciada por fatores do ambiente de crescimento da planta.

Nos estudos com a espécie *Momordica charantia*

- O perfil genético de *M. charantia* também mostrou que há uma similaridade alta entre as populações desta espécie, visto que os índices de similaridade variaram de 0,651 a 0,851. E essa similaridade não corresponde à localização geográfica, podendo estar relalcionada com as formas de reprodução e dispersão de sementes da espécie, que apresenta uma variedade de insetos polinizadores e animais dispersores o que pode contribuir com o fluxo gênico entre as populações e consequentemente com a redução da variação genética interpopulacional;

- As análises fitoquímicas das diferentes populações de *M. charantia*, realizadas por espectrofotometria e HPLC, mostraram a ocorrência de variação quantitativa significativa nos teores de compostos fenólicos entre as esssas populações. Entretanto, essa diferença interpopulacional foi pequena ao compararmos com a espécie *V. curassavica*;

- A avaliação da atividade antioxidante dos extratos de *M. charantia* realizada pelos ensaios ABTS, DPPH e atividade quelante do Fe^{2+}, demonstrou que esta espécie apresenta uma atividade antioxidante significativa apenas no ensaio da atividade quelante do Fe^{2+};

- As análises multivariadas utilizadas para comparar o perfil genético e químico de populações de *M. charantia* segerem que, a produção de compostos fenólicos e sua atividade antioxidantesão influenciadas da mesma maneira por fatores genéticos e ambientais, pois, apesar da não correlação entre os agrupamentos genético e químico, a similaridade foi alta entre as populações desta espécie;

- Assim como observado em *V. curassica*, *M. charantia* também apresenta uma citotoxicidade seletiva para células tumorais, sarcoma 180. Entretanto, não há uma grande variação do efeito antiproliferativo entre as populações, sugerindo que nesta espécie a atividade antitumoral não é influenciada pela localização geográfica.

REFERÊNCIAS

ABDIN, M. Z.; ALAM, P. Genetic engineering of artemisinin biosynthesis: prospects to improve its production. **Acta Physiol Plant**, v. 37 , p. 1-12, 2015.

AGUIAR, J. R. et al. Disparities in cancer epidemiology and care delivery among Brazilian indigenous populations. **Einstein (São Paulo),** v. 14, p. 330-337, 2016.

AHAMAD, J.; AMIN, S.; SHOWKAT, R. M. *Momordica charantia* Linn. (Cucurbitaceae): Review on Phytochemistry and Pharmacology. **Research Journal of Phytochemistry,** v. 11, p. 53-65, 2017.

ALMEIDA, V. L. D. et al. Câncer e agentes antineoplásicos ciclo-celular específicos e ciclo-celular não específicos que interagem com o DNA: uma introdução. **Química Nova,** v. 28, p. 118-129, 2005.

ALQAHTANI, A. et al. Seasonal Variation of Triterpenes and Phenolic Compounds in Australian *Centella asiatica* (L.) Urb. ***Phytochem. Anal.***, v. 26, p. 436–443, 2015.

ALSHEHRI, M. A. Anticancer activity of methanolic extarct of Momordica charantia against human colon, liver and breast cancer cell lines- In vitro. **Journal of Biology, Agriculture and Healthcare**, v. 6, n. 6, 2016.

AMATATONGCHAI, M. et al. Simple flow injection for screening of total antioxidant capacity by amperometric detection of DPPH radical on carbon nanotube modified-glassy carbon electrode. **Talanta,** v. 97, p. 267-272, 2012.

ANGELO, P. M.; JORGE, N. Compostos fenólicos em alimentos – Uma breve revisão. **Revista Instituto Adolfo Lutz**, v. 66, n. 1, p. 1–9, 2007.

ARCHAK, S. et al. Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (Anacardium occidentale L.) accessions of India. **Genome,** v. 46, n. 3, p. 362-369, 2003.

AUDIC, Y.; HARTLEY, R. S. Post-transcriptional regulation in cancer – Review. **Biology of the Cell,** v. 96, p. 479–498, 2004.

AZEVEDO, E. **Guia de Plantas Medicinais: Manual do botânico-fitoterapeuta.** Editora: Vegetall. 109p. 2017.

BALASUNDRAM N, SUNDRAM K, SAMMAN S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. **Food Chemistry**, v. 99, p. 191-203, 2006.

BALDIVIA, D.D.S. et al. Evaluation of In Vitro Antioxidant and Anticancer Properties of the Aqueous Extract from the Stem Bark of *Stryphnodendron adstringens*. **Int. J. Mol. Sci.** v. *1*9, p. 2432, 2018.

BALEA et al. Polyphenolic Compounds, Antioxidant, and Cardioprotective Effects of Pomace Extracts from Fetească Neagră Cultivar. **Oxidative Medicine and Cellular Longevity,** v. 2018.

BARBOSA, K. B. F. et al. Estresse oxidativo: conceito, implicações e fatores modulatórios. **Revista de Nutrição,** v. 23, p. 629-643, 2010.

BARREIROS, A. L. B. S.; DAVID, J. M.; DAVID, J. P. Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. **Quím. Nova**, São Paulo , v. 29, p. 113-123, 2006.

BARRETT, S.C.H. & RICHARDS, J. H. Heterostyly in tropical plants. **Memoirs of the New York Botanical Garden 55**. p. 35-61,1990.

BATTESTIN, V.; KATSUMI, L.; ALVES, G. Fontes e aplicações de taninos e tanases em alimentos. **Alimentos e Nutrição**, v. 15, p. 63-72, 2004.

BAYEUX, M.; FERNANDES, A.; FOGLIO, M.; CARVALHO, J. Evaluation of the antiedematogenic activity of artemetin isolated from *Cordia curassavica* DC. **Braz. J. Med. Biol. Res.** v. 35, p. 1229-1232, 2002.

BENDAOUD, H. et al. Chemical Composition and Anticancer and Antioxidant Activities of *Schinus Molle* L. and *Schinus Terebinthifolius* Raddi Berries Essential Oils. **Journal of Food Science,** v. 75, n. 6, p. 2010.

BENZIE, I. F. F.; DEVAKI, M. The ferric reducing/antioxidant power (FRAP) assay for non-enzymatic antioxidant capacity: concepts, procedures, limitations and applications. In: Apak, R.; Capanoglu, E.; Shahidi, F. (Ed.). **Measurement of Antioxidant Activity & Capacity,** pp.77-106, 2017.

BERTAN, I. et al. Comparação de métodos de agrupamento na representação da distância morfológica entre genótipos de trigo. **Revista Brasileira de Agrociência,** v. 12, n. 3, p. 279-286, 2006.

BIANCHI, M. D. L. P.; ANTUNES, L. M. G. Radicais livres e os principais antioxidantes da dieta. **Revista de Nutrição,** v. 12, p. 123-130, 1999.

BOLINA, C. O. **Metabolismo, desenvolvimento e composição química de *Varronia curassavica* Jacq.** EM FUNÇÃO DA SUPRESSÃO DA IRRIGAÇÃO. Tese (Doutorado). Faculdade de Ciências Agronômicas da UNESP, Botucatu - SP, 2015.

BOLZANI, V. S.; VALLI, M.; PIVATTO, M.; VIEGAS JÚNIOR, C. Natural products from Brazilian biodiversity as a source of new models for medicinal chemistry. **Pure and Applied Chemistry**, v. 84, n.9, p.1837-1937, 2012.

BOUGHTON-SMITH, N. K.; HAWKEY, C. J.; WHITTLE, B. J. Biosynthesis of lipoxygenase and cyclo-oxygenase products from [14C]-arachidonic acid by human colonic mucosa. **Gut,** v. 24, n. 12, p. 1176-1182, 1983.

BRASIL. Ministério da Saúde. Instituto Nacional do Câncer José de Alencar Gomes da Silva (INCA). **Estimativas 2018: incidência de câncer no Brasil.** Rio de Janeiro (RJ): INCA; 2018.

_______. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. **Política Nacional de Atenção Básica.** 4 ed. Brasília: Ministério da Saúde, p. 68, 2007.

_______. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. **Política Nacional de Práticas Integrativas e Complementares no SUS – PNPIC – SUS.** Brasília: Ministério da Saúde, p. 92, 2006.

_______. Ministério da Saúde. Secretaria de Ciência, Tecnologia e Insumos Estratégicos. Departamento de Assistência Farmacêutica. Atenção à Saúde.

Departamento de Atenção Básica. **Política Nacional de Plantas Medicinais e Fitoterápicos.** Brasília: Ministério da Saúde, p. 60, 2006.

BRITO, F. A. et al. Genetic diversity analysis of *Varronia curassavica* Jacq. accessions using ISSR markers. **Genetics and Molecular Research,** v. 15, p. 1-10, 2016.

BURDA, S.; OLESZEK, W. *Antioxidant and Antiradical Activities of Flavonoids.* ***Journal of Agricultural and Food Chemistry,*** *v. 49, p. 2774–2779,* 2001.

CAMACHO, M. et al. Prostaglandin E2 pathway in head and neck squamous cell carcinoma. **Head & Neck,** v. 30, n. 9, p. 1175-1181, 2008.

CAROLLO, C. A. **Análise fitoquímica e avaliação dos efeitos dos tipos de adubação, da radiação e do estresse hídrico, no acúmulo de metabólitos secundários em espécies do gênero *Mikania*.** 2008. 228f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2008.

CARVALHO, P. M. et al. Chemical composition and antimicrobial activity of the essential oil of *Cordia verbenacea* D.C. **Journal of Ethnopharmacology,** v. 95, n. 2, p. 297-301, 2004.

CERÓN-GARCÍA, A. et al. Oligoglucan Elicitor Effects During Plant Oxidative Stress. In: Bubulya, P (Eds). **Cell Metabolism - Cell Homeostasis and Stress Response**. InfotechOpen, p. 1-13, 2012.

CHALKER-SCOTT, L.; FUCHIGAMI, L. H. **The role of phenolic compounds in plant stress responses**. In: LI, P. H. Low Temperature Stress Physiology 1n Crops. Editora CRC Press, p. 68-76, 1989.

CHAO, C. Y. et al. Anti-inflammatory effect of *Momordica charantia* in sepsis mice. **Molecules,** 19(8), 12777-88, 2014.

CHEYNIER, V. Phenolic compounds: from plants to foods. **Phytochemistry Reviews,** v. 11, p. 153-177, 2012.

CHOI, J. S.; KIM, H. Y.; SEO, W. T.; LEE, J. H; CHO, K. M. Roasting enhances antioxidant effect of bitter melon (*Momordica charantia* L.) increasing in flavan-3-ol and phenolic acid contents. **Food Sci. Biotechnol.** v. 21, p. 19-26, 2012.

CIRINO, C. J., LIN, C. M.; MARIANNE, C. S. **Cultivo de plantas medicinais, condimentares e aromáticas,** Curitiba: Emater, p. 128, 1991.

COSTA, J. G. M. et al. Antibacterial activity of *Momordica charantia* (Curcubitaceae) extracts and fractions. **Journal of Basic and Clinical Pharmacy**, India**,** v. 2, n. 1, p. 45-51, 2010.

CORREIA, N. M.; ZEITOUM, V. Controle químico de melão-de-são-caetano em área de cana-soca. **Bragantia**, Campinas , v. 69, n. 2, p. 329-337, 2010.

COWAN, M. M. Plant products as antimicrobial agents. **Clinical Microbiology Reviews,** v. 12; p. 564–582, 1999.

CROZIER, A.; JAGANATH, I. B.; CLIFFORD, M. N. Phenols, Polyphenols and Tannins: An Overview. In: CROZIER, A.; CLIFFORD, M. N.; ASHIHARA, H. (Eds). **Plant Secondary Metabolites**, 2006.

CRUSZ, S. M.; BALKWILL, F. R. Inflammation and cancer: advances and new agents. **Nature Reviews Clinical Oncology,** v. 12, p. 584, 2015.

CUNHA, F. A. B. et al. Cytotoxic and antioxidative potentials of ethanolic extract of *Eugenia uniflora* L. (Myrtaceae) leaves on human blood cells. **Biomedicine & Pharmacotherapy,** v. 84, p. 614-621, 2016.

CUONG, D. M. et al. Identification and Characterization of Phenylpropanoid Biosynthetic Genes and Their Accumulation in Bitter Melon (*Momordica charantia*). **Molecules,** v. 23, n. 2, 2018.

DEGÁSPARI, C.; WASZCZYNSKYJ, N. Propriedades antioxidantes de compostos fenólicos. **Visão Acadêmica,** v. 5, n. 2, p. 33-40, 2004.

DEL RÉ, P. V.; JORGE, N. Especiarias como antioxidantes naturais: aplicações em alimentos e implicação na saúde. **Revista Brasileira de Plantas Medicinais,** v. 14, p. 389-399, 2012.

DIAS, L. A. S. Análises multidimensionais. In: ALFENAS, L.C. (Ed.). **Eletroforese de isoenzimas e proteínas afins: fundamentos e aplicações em plantas e microrganismos.** Viçosa: Editora UFV, 1998. p.405-75.

DINIZ, M. F. F.; OLIVEIRA, R. A. G.; MEDEIROS, A. C. D.; MALTA Jr., A. **Memento fitoterápico: as plantas como alternativa terapêutica aspectos populares e científicos.** João Pessoa: Editora Universitária/UFPB, p. 205, 1997.

EL-SAID, S. M.; AL-BARAK, A. S.. Extraction of Insulin like Compounds from Bitter Melon Plants. **American Journal of Drug Discovery and Development,** v. 1, p. 1-7, 2011.

FEIJÓ, E. V. R. D. S.; OLIVEIRA, R. A. D.; COSTA, L. C. D. B. Light affects Varronia curassavica essential oil yield by increasing trichomes frequency. **Revista Brasileira de Farmacognosia,** v. 24, p. 516-523, 2014.

FERNANDES, E. S. et al. Anti-inflammatory effects of compounds alpha-humulene and (-)-trans-caryophyllene isolated from the essential oil of *Cordia verbenacea*. **Eur J Pharmacol,** v. 569, n. 3, p. 228-36, 2007.

FERNANDES, T. M. **Plantas medicinais: memória da ciência no Brasil.** Rio de Janeiro: EDITORA FIOCRUZ, p. 260, 2004.

FLORA, S. J. S. Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. **Oxidative medicine and cellular longevity,** v. 2, n. 4, p. 191-206, 2009.

GANDERS, F. R. The biology of heterostyly. **New Zealand Journal of Botany,** v. 17, n. 4, p. 607-635, 1979.

GARCIA-BUENO, B.; LEZA, J. C. Inflammatory/anti-inflammatory mechanisms in the brain following exposure to stress. **Rev Neurol,** v. 46, n. 11, p. 675-83, 2008.

GASPARETTO, A. et al. Seasonal variation in the chemical composition, antimicrobial and mutagenic potential of essential oils from *Piper cernuum*. **Industrial Crops and Products,** v. 95, p. 256-263, 2017.

GILBERT, B; FAVORETO, R. *Cordia verbenaceae* DC Boraginaceae. **Revista Fitos,** v. 7, 2012.

GOBBO-NETO, L.; LOPES, N. P. Plantas medicinais: Fatores de influência no conteúdo de metabólitos secundários. **Quim. Nova***,* v. 30, n. 2, p. 374-381, 2007.

GOLENIOWSKI, M. et al. Phenolic Acids. In: K.G. Ramawat and J.M. Me´rillon (eds.). **Natural Products**, Springer- Verlag Berlin Heidelberg, p.1951-1973, 2013.

GOMES, C. A. et al. Anticancer Activity of Phenolic Acids of Natural or Synthetic Origin: A Structure−Activity Study. **Journal of Medicinal Chemistry,** v. 46, n. 25, p. 5395-5401, 2003.

GOTTSCHLING, M.; HILGER, H.H.; WOLF, M.; DIANE, N. Secondary Structure of the ITS1 Transcript and its Application in a Reconstruction of the Phylogeny of Boraginales. **Plant Biology,** v. 3, p. 629-636, 2001.

GOUVEA, D. R. et al. Seasonal variation of the major secondary metabolites present in the extract of *Eremanthus mattogrossensis* Less (Asteraceae: Vernonieae) leaves. **Química Nova,** v. 35, p. 2139-2145, 2012.

GROVER, A.; SHARMA, P. C. Development and use of molecular markers: past and present. **Critical Reviews in Biotechnology,** v. 36, p. 290–302, 2014.

GÜLÇIN, İ. Antioxidant activity of food constituents: an overview. **Archives of Toxicology,** v. 86, n. 3, p. 345-391, 2012.

HAGE MELIN L.I.S. **Estudo das interações entre fosfolipases A2 e o inibidor vegetal, ácido rosmarínico de *Cordia verbenaceae* (Boraginaceae) cocristalização e modelagem molecular.** 2009. 171 p. Universidade de São Paulo. Faculdade de Ciencias de Ribeirao Preto. Ribeirao Preto, 2009.

HAN, X. Z.; SHEN, T.; LOU, H. X. Dietary polyphenols and their biological significance. **Int J Mol Sci,** v. 8, p. 950–988, 2007.

HARB, T. B. et al. Ensaio em microplaca do potencial antioxidante através do sistema quelante de metais para extratos de algas. **Instituto de Biociências, Universidade de São Paulo,** p. 1-5, 2016.

HARDMAN SÁTIRO DE LUCENA FILHO, J. et al. Antimicrobial Potential of *Momordica charantia* L. against Multiresistant Standard Species and Clinical Isolates. **J Contemp Dent Pract.,** v. 16, p. 854-858, 2015.

HAVSTEEN B. H. The biochemistry and medical significance of the flavonoids. **Pharmacology and Therapeutics***,* v.96, p. 67–202, 2002.

HOELTGEBAUM, M. P. et al. Diversidade e estrutura genética de populações de *Varronia curassavica* Jacq. em restingas da Ilha de Santa Catarina. **Revista Brasileira de Plantas Medicinais,** v. 17, p. 1083-1090, 2015.

HOELTGEBAUM, MARCIA P**. CARACTERIZAÇÃO DA AUTOECOLOGIA DE POPULAÇÕES DE *Varronia curassavica* Jacq. (BORAGINACEAE) EM AMBIENTE DE RESTINGA DE SANTA CATARINA.** Tese (Doutorado) - Programa de Pósgraduação em Recursos Genéticos Vegetais, Universidade Federal de Santa Catarina, Florianópolis – SC, 2017.

HOELTGEBAUM, M. P. et al . Reproductive Biology of *Varronia curassavica* Jacq. (Boraginaceae). **An. Acad. Bras. Ciênc.**, Rio de Janeiro , v. 90, n. 1, p. 59-71, Mar. 2018 .

HOLLMAN, P. C. H. Absorption, bioavailability, and metabolism of flavonoids. **Pharmaceutical Biology***,* v. 42, p. 74–83, 2004.

HOLLMAN, P. C.; KATAN, M. Dietary Flavonoids: Intake, Health Effects and Bioavailability. **Food and Chemical Toxicology,** v. 37, n. 9–10, p. 937–942,1999.

HOSSAIN, S. J. et al. Total Phenolic Content, Antioxidative, Anti-amylase, Anti-glucosidase, and Antihistamine Release Activities of Bangladeshi Fruits. **Food Science and Technology Research,** v. 14, n. 3, p. 261-268, 2008.

HUANG, L. D; BACKHOUSE, D. Effects of Fusarium species on defence mechanisms in sorghum seedlings. **N.Z Plant Protection,** v.57, p.121-124, 2004.

HUANG, M.; FERRARO, T. Phenolic Compounds in Food and Cancer Prevention. In: Huang, M.; Ho, C.; Lee, C. Y. **Phenolic Compounds in Food and Their Effects on Health II.** 8-34, 1992.

HUANG, W.-Y.; CAI, Y.-Z.; ZHANG, Y. Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. **Nutrition and cancer,** v. 62, n. 1, p. 1–20, 2009.

HUFFAKER, A. et al. Novel acidic sesquiterpenoids constitute a dominant class of pathogen-induced phytoalexins in maize. **Plant physiology,** v.156, p.2082-2097, 2011.

JAAFAR, H. Z. E.; IBRAHIM, M. H.; FAKRI, N. F. M. Impact of soil field water capacity on secondary metabolites, phenylalanine ammonia-lyase (PAL), maliondialdehyde (MDA) and photosynthetic responses of Malaysian Kacip Fatimah (*Labisia pumila* Benth). **Molecules,** v. 17, p. 7305– 7322, 2012.

JIA, S. et al. Recent Advances in *Momordica charantia*: Functional Components and Biological Activities. **International journal of molecular sciences,** v. 18, n. 12, p. 2555, 2017.

JOMOVA, K.; VONDRAKOVA, D.; LAWSON, M.; VALKO, M. Metals, oxidative stress and neurodegenerative disorders. **Molecular and Cellular Biochemistry,** v. 345, p. 91-104, 2010.

JORGE, L.I.F.; SAKUMA, A. M.; INOMATA, E. I. Análise histológica e bioquímica de *Momordica charantia* L. (melão de são Caetano). **Revista do Instituto Adolfo Lutz, São Paulo,** v. 52, p. 23-26, 1992.

KANTARJIAN, H. M.; O'BRIEN, S.; CORTES, J. Homoharringtonine/omacetaxine mepesuccinate: the long and winding road to food and drug administration approval. **Clinical lymphoma, myeloma & leukemia,** v. 13, n. 5, p. 530-533, 2013.

KENNY, O.; SMYTH, T. J; HEWAGE, C. M.; BRUNTON, N. P. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (Trigonella foenum-graecum) seeds and bitter melon (*Momordica charantia*) fruit. **Food Chem.** v. 141; p. 4295-4302, 2013.

KHATIB, A. al. Discrimination of Three Pegaga (Centella) Varieties and Determination of Growth-Lighting Effects on Metabolites Content Based on the Chemometry of H-1 Nuclear Magnetic Resonance Spectroscopy. **J. Agric. Food Chem.,** *v. 60,* p. 410–417, 2011.

KOVÁČIK, J.; BAČKOR, M. Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. **Plant Soil**, v. 297, p. 255–265, 2007.

KREMER, A. A. U. et al. Monitoring genetic diversity in tropical trees with multilocus dominant markers. **Heredity,** v. 95, p. 274– 280, 2005.

KUMAR, S.; PANDEY, A. K. Chemistry and Biological Activities of Flavonoids: An Overview. **The Scientific World Journal,** v. 2013, p. 16, 2013.

KUMMER, C. L.; COELHO, T. C. R. B. Antiinflamatórios não esteróides inibidores da ciclooxigenase-2 (COX-2): aspectos atuais. **Revista Brasileira de Anestesiologia,** v. 52, p. 498-512, 2002.

KUNDU, J. K.; SURH, Y.-J. Inflammation: Gearing the journey to cancer. **Mutation Research/Reviews in Mutation Research,** v. 659, n. 1, p. 15-30, 2008.

KWATRA, D. et al. Bitter melon extracts enhance the activity of chemotherapeutic agents through the modulation of multiple drug resistance. **Journal of Pharmaceutical Sciences ,** v. 102 , p. 4444 – 54, 2013.

LEAMAN, D. Conservation, Trade, Sustainability and Exploitation of Medicinal Plant Species. In: Saxena, P. K. **Development of Plant-Based Medicines: Conservation, Efficacy and Safety.** SPRINGER-SCIENCE+BUSINESS MEDIA. Germany, p. 269, 2001.

LEE, S. H. et al. Phenolic Acid, Carotenoid Composition and Antioxidant Activity of Bitter Melon (*Momordica charantia* L.) at Different Maturation Stages. **Int. J. Food Prop.,** 2016.

LI, C.-F. et al. Global transcriptome and gene regulation network for secondary metabolite biosynthesis of tea plant (*Camellia sinensis*). **BMC Genomics,** v. 16, n. 1, p. 560, 2015.

LI, C.-J. et al. *Momordica charantia* Extract Induces Apoptosis in Human Cancer Cells through Caspase- and Mitochondria-Dependent Pathways. **Evidence-based complementary and alternative medicine,** v. 2012, p. 261971-261971, 2012.

LIMA, A. R. et al . Compostos bioativos do café: atividade antioxidante in vitro do café verde e torrado antes e após a descafeinação. **Quím. Nova**, São Paulo , v. 33, n. 1, p. 20-24, 2010.

LIMA, L. F. P. **Estudo taxonômicos e morfopolínicos em Cucurbitaceas Brasileiras.** Tese de Doutorado. Universidade Federal do Rio Grande do Sul, Rio Grande do Sul, 2010.

LIN, D. et al. An Overview of Plant Phenolic Compounds and Their Importance in Human Nutrition and Management of Type 2 Diabetes. **Molecules,** v. 15, p. 1374, 2016.

LIU, L. et al. The antiatherogenic potential of oat phenolic compounds. **Atherosclerosis,** v. 175, n. 1, p. 39-49, 2004.

LIU, Y. et al. A Geranylfarnesyl Diphosphate Synthase Provides the Precursor for Sesterterpenoid (C25) Formation in the Glandular Trichomes of the Mint Species *Leucosceptrum canum*. The Plant Cell, v. 28, n. 3, p. 804, 2016.

LONG, C. et al. Bioactive flavonoids of *Tanacetum parthenium* revisited. **Phytochemistry**, v. 64, n. 2, p. 567–569, 2003.

LÓPEZ-ALARCÓN, C.; DENICOLA, A. Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. **Analytica Chimica Acta*,*** v. 763, p. 1–10, 2013.

LORENZI, H.; MATOS, F. J. A. **Plantas medicinais no Brasil: nativas e exóticas.** 2. ed. Nova Odessa: Instituto Plantarum. 2008.

LU, K. H. et al. Wild bitter gourd protects against alcoholic fatty liver in mice by attenuating oxidative stress and inflammatory responses. **Food Funct.,** v. 5, p. 1027-37. 2014.

LYU, S. W. et al. Effects of mixtures of phenolic acids on phosphorus uptake by cucumber seedlings. **Journal of Chemical Ecology,** v. 16, n. 8, p. 2559-2567, 1990.

LOPES, A. A.; OLIVEIRA, A. M.; PRADO, C. B. C. Principais genes que participam da formação de tumores. **Revista de Biologia e Ciências da Terra**, v. 2, 2002.

MOHARRAM, A. H.; YOUSSEF, M. Methods for Determining the Antioxidant Activity: A Review. Biochemistry & Analytical Biochemistry, p. 31-42, 2014.

MADDOX, C. E.; LAUR, L. M.; TIAN, L. Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. **Current microbiology,** v. 60, n. 1, p. 53-58, 2010.

MAIA, R. R. et al. Efeito antimicrobiano do extrato de *Momordica charantia* linn isolado e em associação com antibióticos sobre *Staphylococcus aureus* multirresistentes. *v. 4,* **Agropecuária Científica no Semi-árido,** 2008.

MALIK, Z. A.; SINGH, M.; SHARMA, P. L. Neuroprotective effect of *Momordica charantia* in global cerebral ischemia and reperfusion induced neuronal damage in diabetic mice. **J Ethnopharmacol,** v. 133, n. 2, p. 729-34, 2011.

MANABE, M. et al. Induction of Anti-inflammatory Responses by Dietary *Momordica charantia* L. (Bitter Gourd). **Bioscience, Biotechnology, and Biochemistry,** v. 67, n. 12, p. 2512-2517, 2003.

MATIAS, E. F. F. et al. Biological Activities and Chemical Characterization of *Cordia verbenacea* DC. as Tool to Validate the Ethnobiological Usage. **Evidence-Based Complementary and Alternative Medicine,** v. 2013, p. 7, 2013.

MATOS, F. J. A. **O formulário fitoterápico do professor Dias da Rocha: Informações sobre o emprego na medicina caseira de plantas do Nordeste, especialmente do Ceará.** 2.ed. Fortaleza: EUFC, 1997. 260p.

MESQUITA, M. L. et al. Cytotoxic activity of Brazilian Cerrado plants used in traditional medicine against cancer cell lines. **Journal of Ethnopharmacology,** v. 123, n. 3, p. 439-445, 2009.

MELO, E.A.; GUERRA, N.B. Ação antioxidante de compostos fenólicos naturalmente presentes em alimentos. **Boletim da Sociedade Brasileira de Ciências e Tecnologias de Alimentos,** v.36, n.1, p.1-11, 2002.

MELLO, J. C. P.; SANTOS, S. C. Taninos. IN: SIMÕES, C. M. O.; SCHENKEL, E. P.; MELLO, J. C. P.; MENTZ, L. A.; PETROVICK, P. R. (Orgs.). **Farmacognosia:**

Do produto natural ao medicamento. 1. Ed. Porto Alegra: Artmed. p. 235-248, 2017.

MEYER, A. DA S. **Comparação de coeficientes de similaridade usados em análises de agrupamento com dados de marcadores moleculares dominantes**. Piracicaba: Escola Superior de Agricultura Luiz de Queiroz, 2002.

MILLER, J.S. New Boraginales from tropical America 5: New names anda typifications for neotropical species os *Cordia* and *Varronia*. **A Journal for Botanical Nomenclature,** v.17, n. 3, p. 372-375, 2007.

MILLER, J. S.; GOTTSCHLING, M. Generic classification in the Cordiaceae (Boraginales): resurrection of the genus *Varronia* P. Br. **Taxon,** v. 56, p. 163-169, 2007.

MILES, E. A.; ZOUBOULI, P.; CALDER, P. C. Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. **Nutrition,** v. 21, n. 3, p. 389-394, 2005.

MONTANARI JR., I. **Variablidade genética em uma população de *Cordia verbenacea* DC. para características agronômicas e fitoquímicas.** Tese (Doutorado) - Faculdade de Ciências Agronômicas, Universidade Estadual Paulista, Botucatu, 2011.

MONTANARI JR., I. Cultivo comercial de erva-baleeira. **Revista Agroecologia Hoje,** v. 3, n.1, p. 14-15, 2000.

MONTEIRO, J. M. et al. Taninos: uma abordagem da química à ecologia. **Química Nova,** v. 28, p. 892-896, 2005.

MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. **J Immunol Methods.,** v. 65, p. 55-63, 1983.

NABAVI, S. M. et al. Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering. **Biotechnology Advances**, p. 30181-2, 2018.

NEWMAN, D. J.; CRAGG, G. M. Natural Products as Sources of New Drugs from 1981 to 2014. **Journal of Natural Products,** v. 79, n. 3, p. 629-661, 2016.

NIMSE, S. B.; PAL, D. Free radicals, natural antioxidants, and their reaction mechanisms. **RSC Advances,** v. 5, n. 35, p. 27986-28006, 2015.

NIZIO, D. A. D. C. et al. Chemical diversity of native populations of *Varronia curassavica* Jacq. and antifungal activity against Lasiodoplodia theobromae. **Industrial Crops and Products,** v. 76, p. 437-448, 2015.

NOGALES, M. et al. Contribution by vertebrates to seed dispersal effectiveness in the Galapagos Islands: a community-wide approach. **Ecology,** v. 98, n. 8, p. 2049-2058, 2017.

NOWICKE, J.W.; MILLER, J.S. Pollen morphology of the Cordioideae (Boraginaceae). Auxemma, *Cordia* and *Patagonula.* **Plant Systematics and Evolution,** v.5, p.103-121, 1990.

NUNES, A. R. et al. Photoprotective potential of medicinal plants from Cerrado biome (Brazil) in relation to phenolic content and antioxidant activity. **Journal of Photochemistry and Photobiology B: Biology,** v. 189, p. 119-123, 2018.

OLIVEIRA, A. C. D. et al. Fontes vegetais naturais de antioxidantes. **Química Nova,** v. 32, p. 689-702, 2009.

OLIVEIRA, D. M. D.; BASTOS, D. H. M. Biodisponibilidade de ácidos fenólicos. **Química Nova,** v. 34, p. 1051-1056, 2011.

OLIVEIRA, G. L. S. Determinação da capacidade antioxidante de produtos naturais in vitro pelo método do DPPH": estudo de revisão. **Revista Brasileira de Plantas Medicinais,** v. 17, p. 36-44, 2015.

OLIVEIRA, M. et al. Phytochemical profile and biological activities of *Momordica charantia* L. (Cucurbitaceae): A review. **African Journal of Biotechnology,** v. 17, p. 829-846, 2018.

OUERGHEMMI, I. et al. Antioxidant and antimicrobial phenolic compounds from extracts of cultivated and wild-grown Tunisian Ruta chalepensis. **Journal of Food and Drug Analysis,** v. 25, n. 2, p. 350-359, 2017.

OZCAN, T. et al. Phenolics in Human Health. **International Journal of Chemical Engineering and Applications,** v. 5, n. 5, p. 393–396, 2014.

PAIVA, C. L. **Acidos fenólicos e aminas bioativas livres e conjugadas em sorgo: teores e atividade antioxidante.** 2014. Tese (Doutorado). Programa de Pós-Graduação em Ciência de Alimentos da Universidade Federal de Minas Gerais, Minas Gerais – MG, 2014.

PAGARE, S. et al. Secondary metabolites of plants and their role: Overview. **Current Trends in Biotechnology and Pharmacy,** p. 293-304, 2015.

PANCHE, A. N.; DIWAN, A. D.; CHANDRA, S. R. Flavonoids: an overview. **Journal of nutritional science,** v. 5, p. e47-e47, 2016.

PARISOTTO, E. B. et al. The antitumor activity of extracts from Cordia verbenacea D.C. obtained by supercritical fluid extraction. **The Journal of Supercritical Fluids,** v. 61, p. 101-107, 2012.

PASSOS, D. et al. Frugivory of *Momordica charantia* (Cucurbitaceae) by *Ameivula ocellifera* (Squamata:Teiidae) in coastal area of northeastern Brazil. *Salamandra,* v. 49, p. 234-236, 2013.

PASSOS, G. F. et al. Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*. **J Ethnopharmacol,** v. 110, n. 2, p. 323-33, 2007.

PATRA, B. et al. Transcriptional regulation of secondary metabolite biosynthesis in plants. **Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms,** v. 1829, n. 11, p. 1236-1247, 2013.

PENALBA, T.; RITA, M. Efectos de los extractos liofilizados de *Momordica charantia* sobre la glucemia em preparaciones caninas anestesiados e conscientes. **Scientia,** v.3, n.2, p.7-16, 1988.

PENG, C. et al. Biology of Ageing and Role of Dietary Antioxidants. **BioMed Research International,** v. 2014, p. 13, 2014.

PIETTA, P.-G. Flavonoids as Antioxidants. **Journal of Natural Products,** v. 63, n. 7, p. 1035-1042, 2000.

PIO CORREA, M. **Dicionário das plantas úteis do Brasil e das exóticas cultivadas.** Rio de Janeiro: Ministério da Agricultura, 1984.

PIRES, I. F. B. et al. Plantas medicinais: cultivo e transmissão de conhecimento em comunidade cadastrada na Estratégia Saúde da Família. **Revista Brasileira de Pesquisa em Saúde**, 18(4): 37-45, 2016.

PRINSLOO, G.; NOGEMANE, N. The effects of season and water availability on chemical composition, secondary metabolites and biological activity in plants. **Phytochem Rev.,** v. 17, p. 889–902, 2018.

PRIOR, R. L., WU, X., & SCHAICH, K. Standardized methods for the determination of antioxidante capacity and phenolics in foods and dietary supplements. **Journal of Agricultural and Food Chemistry,** 53, 4290–4302, 2005.

RAINA, K.; KUMAR, D.; AGARWAL, R. Promise of bitter melon (Momordica charantia) bioactives in cancer prevention and therapy. **Seminars in Cancer Biology,** v. 40-41, p. 116-129, 2016.

RALSTON, R. A.; SCHWARTZ, S. J.; HOSTETLER, G. L. Flavones: Food Sources, Bioavailability, Metabolism, and Bioactivity. **Advances in Nutrition,** v. 8, n. 3, p. 423-435, 2017.

RAKOCZY-TROJANOWSKA, M.; BOLIBOK, H. Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. **Celular & Molecular Biology Letters,** v.9, n.2, p.221-38, 2004.

REDDY, M. P.; SARLA, N.; SIDDIQ, E. A. Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. **Euphytica,** v. 128, p. 9–17, 2002.

REZAEIZADEH, A. ET AL. Determination of antioxidant activity in methanolic and chloroformic extracts of *Momordica charantia.* **African Journal of Biotechnology,** v. 10, p. 4932-4940, 2011.

RIBEIRO, M. V. et al. Diversidade genética entre acessos de espinheira-santa (*Maytenus ilicifolia* Mart. ex Reis.) coletados no estado do Rio Grande do Sul, Brasil. **Revista Brasileira de Plantas Medicinais,** v. 12, p. 443-451, 2010.

SAMPAIO, B. L.; EDRADA-EBEL, R.; BATISTA, F.; COSTA, D. Effect of the environment on the secondary metabolic profile of *Tithonia diversifolia* : a model for environmental metabolomics of plants. **Nat Publ Gr,** p. 1–11, 2016.

SÁNCHEZ-MORENO, C. Compuestos polifenólicos: efectos fisiológicos: actividad antioxidante. **Alimentaria,** v. 329, p. 29-40, 2002.

SANTANA-GÁLVEZ, J.; JACOBO-VELÁZQUEZ, D. A. Classification of Phenolic Compounds. In: Nollet, L. M. L.; Gutierrez-Uribe, J. (Ed.). **Phenolic Compounds in Food: Characterization and Analysis.** CRC Press, Taylor & Francis Group, New York, p. 3-21, 2018.

SANTOS, K. K. et al. Trypanocide, cytotoxic, and antifungal activities of *Momordica charantia*. **Pharm Biol.,** v. 50, p. 162-6, 2012.

SANTOS, V. F. **Estudos das alterações da parede celular durante ativação de mecanismos de defesa em *Momordica charantia* como fator de produção de metabólitos secundários bioativos.** 2013. Dissertação (Mestrado) –

Faculdade de Ciências Farmacêuticas de Ribeirão Preto- Programa de Pós-Graduação em Biociências Aplicadas à Farmácia. Ribeirão Preto - São Paulo, 2013.

SEBBENN, A. M. et al. Modelling the long-term impacts of selective logging on genetic diversity and demographic structure of four tropical tree species in the Amazon forest. **Forest Ecology and Management,** v. 254, n. 2, p. 335-349, 2008.

SERTIE, J. A. et al. Pharmacological assay of *Cordia verbenacea* V: oral and topical anti-inflammatory activity, analgesic effect and fetus toxicity of a crude leaf extract. **Phytomedicine,** v. 12, n. 5, p. 338-44, 2005.

SERTIE, J. A. A. et al. Pharmacological Assay of *Cordia verbenacea* ; Part 1. Anti-Inflammatory Activity and Toxicity of the Crude Extract of the Leaves. **Planta Med.,** v. 54, p. 7-10, 1988.

SEUNG MI, S. et al. Anti-Inflammatory Effect of Bitter Melon (*Momordica charantia*) in RAW 264.7 Cell. **Cancer prevention research,** v. 17, n. 1, p. 56-61, 2012.

SHENG, H. et al. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. **Cancer Research,** v. 58, n. 2, p. 362, 1998.

SHODEHINDE, S. A. et al. Phenolic Composition and Evaluation of Methanol and Aqueous Extracts of Bitter Gourd (*Momordica charantia* L) Leaves on Angiotensin-I-Converting Enzyme and Some Pro-oxidant-Induced Lipid Peroxidation in vitro. **J. Evid. Based. Complement. Altern. Med.** 21(4):1-10, 2016.

SETIA, S.; SANYA, S. N. Downregulation of NF-jB and PCNA in the regulatory pathways of apoptosis by cyclooxygenase-2 inhibitors in experimental lung cancer. **Mol Cell Biochem,** v. 369, p. 75–86, 2012.

SCHENKEL, E. P.; GOSMANN, G.; PETROVISCK, P. R. Produtos de origem vegetal e o desenvolvimento de medicamentos. In: SIMÕES, C. M. O et al. **Farmacognosia: da planta ao medicamento.** 4ª edição Ed. UFSC, 2002.

SHIM, S.-Y.; KIM, H.-S. Oxidative stress and the antioxidant enzyme system in the developing brain. **Korean journal of pediatrics,** v. 56, n. 3, p. 107-111, 2013.

SHOBHA, C. et al. In vitro anti-cancer activity of ethanolic extract of *Momordica charantia* on cervical and breast cancer cell lines. **International Journal of Health & Allied Sciences,** v. 4, n. 4, p. 210-217, 2015.

SOARES, S. E. Ácidos fenólicos como antioxidantes. **Revista de Nutrição,** v. 15, p. 71-81, 2002.

STAPF, M. N. S. Nomenclatural notes on *Varronia* (Boraginaceae s.l.) in Brazil. **Rodriguésia**, v. 61, p. 133-135, 2010.

SUCUPIRA, N. R., SILVA, A. B., PEREIRA, G., & COSTA, J. N. Métodos para determinação da atividade antioxidante de frutos. **Revista Unopar Científica Ciências Biológicas e da Saúde,** 14(4), 263-269, 2012.

TAN, M. J. et al. Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. **Chem Biol,** v. 15, n. 3, p. 263-73, 2008

TAN, S. et al. Bitter Melon (*Momordica charantia L.)* bioactive composition and health benefits: A review. **Food Reviews International,** v. 32, 2015.

TCHEGHEBE OLIVIER, T. et al. Ethnobotanical uses, phytochemical and pharmacological profiles, and cultural value of *Momordica chlarantia* L. : An overview. **Global Journal of Medicinal Plant Research,** v. 4, p. 23-39, 2016.

TEIXEIRA, J. et al. Hydroxycinnamic Acid Antioxidants: An Electrochemical Overview. **BioMed Research International,** v. 2013, p. 11, 2013.

TEOW, C. C.et al, Truong, V.-D., McFeeters, R. F., Thompson, R. L., Pecota, K. V., & Yencho, G. C.. *Antioxidant activities, phenolic and β-carotene contents of sweet potato genotypes with varying flesh colours. Food Chemistry, 103(3), 829–838,* 2007.

TICLI, F. K. et al. Rosmarinic acid, a new snake venom phospholipase A2 inhibitor from *Cordia verbenacea* (Boraginaceae): antiserum action potentiation and molecular interaction. **Toxicon,** v. 46, n. 3, p. 318-327, 2005.

TOMÁS-BARBERÁN F. A.; ESPÍN J. C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. **Journal of the Science of Food and Agriculture, v. 81,** p. 853 876, 2001.

THIRUVENGADAM M. et al. Establishment of Momordica charantia hairy root cultures for the production of phenolic compounds and determination of their biological activities. **Plant Cell Tissue Organ Cult.**, v. 118, p. 545- 557, 2014.

TURCHETTO-ZOLET, A. C.; TURCHETTO; C.; ZANELLA, C. M.; PASSAIA, GISELE. **Marcadores Moleculares na Era genômica:** Metodologias e Aplicações. Ribeirão Preto: Sociedade Brasileira de Genética, 2017. 181 p.

TZIN, V.; GALILI, G.; AHARONI, A. Shikimate Pathway and Aromatic Amino Acid Biosynthesis. **Annu Rev Plant Biol.,** v. 63, p. 73-105, 2012.

VARSHNEY, R. K. et al. A.Single nucleotide polymorphisms in rye (*Secale cereale* L.): discovery, frequency, and applications for genome mapping and diversity studies. **TAG Theoretical and Applied Genetics,** v.114. p.1105-1116, 2007.

VENTRELLA, M. C., MARINHO, C. R. Morphology and histochemistry of glandular trichomes of *Cordia verbenacea* DC. (Boraginaceae) leaves. Revista Brasileira de Botânica, São Paulo, v. 31, p. 457-467, 2008.

VERMA, N.; SHUKLA, S. Impact of various factors responsible for fluctuation in plant secondary metabolites. **Journal of Applied Research on Medicinal and Aromatic Plants,** v. 2, p. 105-113, 2015.

VIEIRA, D. D.; CONCEIÇÃO, A. S.; MELO, J. I. M.; STAPF, M. N. S. A família Boraginaceae sensu latu na APA Serra Branca/Raso da Catarina, Bahia, Brasil. **Rodriguésia,** v. 64, n. 1, p.151-168. 2013.

VILAR, M. S. et al. Assessment of Phenolic Compounds and Anti-Inflammatory Activity of Ethyl Acetate Phase of *Anacardium occidentale* L. Bark. **Molecules,** v. 21, n. 8, 2016.

VICTORIO, C. P. et al . Vasodilator activity of extracts of field *Alpinia purpurata* (Vieill) K: Schum and *A. zerumbet* (Pers.) Burtt et Smith cultured in vitro. **Braz. J. Pharm. Sci.**, São Paulo , v. 45, n. 3, p. 507-514, 2009.

VON MINCKWITZ, G.; MARTIN, M. Neoadjuvant treatments for triple-negative breast cancer (TNBC). **Annals of Oncology,** v. 23, p. vi35-vi39, 2012.

VUILLEUMIER, B. S. The origin and evolutionary development of heterostyly in the angiosperms. **Evolution,** v. 21, n. 2, p. 210-226, 1967.

WALTERS, T. W.; DECKER-WALTERS, D. S. Balsam-Pear (*Momordica charantia*, Cucurbitaceae). **Economic Botany,** v. 42, n. 2, p. 286-288, 1988.

WARD, L. S. Entendendo o Processo Molecular da Tumorigênese. **Arquivos Brasileiros de Endocrinologia & Metabologia,** v. 46, p. 351-360, 2002.

WEAVER, B. A. How Taxol/paclitaxel kills cancer cells. **Molecular biology of the cell,** v. 25, n. 18, p. 2677-2681, 2014.

WEI, L. et al. Increase in the free radical scavenging capability of bitter gourd by a heat-drying process. **Food Funct.,** v. 4, p. 1850-5, 2013.

WONG, T. Y. et al. Tannins and Human Health: A Review AU - Chung, King-Thom. **Critical Reviews in Food Science and Nutrition,** v. 38, n. 6, p. 421-464, 1998.

WUNDERLIN, R.P. Family 182. Cucurbitaceae. In: Woodson Jr., R.E. & Schery, R.W. (eds.). Flora of Panama. Part IX. **Annals of the Missouri Botanical Garden**, 285-366, 1978.

XU, D.-P. et al. Natural Antioxidants in Foods and Medicinal Plants: Extraction, Assessment and Resources. **International journal of molecular sciences,** v. 18, n. 1, p. 96, 2017.

YAJID, A. I. et al. Potential Benefits of *Annona muricata* in Combating Cancer: A Review. **The Malaysian journal of medical sciences : MJMS,** v. 25, n. 1, p. 5-15, 2018.

YANG, H.-Y.; LEE, T.-H. Antioxidant enzymes as redox-based biomarkers: a brief review. **BMB reports,** v. 48, n. 4, p. 200-208, 2015.

YANG, L. et al. Recent advances in biosynthesis of bioactive compounds in traditional Chinese medicinal plants. **Science bulletin,** v. 61, p. 3-17, 2016.

YANG, S. L.; WALTERS, T. W. Ethnobotany and the economic role of the Cucurbitaceae of China. **Economic Botany,** v.46, n.4, p.349-367, 1992.

YU, Z. X. et al.The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua.* **Mol Plant,** 5 (2012), pp. 353-365.

YUAN, Z.-L.; DAI, C.-C.; CHEN, L.-Q. Review- Regulation and accumulation of secondary metabolites in plant-fungus symbiotic system. **African Journal of Biotechnology,** v. 6, 2007.

ZADERNOWSKI, R.; CZAPLICKI, S.; NACZK, M. Phenolic acid profiles of mangosteen fruits (*Garcinia mangostana*). **Food Chemistry,** v. 112, n. 3, p. 685-689, 2009.

ZIETKIEWICZ, E.; RAFALSKI, A.; LABUDA, D. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. **Genomics,** v.20, p.176-183. 1994.

ZHONG, Y.; SHAHIDI, F. 12 - Methods for the assessment of antioxidant activity in foods11This chapter is reproduced to a large extent from an article in press by the authors in the Journal of Functional Foods. In: SHAHIDI, F. (Ed.). **Handbook of Antioxidants for Food Preservation**: Woodhead Publishing, p.287-333, 2015.

ZHU, K. et al. Oncogenes and tumor suppressor genes: comparative genomics and network perspectives. **BMC genomics,** v. 16, p. S8-S8, 2015.

ZHU, Y.; DONG, Y.; QIAN, X.; CUI, F.; GUO, Q.; ZHOU, X.; WANG, Y.; ZHANG, Y.; XIONG, Z. Effect of Superfine Grinding on Antidiabetic Activity of Bitter Melon Powder. **Int. J. Mol. Sci.,** *v. 13*, p. 14203-14218, 2012.

ZOCOLER, A.M. D; MOURÃO, M. K.S. DOS; MELLO, J.C.P.; MARQUES, L.C. Contribuição ao controle de qualidade farmacognóstico das folhas e caules de melão-de-São Caetano (*Momordica charantia* L. - Cucurbitaceae). **Acta Farmacéutica Bonarense,** n.25, v.1, p.22-27, 2006. . 12 Mar. 2011.

ZUANAZZI, J. A. S.; MONTANHA, J. A.; ZUCOLOTTO, S. A. Flanonoides. IN: SIMÕES, C. M. O.; SCHENKEL, E. P.; MELLO, J. C. P.; MENTZ, L. A.; PETROVICK, P. R. (Orgs.). **Farmacognosia: Do produto natural ao medicamento.** 1. Ed. Porto Alegra: Artmed. p. 209-235, 2017.