UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ENGENHARIA DE ALIMENTOS

IRAMAIA ANGELICA NERI NUMA

EVALUATION OF FUNCTIONAL PROPERTIES OF IRIDOIDS BLUE-BASED PIGMENTS FROM GENIPAP (Genipa americana L.)

AVALIAÇÃO DAS PROPRIEDADES FUNCIONAIS DOS IRIDÓIDES OBTIDOS DO JENIPAPO *(Genipa americana* L.*)*

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Thesis presented to the School of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Food Science

Tese apresentada a Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Ciência de Alimentos

Orientadora: Profª Drª Gláucia Maria Pastore

Coorientadora: Profª Drª Ana Lucia Tasca Gois Ruiz

ESTE EXEMPLAR CORRESPONDE A VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA IRAMAIA ANGELICA NERI NUMA, ORIENTADA PELA PROFª DRª GLÁUCIA MARIA PASTORE E COORIENTADA PELA PROFª DRª ANA LUCIA TASCA GOIS RUIZ

CAMPINAS

2018

Agência(s) de fomento e nº(s) de processo(s): CNPq, 481670/2013-0; FAPESP, 2015/50333-1
ORCID: https://orcid.org/0000-0002-2151-417X

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

Neri Numa, Iramaia Angelica, 1978-
N356a Avaliação das propriedades funcionais dos iridóides obtidos do jenipapo (*Genipa americana* L.) / Iramaia Angelica Neri Numa. – Campinas, SP : [s.n.], 2018.

Orientador: Glaucia Maria Pastore.
Coorientador: Ana Lúcia Tasca Gois Ruiz.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Pigmentos funcionais. 2. Genipina. 3. Genotoxicidade. 4. Micropartícula lipídica sólida. 5. Apoptose. I. Pastore, Glaucia Maria. II. Ruiz, Ana Lúcia Tasca Gois. III. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Evaluation of functional properties of iridoids blue-based pigments from genipap (*Genipa americana* L.)
Palavras-chave em inglês:
Functional pigments
Genipin
Genotoxicity
Solid lipid microparticle
Apoptosis
Área de concentração: Ciência de Alimentos
Titulação: Doutora em Ciência de Alimentos
Banca examinadora:
Glaucia Maria Pastore [Orientador]
Adriana Dillenburg Meinhart
Adriana Pavesi Arisseto Bragotto
Ana Paula Dionísio
Izabela Dutra Alvim
Data de defesa: 30-05-2018
Programa de Pós-Graduação: Ciência de Alimentos

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A ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica da Iramaia Angelica Neri Numa.

Dedico esta tese a Deus, fonte inesgotável de amor e sabedoria. Quem me capacitou e sustentou-me durante toda a jornada, sem Ele nada seria possível.

AGRADECIMENTOS

Em primeiro lugar agradeço a Deus, por me ajudar a seguir em frente com os meus objetivos e não desanimar diante das dificuldades.

À Helena minha filha amada, pela compreensão nas exaustivas horas de estudo.

Ao meu esposo pelo apoio e compreensão.

Aos meus pais quem, por todo o apoio e amor que sempre dedicaram a mim. Eles sempre me mostraram o quanto era importante estudar, mesmo não tendo eles a mesma oportunidade no passado.

À professora Dra. Gláucia M. Pastore, pela orientação, pelos ensinamentos transmitidos, pelo enorme incentivo, pela amizade, pela confiança em meu trabalho.

À professora Dra. Ana Lucia T. Gois Ruiz, pela solicitude, disposição e ajuda na interpretação dos resultados, correções e sugestões que foram fundamentais para a conclusão desse trabalho.

Aos membros da banca, titulares e suplentes (Dra. Adriana D. Meinhart, Profa. Dra. Adriana P. A. Bragotto, Dra. Ana Paula Dionisio, Profa. Dra. Luciana Azevedo, Dra. Izabela D. Alvim, Prof. Dr. Jose Pedrazzoli Jr. e Profa. Dra. Maria Ines G. Rodrigues) pelas correções e contribuições que ajudaram a melhorar meu trabalho.

A Dra. Roberta Roesler e Profa. Dra. Cintia Baú Betim Cazarin pelas sugestões durante a qualificação para a defesa.

Aos docentes do Depto. de Ciências de Alimentos, que tão prontamente nos transmitiram seus conhecimentos.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico pelo auxílio financeiro (Processo CNPq 481670/2013-0).

A Fundação de Amparo à Pesquisa de São Paulo do Estado de São Paulo pelo auxílio financeiro (Processo FAPESP 2015/50333-1).

Ao meu “bródi” Bruno Paulino pela amizade (muitas risadas juntos), apoio e incentivo incondicional. Obrigada por sempre torcer pelas minhas conquistas!

A Ana Paula Pereira e Renata A. Soriano Sancho pela amizade e companheirismo de sempre.

Ao Célio Angolini pela presteza, competência e inestimável auxílio no desenvolvimento deste trabalho.

Ao Prof. Dr. Juliano L. Bicas, Profa. Dra. Mirian D. Hubinger, Dra. Adriana Della Torre, Dr. Bruno N. Paulino, Dr. Celio F.F. Angolini, Dra. Marina G. Pessoa, Dra. Vivian B. Oriani, Gilberto C. Franchi Jr., Gustavo A. Pereira e Henrique S. Arruda pela colaboração na execução do trabalho.

A todos os colegas do Laboratório de Bioaromas, principalmente Nadir, Dora e Débora por todo o auxílio, companheirismo e amizade.

À Camila e Andreia da secretaria de pós-graduação, pelo apoio neste período.

EPÍGRAFE

Velhas árvores

Olha estas velhas árvores, mais belas
Do que as árvores novas, mais amigas:
Tanto mais belas quanto mais antigas,
Vencedoras da idade e das procelas...

O homem, a fera, e o inseto, à sombra delas
Vivem, livres de fomes e fadigas,
E em seus galhos abrigam-se as cantigas
E os amores das aves tagarelas.

Não choremos, amigo, a mocidade!
Envelheçamos rindo! envelheçamos
Como as árvores fortes envelhecem:

Na glória da alegria e da bondade,
Agasalhando os pássaros nos ramos,
Dando sombra e consolo aos que padecem!

Olavo Bilac, in "Poesias"

RESUMO

As propriedades tintoriais do jenipapo (*Genipa americana* L.) são conhecidas desde a antiguidade, mas nos últimos anos houve um aumento de interesse nos pigmentos azuis a base de iridóides *(IBBP= iridioid blue-based pigments,* particularmente genipina) como uma alternativa natural para substituir os corantes alimentícios sintéticos. Apesar disto, a caracterização dos constituintes químicos, bem como a avaliação do perfil toxicológico dessas substâncias ainda são incipientes, principalmente no que diz respeito a estabilidade da cor e à possibilidade de desencadear efeitos colaterais após serem metabolizados. Portanto, os objetivos deste estudo foram baseados em quatro pilares: 1) desenvolver e validar um método por UHPLC-DAD para separar e identificar os iridóides responsáveis pela cor (genipina, geniposideo e ácido geniposidico) presentes no mesocarpo e endocarpo dos frutos "verde" e maduro do jenipapo, 2) avaliar a influência do pH e temperatura na estabilidade da cor do extrato IBBP utilizando o método de delineamento central composto rotacional com duas variáveis (pH e temperatura), 3) produzir e caracterizar micropartículas sólidas lipídicas a partir do extrato IBBP utilizando a técnica de *spray chilling* e 4) avaliar o potencial biológico do extrato IBBP e de sua micropartícula, através da avaliação da bioacessibilidade de seus componentes-alvo, da atividade antiproliferativa e avaliação da genotoxicidade *in vitro*. Assim, os principais parâmetros de validação (linearidade, limite de quantificação, limite de detecção e sensibilidade) foram determinados e demonstraram que o método analítico por UHPLC-DAD pode ser aplicado para a quantificação dos compostos iridóides alvo, além de evidenciar as diferenças na composição química em função do estágio de maturação do fruto de jenipapo. Desta maneira, onze iridóides glicosídicos foram detectados sendo os compostos majoritários genipina, genipina 1-β-gentiobiosídeo, geniposideo seguidos pelo gardenosídeo, 6''-O-p-Cumaroil-1-β-gentiobiosídeo ácido geniposídico, 6''-O-p-Cumaroil genipina-gentiobiosídeo, 6'-O-p-Cumaroil- ácido geniposídico e 6'-O-feruloil-ácido geniposídico. Também foram obtidos dados que demostraram a estabilidade da cor do extrato IBBP em temperaturas e pH baixos (5-20 ^{o}C e 3.0 – 4.0, respectivamente), sugerindo ser compatível para corar alimentos ácidos. Além disso, o extrato IBBP mostrou um grande potencial biológico, apresentando potente atividade antioxidante e fraca atividade antiproliferativa frente algumas linhagens tumorais humanas, quando utilizado uma dose de 25 µg/mL do extrato. Também foi possível demonstrar um limite de segurança genotóxica *in vitro*, o qual deverá ser confirmado por estudos complementares. Adicionalmente, nossos resultados indicam que a técnica de spray chilling teve um fraco

desempenho em evitar a degradação do conteúdo de iridóides presentes na micropartícula. Apesar disso, a micropartícula de IBBP parece ter induzido a apoptose em linhagens de células leucêmicas na dose (25 μg/mL) estudada. Este estudo descreve, pela primeira vez o encapsulamento de pigmentos azuis a base de iridóides obtidos do jenipapo, bem como a realização de testes de bioacessibilidade através da digestão simulada e testes biológicos *in vitro*. Sendo assim, o assunto abordado representa um *hotspot* de inovação para a exploração estratégica do jenipapo como uma nova fonte de pigmentos naturais azuis com apelo funcional, além de estimular novas linhas de pesquisa em relação aos estudos abrangentes sobre eficiência de encapsulamento, toxicidade, metabolismo e distribuição.

ABSTRACT

The colouring properties of *Genipa americana* L. have been known since ancient times but in the last few years we have witnessed a growing interest in their iridoid blue-based pigments (IBBP, particularly genipin) as a natural alternative to synthetic ones for food applications as well as to exert desirable biological effects on human health. However, the characterization of the chemical constituents as well as genipap IBBP toxicity studies are still scarce, mainly with regard to the colour stability and the possibility of triggering side effects or toxicity after metabolization. Therefore, the goal of our study was based on four pillars: 1) to develop and validate an UHPLC-DAD method to separate and identify target iridoid compounds (genipin, geniposide and geniposidic acid) from genipap, 2) to assess the influence of pH and temperature on colour stability of IBBP extract using a Central Composite Design with two variable (pH and temperature), 3) to produce and to characterize the IBBP solid lipid microparticles (IBBP-SLM) through the spray chilling technique and 4) to evaluate the biological profiles of both IBBP extract and its microparticle (IBBP-SLM) through of the bioacessibility of its target components, antiproliferative activity and *in vitro* genotoxicity evaluation. Thus, the mainly validation parameters (linearity, limit of quantification, limit of detection and sensitivity) were determined and demonstrated that analytical method by UHPLC-DAD can be applied for the quantification of target iridoid compounds, evidencing the chemical composition differences according ripeness stage of genipap fruit. In this way, eleven glucoside iridoids were detected and the main compounds identified were genipin, genipin 1-β-gentiobioside, geniposide, followed by gardenoside, 6''-O-p-Coumaroyl-1-β-gentiobioside geniposidic acid, 6’’-O-p-Coumaroylgenipin-gentiobioside, 6’-O-p-Coumaroyl-geniposidic acid and 6'-O-feruloyl-geniposidic acid. We observed that the colour of IBBP extract was more stable at 5-20 ^{o}C and low temperature (3.0 - 4.0), suggesting that it is compatible for colouring acidic foods. In addition, the IBBP extract showed a great biological potential, showing a potent antioxidant activity and a weak antiproliferative activity (at 25 μg/mL) against some human tumor lines. It was also possible to demonstrate an *in vitro* genotoxic safety limit, which should be confirmed by complementary studies. Farther, our results indicated that the spray chilling technique for the production of lipid microparticles containing IBBP extract had a weak performance in protecting the IBBP from degradation. Nevertheless, the IBBP-SLM seemed to exert desirable effects and apoptosis induction (at 25 μg/mL dosage). This study describes for the first time the encapsulation iridoids blue-based pigments belonging to

genipap has been as well as conducting bioaccessibility tests through simulated *in vitro* digestion and biological assays. Thus, this study represents an innovation hotspot in the strategic exploration of genipap fruit as a novel source of blue natural colorants with functional appeal, besides stimulating new lines of research regarding comprehensive studies on encapsulation efficiency, toxicity, metabolism and distribution.

RIASSUNTO

Le proprietà di tintura del genipapo (*Genipa americana* L.) sono note fin dall'antichità, ma negli ultimi anni c'è stato un maggiore interesse per i pigmenti a base di iridioidi blu (IBBP= *iridioid blue-based pigments,* in particolare la genipina), come una alternativa naturale per sostituire i coloranti alimentari sintetici. Nonostante ciò, la caratterizzazione dei costituenti chimici e la valutazione del profilo tossicologico di queste sostanze sono ancora incipienti, soprattutto per quanto riguarda la stabilità del colore e la possibilità di innescare effetti collaterali dopo essere stati metabolizzati. Pertanto, gli obiettivi di questo studio erano basati su quattro pilastri: 1) per sviluppare e convalidare un metodo di UHPLC-DAD per separare e identificare gli iridioidi responsabile per la produzione di colore (genipina, geniposide e acido geniposidico) presenti nel mesocarpo e endocarpo dei frutti immaturi e maturi del genipapo, 2) valutare l'influenza del pH e della temperatura sulla stabilità cromatica dell'estratto di IBBP usando il metodo di delineazione centrale composita rotazionale con due variabili (pH e temperatura), 3) per produrre e caratterizzare microparticelle lipidiche solide dall'estratto IBBP usando la tecnica dello *"spray chilling"* e 4) per valutare il potenziale biologico dell'estratto IBBP e della sua microparticella (IBBP-SLM) valutando la bioaccessibilità dei suoi componenti e anche l´attività antiproliferativa e la valutazione di genotossicità *in vitro*. Pertanto, sono stati determinati i principali parametri di convalida (linearità, limite di quantificazione, limite di rilevazione e sensibilità) e dimostrato che il metodo analitico dell'UHPLC-DAD può essere applicato per la quantificazione dei composti iridoidi, nonché per evidenziare le differenze di composizione chimica secondo la fase di maturazione del genipapo. In questo modo, sono stati rilevati undici iridodi glicosidici, con i principali composti come la *genipin, genipin 1-β-gentiobioside, geniposide*, seguite dal *gardenoside, 6''-O-p-Coumaroyl-1-β-gentiobioside geniposidic acid, 6''-O-p-Coumaroylgenipin-gentiobioside, 6'-O-p-Coumaroyl-geniposidic acid* e dal *6'-O-feruloyl-geniposidic acid*. Sono stati ottenuti anche dati che hanno dimostrato la stabilità del colore dell'estratto di IBBP a basse temperature e pH (5-20 ^{o}C e 3.0 - 4.0, rispettivamente), suggerendo di essere compatibile per colorare gli alimenti acidi. Inoltre, l'estratto di IBBP ha mostrato un grande potenziale biologico, presentando una potente attività antiossidante e una debole attività antiproliferativa (25 µg/mL di dosaggio) nei confronti di alcune linee tumorali umane. È stato anche possibile dimostrare un limite di sicurezza genotossico *in vitro*, che dovrebbe essere confermato da studi complementari. D'altra parte, i nostri risultati

indicano che la tecnica dello *spray chilling* ha avuto scarso rendimento nell'evitare la degradazione del contenuto di iridóidi presenti nella IBBP-SLM. Tuttavia, l'IBBP-SLM sembra esercitare effetti desiderabili e induzione dell'apoptosi (25 μg/mL di dosaggio). Questo studio descrive per la prima volta l'incapsulamento di pigmenti blu a base degli iridoidi ottenuti del genipapo, oltre a condurre test di bioaccessibilità mediante digestione simulata e analisi biologiche *in vitro*. Pertanto, l'argomento affrontato rappresenta un punto di svolta per l'innovazione per l'esplorazione strategica del genipapo come nuova fonte di pigmenti blu naturali con richiamo funzionale, oltre a stimolare nuove linee di ricerca in relazione agli studi completi sull'efficienza dell'incapsulamento, tossicità, metabolismo e distribuzione.

SUMÁRIO

INTRODUÇÃO GERAL

A Biodiversidade brasileira é uma das mais ricas do mundo e está representada principalmente pelos Biomas Amazônia, Mata Atlântica e Cerrado, apesar da sua composição química não ser totalmente conhecida e talvez nunca venha a ser, tal a sua magnitude e complexidade (Coradin et al., 2011). Assim, as espécies nativas da nossa flora têm sido objeto de estudos prospetivos e tecnológicos no que diz respeito a elucidação de moléculas biologicamente relevantes para a aplicação em suplementos alimentares, cosméticos, agroquímicos e a descoberta de novos fármacos (Bessa et al., 2013, Bolzani, 2016, Valli et al., 2016).

Tais substâncias são produtos do metabolismo secundário das plantas, essenciais aos processos biológicos de regulação celular, comunicação química e defesa (Lee & Paek, 2014). Os metabolitos secundários são sintetizados em diferentes compartimentos celulares, através de quatro rotas biossintéticas: acetato malonato, ácido mevalônico (MEV), metileritritol fosfato (MEP) e do ácido chiquímico (Rezende et al., 2016 – botânica no inverno). Os produtos resultantes dessas vias metabólicas, diferem tanto estruturalmente quanto ao grupo funcional, sendo eles: terpenos, compostos fenólicos e compostos nitrogenados (alcalóides) (Santos et al., 2010, Rezende., 2016 – botânica no inverno).

A família Rubiaceae é conhecida pela importância econômica (*Coffea arabica*, café), farmacêutica (*Uncaria tomentosa*, unha de gato) e ornamental (*Gardenia sp*, jasmim) e pelos inúmeros metabolitos secundários com atividade biológica, sendo os mais representativos: iridóides, alcalóides, triterpenos, compostos fenólicos, taninos, saponinas, esteroides e glicosídeos cardiotônicos (Wiart, 2007, Tomaz et al., 2008, Simpson, 2011, Verma, 2011, Kouam et al., 2013, Martins & Nunez, 2015).

Espécies como *Genipa americana* L. (Rubiaceae), comumente conhecida por jenipapo, são empregadas na medicina popular para tratar icterícia, afecções dos tratos digestório e respiratório, em casos de sífilis, gonorréia e também como analgésico. Além disso, o jenipapo possui uma característica peculiar que é quando os frutos verdes são macerados, fornecem um líquido transparente a princípio, o qual torna-se preto-azulado quando oxidado. Este é utilizado para pintar a pele, tecidos e objetos de cerâmica (UNCTAD, 2005, Lorenzi, 2008, Renhe et al., 2009, Horák et al., 2014).

Dentro deste contexto, a utilização dos corantes naturais em detrimento dos artificiais, assim como a demanda dos pigmentos azuis naturais têm sido cada vez mais requeridos na indústria de alimentos, especialmente para produtos de confeitaria e bebidas (Carocho et al., 2015). No entanto, as tonalidades azuis oriundas de fontes naturais são escassas (Jespersen, Stromdahl, Olsen, & Skibsted, 2005, Sigurdson, Tang, & Giusti, 2017). Hoje em dia, a maioria dos corantes azuis naturais aprovados para uso em alimentos são provenientes das antocianinas (flores, frutas e vegetais) ou ficocianina (cianobactérias), apesar das ressalvas de estabilidade de cor quando comparadas com os corantes sintéticos disponíveis no mercado (azul brilhante FD&C no. 1 e azul de ingotina FD&C no. 2) (Brauch, 2016, Buchweitz, 2016, Wrolstad & Culver, 2012).

Não obstante, os trabalhos sobre a composição química de metabólitos especiais do jenipapo ainda são escassos e, dentro desse pequeno universo, verifica-se uma predominância na investigação de sustâncias da classe dos iridóides (genipina, ácido genípico, ácido genipínico, ácido geniposídico, geniposídeo, tarenosídeo, gardenosídeo, genamesídeos A-D, genipina-gentibiosideo, genipaol, gardeniol, éster metílico do ácido desacetilasperulosídico e shanzhisídeo). Evidencia-se a genipina que, por se tratar de um agente de reticulação natural (formador de ligação cruzada), menos citotóxico, que vem mostrando ser promissor em diversas pesquisas para o desenvolvimento de biomateriais (Souza & Lorenzi, 2008, Barbosa, 2008, Aramwit *et al.,* 2010, Manickam *et al.,* 2014, Know *et al.,* 2016)

No que se refere as propriedades funcionais, a genipina e o geniposídeo extraídos do fruto da *Gardenia jasminoides* Ellis, outra representante das rubiáceas rica em iridóides, têm sido associados aos efeitos farmacológicos (antioxidantes, anti-inflamatórios, antitrombóticos, neuroprotetores, inibição da peroxidação lipídica etc.). (Kojima et al., 2011, Lee et al., 2014, Chen et al., 2015, Bentes et al., 2015, Brauch, 2016b). Atualmente, o uso da genipina é explorado para fins alimentícios e farmacêuticos em países da Ásia Oriental como o Japão e a Coréia (Bentes et al., 2015).

Por outro lado, apesar do jenipapo conter estas mesmas substâncias, continua pouco explorado, não havendo comprovação científica sobre suas possíveis propriedades biológicas. Assim, o objetivo do presente estudo foi avaliar as propriedades biológicas do

extrato de jenipapo rico em iridóides (IBBP, *iridoid blue-based pigments*) bem como a sua segurança e benefícios para a saúde enquanto corante a ser destinado para consumo.

OBJETIVOS

1. Objetivo Geral

Avaliação do potencial funcional do extrato rico em iridóides obtido do fruto de jenipapo *(Genipa americana* L.*)*, enfatizando as suas propriedades antioxidante, antiproliferativa e antigenotóxica.

2. Objetivos Específicos

1.1. Desenvolver e validar um método por UHPLC-DAD para separar e identificar os iridóides genipina, geniposideo e ácido geniposídico presentes no jenipapo,

1.2. Avaliar a composição de iridóides dos extratos obtidos do mesocarpo e endocarpo dos frutos "verde" e maduro por UHPLC-ESI-MS/MS e selecionar a fração com maior teor de genipina (extrato IBBP),

1.3. Avaliar a influência do pH e temperatura na estabilidade de cor e teores de genipina do extrato IBBP,

1.4. Preparar micropartícula sólida lipídica a partir do extrato IBBP por *spray chilling*,

1.5. Avaliar a influência da digestão *in vitro* sobre o potencial antioxidante do extrato IBBP e sua micropartícula (IBBP-SLM) através do método de ORAC e perfil de iridóides,

1.6. Avaliar a atividade antiproliferativa do extrato IBBP em culturas de células tumorais,

1.7. Avaliar o potencial mutagêncio e genotóxico do IBBP em células de ovário de *hamster* chinês (CHO-K1) através do teste do cometa na ausência e presença de ativação metabólica,

1.8. Comparar os efeitos do extrato IBBP e IBBP-SLM em células leucêmicas humanas através de testes de viabilidade celular e indução da apoptose.

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

GENIPIN: A NATURAL BLUE PIGMENT FOR FOOD AND HEALTH PURPOSES

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Artigo publicado na Trends in Food Science & Technology

Fator de Impacto 5.191 - Qualis A1

(autorização no anexo 1)

Abstract

Genipin can be obtained from *Gardenia sp* or *Genipa americana* L. and represents an alternative of natural blue colorant in food industry. It also has been widely used as a cross-linker agent of structural biopolymers suitable for encapsulation technologies and biomaterials engineering as well as for food component replacers, drug/bioactive compounds delivery vehicle or enzyme immobilization. In addition, genipin possess a wide spectrum of health effects playing a significant role in the anti-inflammatory, antidiabetic and antithrombotic processes, amongst others. In other hand, the major challenge for the industrial use of genipin is its stability, but, due to the advantages of low toxicity, biocompatibility, permeability, and similarity with the extracellular matrix and intrinsic cellular interaction, worth making continuous efforts to overcome this drawback. Thus, genipin-derivatives comply with legal safety barriers regarding the toxicity. In this context, the strategic exploration of novel sources of genipin as well as methodology of extraction and optimization, recovery and purification process, represents an innovation hotspot.

Keywords: iridoids, food additives, genipin, blue pigments, crosslinking, bioactive carries

1. Introduction

Natural and synthetic pigments are widely used to colour foodstuffs in order to make the processed food more attractive to consumers (Carocho et al., 2015, Stich, 2016, Spence, 2015). Whether natural or synthetic, all colour additives must meet stringent requirements before their use in spite of having the trend the towards replacement of synthetic ones for healthier alternatives as well as ecofriendly and biodegradable commodities (Newsome et al., 2014; Martins et al., 2016).

Concerning natural pigments, they can be obtained from plants, microorganisms and insects or animals tissues (Zhang et al., 2014). Structurally, natural pigments can be classified in different groups that comprises several compounds with specific characteristics, such as isoprenoid derivatives (carotenoids and iridoids), benzopyran derivatives (oxygenated heterocyclic compounds like anthocyanins and others flavonoid piments), quinones (benzoquinone, naphtoquinone, and anthraquinone), tetrapyrrole derivatives (chrolophylls and heme colours), N-heterocyclic compounds different from tetrapyrroles (purines, pterins, flavins, phenazines, phenoxazines, and betalains) and melanins (Delgado-Vargas et al., 2000). Among natural pigments from plant sources, the main are either water- or lipid- soluble represented by carotenoids, chlorophylls, anthocyanins and betalains wich differ both in structure and metabolic pathway (Tanaka et al., 2008; Zhang et al., 2014; Buchweitz, 2016).

From the commercial point of view, anthocyanins, betalains and carotenoids are used in large scale for red, orange and yellow hues while natural green and blue colorants are few, thus making room to search for novel pigment sources such as unexploited fruits and vegetables (Brauch, 2016; Buchweitz, 2016).

Furthermore, these compounds have drawn considerable attention to the food and pharmaceutical segments, not only because of their coloring properties, but due to their biological activities such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective activities (Pangestuti & Kim, 2011; Rodriguez-Amaya, 2016). For this reason, much effort has been focused on improving colorant extraction, purification and stability as well as to satisfy the related hygiene and safety criteria as prerequisite to the approval of food regulatory authorities (Shahidi et al., 2013; Rodriguez-Amaya, 2016).

Thus, being motivated by the possible applications of genipin isolated not only from Gardenia sp but also from genipap (*Genipa americana* L.) as a precursor of natural blue pigment, the proposed review emphasized their potential health benefits for the food and pharmaceutical areas.

2. A brief overview of food colour additives

Since ancient times, foods appearance represents an important role in the lives of people, mainly when early humans searched for food and they had to learn how to identify edible or ripe fruits, to avoid toxic and spoiled objects (Adam Burrows, 2009; Stich, 2016). Now in much the same way, the practice of adding colours to food is intended to serve as a visual cue to quality, to look more appealing and meet consumer expectations (Spence, 2015). In addition, archaeological finds have shown that ancient cultures used dyes from plant, animal or mineral sources relates to the use of both for colouration or medical purposes (Adam Burrows, 2009; Mapari et al., 2010).

One of the earliest records of the use of food colorants relates to the use of wine in order to improve a products appearance in 400 B.C. (Stich, 2016). The Egyptians coloured food, drugs and cosmetics adding saffron, turmeric and paprika as far back as 1.500 B.C., while the Romans obtained the royal purple colour for food purposes using mucus gland excretions from sea snails (Downham & Collins, 2000).

Up to the middle of the 19th century food colorants were employed to disguise inferior products or as an attractive effect to certain foodstuffs (Downham & Collins, 2000). The British chemist Sir William Henry Perkin created the first synthetic dye, mauveine, in 1856 by oxidizing aniline. By the end of the century, hundreds of bright new dyes, including coal tar derivatives were indiscriminately used in jellies, butter, cheese, ice cream, sausages, pasta, and wine (Adam Burrows, 2009; Orna, 2013).

Although the synthetic colour additives were harshly criticized, the regulatory advances in the area of food happened only gradually and were marked by important events such as “the Pure Food and Drugs Act” (1906) and “the Food, Drug and Cosmetic Act” (1938) whose focus was restricted to the abuse of food additives, misbranded and adulterated foods, drinks and drugs (Adam Burrows, 2009).

In 1956 a Joint Expert Committee on Food Additives (JECFA) was established, administered jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) in order to review around 1500 substances, including food colours (Orna, 2013; Lehto et al., 2017). Since then, all food additives production must have a demonstrated useful purpose and undergo rigorous scientific safety assessment before they can be permitted for use (Orna, 2013; Lehto et al., 2017). Nowadays, industries in countries who follow the safety standards established for the use of food additive is regulated by Joint FAO/WHO Expert Committee on Food Additives (JECFA), European Union (UE), and Codex Alimentarius commission (Cheeseman, 2014; Lehto et al., 2017).

3. Sources of natural blue colorant for the food industry

As is well known, the food industry search constantly for new technologies to produce new foodstuffs with better stability, flavour, texture and appearance. Despite growing interest in the use of natural blue colorants, especially for confectionery and beverages, natural pigments sources are scant and have difficulty matching the colour properties of the synthetic ones (Jespersen et al., 2005; Sigurdson et al., 2017). In addition, the photochemical properties of pigments can be responsible for blue colour perception due to the presence of π-bond conjugation, aromatic ring systems, heteroatoms, and ionic charges (Newsome et al., 2014; Sigurdson et al., 2017).

The production of natural blue pigments has been reported applying biotechnological process or by chemical extraction of vegetal tissues. In last years, the works are focused mainly to production of the phycocyanin spirulina obtained from cyanobacteria and eukaryote algae (Rhodophytes and Cryptophytes), indigo dye and its related analogues from plants belong to the genus Indigofera (Leguminosae family), or microorganisms like *Erwinia chrysantemi*, *Photorhabdus luminescens*, *Streptomyces chromofuscus*, and *Pseudomonas fluorescens* (Erikson, 2008; Sørensen et al., 2013; Kusumawati et al., 2016, Reverchon et al., 2002; Chu et al., 2010; Brachmann et al., 2012; Yu et al., 2013; Adreani et al., 2015).

Nowadays, most approved natural blue hues for food use come from anthocyanins (flowers, fruits or vegetables) or phycocyanin (cyanobacterium), despite caveats of colour

stability when compared to synthetic ones available on the market (Brilliant blue, FD&C no. 1 and Blue Indigotine FD&C no. 2) (Wrolstad & Culver, 2012; Brauch, 2016; Buchweitz, 2016). However, the genipin-based iridoid pigment from members of the Rubiaceae family seems a promising alternative to industrial applications for food products due to the similarity of FD&C Blue no. 2, as showed below (Brauch, 2016; Buchweitz, 2016; Sigurdson et al., 2017).

4. Genipin derived-pigments obtained from Rubiaceae species

Genipin is a colorless iridoid from monoterpenes class, characterized by the presence of a cyclopentanoid unit fused with a dihydropyran ring whose hydroxyl group at the C1 position of the genipin pyran ring can be substituted by 1-2 moieties forming the genipin glycosides genipin-1-O-β-glucoside (geniposide) and genipin-1-O-β-D-gentibioside, respectively (Dinda et al., 2007).

In addition, this iridoid can be obtained from fruits of *Gardenia jasminoides* J. Ellis (gardenia) an evergreen flowering plant from Asia or unripe fruits of *Genipa americana* L. (genipap) naturally growing in the lowlands of the Amazon forest and other parts of Latin America (UNCTAD, Nations Conference on Trade and Development, 2005; Son et al., 2015). Although genipin itself is colourless, in the presence of oxygen, it reacts with primary amines and proteins producing water-soluble bluish-violet pigments **(Figure 1)** despite the mechanism of reaction between genipin and amino groups is complicated and strongly depends on the pH (Buchweitz, 2016; Tokareva et al., 2017).

Particularly, gardenia fruits present the glycosylated form (geniposide) requiring enzymatic treatment with β-glucosidase to release the aglycone while genipap fruits possess free genipin (Bentes, et al., 2015; Brauch, 2016; Ramos-de-la-Peña et al., 2014). Apart from genipin, genipin-derivatives (geniposide and geniposidic acid) also react with other compounds to produce pigments (Wu & Horn, 2013). However, the detailed mechanism of pigments formation is unclear (Brauch, 2016; Buchweitz, 2016; Kumar et al., 2016).

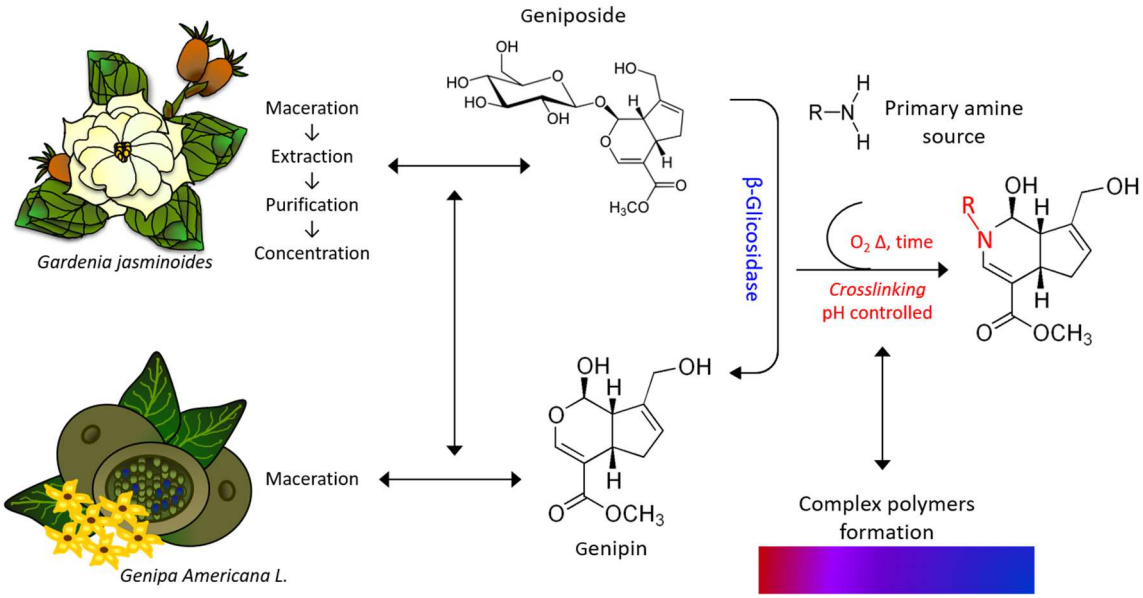

Figure 1. Schematic representation of reaction mechanism of genipin. Genipin is a colorless iridoid that are composed of a cyclopentan –[C]-pyran skeleton. The hydroxil group at the C1 position of the genipin pyran ring can be substituted by one or two sugar moieties forming the genipin glycosides genipin-1-O-β-D-glucoside (geniposide) and genipin-1-O-β-D-gentiobioside, respectivelly. In brief, genipin can be obtained after the hydrolisis of geniposide from gardenia fruit or directly from unripe genipap fruit including maceration, extraction and rather complex purification steps (separation of proteins) followed by concentration process. Adapted from Ramos-de-la-Peña et al.,2014 and Buchweitz, 2016.

As regards to regulatory aspects, the genipin from *G. jasminoides* is available for food and medicinal purposes just in East Asia countries such as Japan and Korea (Lee et al., 2014, Bentes, et al., 2015, Buchweitz, 2016). The blue pigment obtained from *G. americana* or in combination with other fruits is commercially exploited in the United States or European Union as concentrated fruit juices for use as colorants exempt from certification (Brauch, 2016; Sigurdson et al., 2017).

4.1. Factors affecting the formation and stability of genipin-derived colourants

As described above, genipin-based pigments can be obtained after reaction of the aglycone with primary amines in the presence of oxygen. Colour formation, is, therefore,

deeply dependent on factors influencing this reaction, such as pH, temperature, amino acid source and genipin-to-amino source ratio (Brauch, 2016).

Optimum pH range reported includes the pH of unripe fruits of genipap: 4.5 and 5.2 of mesocarp and endocarp, respectively (Brauch, 2016, Wu & Horn, 2013, Bentes & Mercadante, 2014). In lower pH values (pH < 4), the amine groups are in their protonated from, thus, the nucleophilic attack of genipin C3 carbon is inhibited (Mi et al., 2005). During the ripening process, the fruits pH is acid (pH 3), a fact that corroborates with suppressing blue colour formation (Bentes et al., 2015). In higher pH (pH > 10), the reaction is inhibited due hydrolysis of genipin ester group, and in this condition, a red pigment is formed (Brauch, 2016). Differently from pH, the increase of reaction temperature (60 – 85 ^{o}C) affects pigment production positively, although this parameter is variable according to the primary amino source (Wu & Horn, 2013).

A process for obtaining different colorants compounds from genipin using a variety of amino sources has been patented (CA2912820). It was reported that glycine and lysine content lead to the formation of blue/purple colorants, while the presence of valine, methionine or tyrosine results in blue-green colour. Black and green colours are obtained using proline and tryptophan, respectively (Cano et al., 2014). In addition, it was shown that blue pigment formation increases linearly with increasing of amino acid concentration, up to a saturation point, therefore, it is necessary to find the best genipin-to-amino content ratio in order to optimize colorant production (Brauch, 2016). A simple method for production of blue pigments is performed by mixing genipap juice or puree with an amino acid or protein containing substrate, for example, watermelon juice (Wu & Horn, 2013; Brauch, 2016).

Regarding the chemical stability of blue pigments, it is important to mention that their colour is highly affected by the same factors already described (pH, temperature, amino source, and genipin-to-amino source ratio). Generally, blue colorants derived from genipin are shown to be lighter-, pH- and thermo stable than phycocyanin extracts of spirulina, being suitable for colouring food, beverages, cosmetic and personal care products (Wu & Horn, 2013).

Although promising, there are few studies concerning genipin-based colorants stability. The limitation of blue pigments employment in beverages composition is its low pH

(2.8 - 3.2). An 8 week experiment was performed to verify blue pigment stability in three beverages: (i) a dairy-based beverage, (ii) a vitamin-enhanced water, and (iii) a lemonade beverage containing approximately 10% juice. The formulation (i) and (ii) showed a colour fade of 20 - 25%, with colour change to grayish blue and towards green respectively. For lemonade, the colour fade was about 30 - 35% with a change from a grayish blue to green. All three formulations were also exposed to light stability tests and, using simulated daylight, less than 30% fading it was observed in all beverages. In addition, higher concentrations of blue colour juice presented approximately 5% fading after 8 weeks and light stability test showed little colour change (Wu & Horn, 2013).

A blue pigment produced from the reaction of genipap juice with glycine showed low stability when stored for 19 days, at pH 3, with the presence of ascorbic acid. Reductions of 20% and 30% in the absorbance could be observed at λmax after incubation at 6 ^{o}C and 20 ^{o}C, respectively (Lopez et al., 2010; Cano et al., 2014).

Gardenia blue, the pigment obtained after hydrolyses of geniposide and release of free genipin, was compared with other 2 natural pigments, indigo and phycocyanin, and found to be more stable at temperatures 60, 70 and 80 ^{o}C for 8 - 48 hours, at pH 3, 5 and 7. When in aqueous solution, its exposure to light for 24 hours resulted in 50% degradation. Although it exhibited good stability, gardenia blue appeared as greenish-blue in solid applications, therefore, its use is applicable only for liquid products. Phycocianin has lower stability regarding light and high temperature, but its bright blue colour in solid material is judged to be more acceptable than gardenia blue, being preferable for this application (Jespersen et al., 2005).

In another study, blue pigment obtained from gardenia was tested for it stability at light, high temperatures and pH range (5 - 9). The authors vary the amino acids used for colorant formation, using glycine, lysine, or phenylalanine. It was showed that in the three cases, the pigments were more stable in alkaline than acidic or neutral conditions. In order to verify photodegradation, blue pigments were incubated at 4 ^{o}C and in different pH, for 10 h. In this case, the amino acid source interfered in pigment stability. The remaining absorbance of blue pigments obtained from the reaction with glycine were about 75%, while from reaction with phenylalanine the absorbance remaining was 51%, showing that light has more effect than pH in blue pigments stability (Paik et al., 2001).

Due to its wide potential of application as colorant or bioactive compound, further researches is necessary in order to better understand genipin stabilization mechanisms aiming to increase shelf-life of products containing genipin derivatives.

4.2. Genipin-crosslinking properties and applications

The flagship of applications of genipin is in tissue engineering where it is possible to design polymeric network by based-genipin covalent cross-linker using several biopolymers of proteins and polysaccharides containing residues with primary amine groups (chitosan, gelatin, silk protein, collagen, casein, etc) with adequate strength, degradability and biocompatibility (Aramwit et al., 2010; Manickam et al., 2014; De Clercq et al., 2016; Muzzarelli et al., 2016).

As a result, differentiated complexes or conjugated were obtained applicable for the biomaterial and encapsulation technologies (e.g. hydrogel, membranes, fibers, microgels, sponges and nanocapsules) which are suitable for the food industry, pharmacological and biomedical fields. They can be employed as fat replacers in processed food, immobilize enzymes (galactosidase), carrier agents of bioactive compounds (vitamins, carotenoids, flavonoids, prebiotics, etc) and drug delivery vehicle as well as in transdermal applications and protective coatings (Malafaya et al., 2007; Okuro et al., 2015; Reddy et al., 2015; Tokareva et al., 2017). Schematic illustration of crosslinking reaction between genipin and biopolymers containing primary amine groups and possible intramolecular and intermolecular crosslinking structures are exemplified in **Figure 2.**

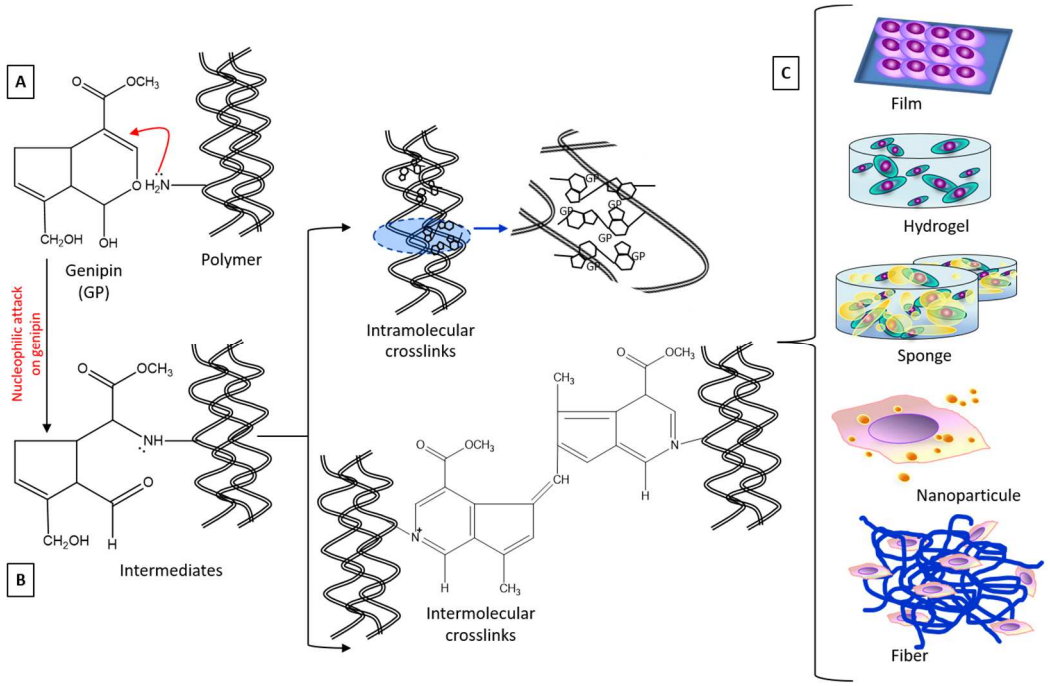

Figure 2. Schematic representation of reaction mechanism of genipin with amino group (A), crosslinking reactions involving genipin with biopolymers (B) and some formed intra- or intermolecular crosslinking structures (C). Adapted from Chang et al., 2001; Kanungo et al., 2014 and Reddy et al 2015.

One approach is the immobilization of multi-enzyme using genipin crosslinking glucose oxidase and catalase or PDCHNF for gluconic acid synthesis or to enhance the protease activity, respectively (Salazar-Leyva et al., 2013; Cui et al., 2017).

In addition, biomaterials crosslinked by genipin also can assist therapies of vascular diseases, diabetes, hepatic dysfunctions, etc. Because genipin-crosslinked scaffold exerts its effect on different cell types targeting several biological tissues, is recommended for healing and tissue regeneration (Wang et al., 2012; Muzzarelli et al., 2015; Manickam 2014).

Due to its unique biocompatibility, genipin can be directly introduced into living tissue, beyond the big advantage of genipin stands out, due to the very low cytoxicity as compared to conventional cross-linkers such as glutaraldehyde and epoxy compounds (5000 – 10000 fold) (Gaudière et al., 2014; Muzzarelli et al., 2015; Kwon et al., 2016; Tokareva et al., 2017).

A recent study assessed whether genipin-crosslinked gelatine microspheres prevents peritoneal adhesions using Balb/c mice model found an excellent biocompatibility and degradation characteristics as well as less postsurgical adhesions and inflammation if compared to control group (De Clercq et al., 2016). In the same way, films obtained from crosslinked chrondroitin sulphate A and poly(L-lysine) promoted promoted cell adhesion, proliferation, and early and late osteogenic differentiation of preosteoblasts (Guadière et al., 2014).

It also been reported that genipin-crosslinked chitosan hydrogels acts as carrier for local antibacterial nanomedicines, promoting faster healing rate, collagen deposition and re-epitheliatization compared with other therapies (Gao et al., 2014). Some material crosslinked by genipin and their and applications are showed in **Table 1.**

Table 1. Biopolymers crosslinked/ scaffold by genipin and their different applications

Polymer	Polymeric system	Application	Reference
Carboxymethyl cellulose/k-carrageenan	Genipin-cross-linked kappa-carrageenan/carboxymethyl cellulose	Hydrogel for carrier nutraceuticals	Muhamad et al., 2011
Chitosan	Catechol modified-chitosan crosslinked by genipin	Hydrogel as rectal drug delivery to treat ulcerative colitis	Xu et al., 2017
	Crosslinked alginate-chitosan	Micropasule with bacterial cells to improve gastrointestinal microflora	Chen et al., 2010
	β-D-Galactosidase–chitosan–genipin	Particles as support matrix for application in food process (β-D-galactosidase immobilization)	Klein et al., 2016
	Chitosan/genipin/poly(N-vinyl-2-pyrrolidone)	Film for drug release in the oral mucosa	Aldana et al., 2012
Gelatin	Genipin-modified gelatin	Nanocarriers for targeted release of anticancer drug	Khan et al., 2016
	Genipin cross-linked 3D gelatin	Scaffolds for tissue engineering	Sánchez et al., 2017
Collagen	Genipin-treat collagen	Promotes odontogenic differentiation of human dental pulpl cells	Kown et al., 2016
Poly(lactic-co-glycolic acid)	genipin-crosslinked PLGA	Improvement of mechanical/repair strenght of damaged connective tissue	Sundararaj et al., 2016
Polyvinyl alcohol-gelatin	Genipin cross-linked polyvinyl alcohol-gelatin	Hydrogel for bone regeneration	Nguyen et al., 2016
Polyethylenimine	Genipin-crosslinked polyethylenimine conjugated globin nanoparticle	Self-fluorescent image probe to tracks tumor cell selective drug delivery	Zhang et al., 2017
Soy protein isolate/whey protein concentrate	Genipin crosslinked- SPI/WPC	Nanoencapsulation of atorvastatin to enhance the antihyperlipidemic potential	Kanoujia et al., 2016

4.3. Therapeutic potential of genipin extract

Genipin and its related iridoid glucosides represents a promissing options for pharmacological purposes, not only due to its crosslinking properties but also to antioxidant, anti-inflammatory and antithrombotic capacities, neuroprotective effects, amongst others (Lee et al., 2014; Chen et al., 2014; Bentes, et al., 2015; Brauch, 2016).

Several studies have been linked genipin oral administration with amelioration of diabetes effects by the inhibition both of hepatic oxidative stress and mitochondrial uncoupling protein-2 expression that markedly decrease blood glucose levels through insulin stimulation (Zhang et al., 2006, Zhou et al., 2009, Qiu et al., 2013, Guan et al., 2013, Hu et al., 2016). In addition, H-NMR-based metabolomic method applied in diabetic rat model revealed that high dose of genipin (100 mg/Kg) improves significantly the disturbance in glucose metabolism as well as lipid and amino acid metabolism (Hu et al., 2016; Shen et al., 2016).

A number of works have also reported that genipin can alleviate depressive symptoms though the antidepressant mechanisms involved are not fully understood. However, a possible mechanism of antidepressant-like effect, has been reported via increasing the brain-derived neurotrophic factor level in the hippocampus, exerting their effect through monoaminergic neurotransmitter system (NE, 5-HT, and 5-HTR) (Tian et al., 2013, Wang et al., 2014, Chen et al., 2015). In the same way, neuroprotective and neuritogenic effects in PC12 neuronal cells and RGC-5 ganglion cells were attributed to genipin action (Yamazaki & Chiba, 2008; Koriyama et al., 2010; Luo et al., 2012).

Some studies also report that genipin has shown potent anti-inflammatory activity based on downregulation of chemokime ligand, chemokime receptor and IFN-induced protein productions in LPS-induced acute systems inflammation, up-regulatin heme oxygenase-1 via PI3-Kinase-JNK1/2-Nrf2 signaling pathway, inhibition expression of iNOS and most IL members, as well as inhibition of cyclooxygenase (COX)-1/-2 enzymes, TNF-α formation and NO production (Jeon et al., 2011; Li et al., 2012; Khanal et al., 2014; Zuo et al., 2017). Genipin has a plethora of other important therapeutic activities which are shown **Table 2**.

Table 2. Health benefits of genipina

Therapeutic application/ health effect	Model	Action mechanism (hypothetical or no)	Reference
Periodontal disease	MMP-1 and MMP-3 production from TNF-α-stimulated HPDLCs	↓ TNF-α-induced MMP-1 and MMP-3 production ↓ TNF-α-induced ERK/AMPK pathway ↓ MMP-1 and MMP-3 release /TNF-α-stimulated HPDLCs ↓ Phosphorylation of signal transduction molecules	Shindo et al., 2014
Osteoporosis	RANKL-induced osteoclast differentiation	↓ RANKL-induced osteoclastogenesis by NF-KB activation ↓c-Fos protein proteolysis ↓IkB degradation	Lee et al., 2014
Gastroprotection	HCl ethanol-induced acute gastritis and indomethacin-induced gastric ulcer in rats	↓ HCl ethanol-induced acute gastritis ↓ Indomethacin-induced gastric ulcer ↑ Prostaglandin E2 (PGE2) in gastritc cancer cells ↑ Apoptosis via p53-independent Egr1/p21 signaling pathway	Ko et al., 2015 Sohn et al., 2017
Septic injury	Molecular mechanism of genipin on immunosuppression in the late phase of sepsis in mice	↑ Anti-apoptotic B-cell lymphoma-2 protein expression ↓ Pro-apoptotic phosphorylated-Bim protein expression in cecal ligation puncture ↓ Lymphocyte apoptosis	Kim et al., 2015

Continue

Table 2. Health benefits of genipina

Therapeutic application/ health effect	Model	Action mechanism (hypothetical or no)	Reference
Anti-inflammatory	Anti-inflammatory mechanisms of genipin against lipopolysaccharide (LPS)-induced proinflammatory responses in RAW264.7 murine macrophages	↑ Upregulatin of heme oxygenase-1 via PI3-Kinase ↑ Downstream activation of JNK1/2 and Nrf2	Jeon et al., 2011
Neuroprotection	Neuroprotective potential of genipin against damage stemming from ROS and RNS production in organotypic hippocampal slice cultures	↓ Cell death ↓ Nitrite levels	Hughes et al., 2014
	Free radical scavenging ability		
Anti-apoptotic and antiproliferative activities	HeLa cells	↑ HeLa cells apoptosis ↑ Cell cycle arrest at G1 phase	Cao et al., 2010
	Cellular Model of Warburg Effect in Breast Cancer	↑UCP2 expressing cell ↓ Cell proliferation of UCP2 ↓ Clonogenic survival in UCP2 ↓ Matrigel invasion	Ayyasamy et al., 2011

Continue

Table 2. Health benefits of genipina

Therapeutic application/ health effect	Model	Action mechanism (hypothetical or no)	Reference
Anti-apoptotic and antiproliferative activities	Human gastric cancer cell line	↑ Apoptosis via p 53-independent Egrl/p21	Ko et al., 2015
	Hepatocellular carcinoma (HCC) Tumor associated macrophages (TAM)	↓ Orthotopic growth of HCC ↓ Infiltration of inflammatory monocytes and TAMs ↓ Migration of TAMs ↓ Expression of macrophage priming-related cytokine IRE1α inactivation	Tan et al., 2017
	SNU719 (Epstein-Barr virus genome-associated gastric carcinoma cell line)	↑Cytotoxicity by methylation on EBV C promoter ↑ Tumor suppressor gene BCL7A ↓ EBV latent/lytic gene regulation	Son et al., 2015

Regarding toxicological aspects, a dearth of information exists on genipin toxicity (Mailloux et al., 2010). However, some findings indicate that genipin can induce DNA damage by *in vitro* models rec-assay and sister chromatid exchange using *Salmonella typhimurium* and *Bacillus subtilis* while it is not found clastogenic effect on Chinese hamster ovary cells (Ozaki et al., 2002; Tsai et al., 2000).

Another drawback is that genipin can acts as a substrate of P-glycoprotein and competitively interact at the drug-binding site of P-glycoprotein (Mailloux et al., 2010, Zhang et al., 2011). In addition, genipin can induces CYP2D6 and inhibit CYP2C19 enzymatic activities requiring caution in drug combination to avoid toxicity-enhancing and effect-reducing with respect to induction or inhibition of genipin on CYP isoenzymes and strong induction on P-glycoprotein (Gao et al., 2014).

Notwithstanding, this was reported that the toxicity of genipin was dose and time dependent when supplemented directly to the cartilage culture medium used in tissue-engineering. The lethal dose was at 2200µM, not lethal at 220 µM and beneficial at 22 µM. Short-term exposure at 220 and 22 µM was sufficient to obtain the benefits on the tissue level without the detrimental effects on the cellular level (Lima et al., 2009). In the same way, genipin suppresses the motility and invasiveness of HepG2 and MHCC97L at non-toxic doses, which may be correlated to the inhibition of genipin on MMP-2 activities in the cells (Wang et al., 2012).

4.4. The use of genipin in forensic science

Another practical application of genipin is to stain of biological tissues, and for visualization of fingerprints as well as to replace ninhidrin in chromatographic identification of amino acids and as dye for colorimetric and fluorimetric analysis (Tokareva et al., 2017)

4.5. Genipin: a promising blue based-iridoid for innovation

In terms of patents granted, in a search from the Questel Intellectual Property Portal using the Internet: <URL: http//www.orbit.com> it was possible to check around 1726 patent claims for genipin-rich material, method of extraction as well as their applications. On

Table 3 there is an overview of genipin purposes. China leads the ranking of patents followed by USA and Korea whose main domains are represented by medical technology (784), pharmaceuticals (689), macro-molecular chemistry/polymers (220), biotechnology (198) and basic materials chemistry (139).

Table 3. Patent overview of genipin for several purposes

Title	Publication number	Publication date	Claim	Technology domain	Applicant/Assignee
Method for preparing functional artificial skin scaffold material	CN105854078	2016-08-17	Method for preparing a functional artificial skin scaffold material	Medical technology	Qingdao University
Genipin cross-linked biogel as well as preparation method and application thereof	CN105778126	2016-08-20	Cross-linked biogel	Macromolecular chemistry, polymers Medical technology Other special machines Pharmaceuticals	Institute of Transfusion Medicine the Acad of Military Medical Sciences
Colorant compounds derived from genipin or genipin containing materials	US 9376569 B2	2016-06-08	Colorant composition	Basic materials chemistry Food chemistry Macromolecular chemistry, polymers Organic fine chemistry	Ecoflora S.A.S.
Preparation method for pectinase and cellulase magnetic composite microspheres	CN105462954	2016-04-06	Cross-linking agent for enzyme immobilization to be applied to the fields of fruit juice, vegetable juice and the like,	Biotechnology Food chemistry	Quingdao Agricultural University
Genipin-rich material and its use	US 8945640 B2	2015-11-03	Extraction	Biotechnology Food chemistry Pharmaceuticals	Wild Flavors, Inc.
Food composition pharmaceutical composition animal medicine and feed composition against obesity with genipin	KR20160116779	2015-03-31	Prevention and treatment of obesity	Pharmaceuticals	Kyungpook National University Industry Academic Coop Found, Seoul National University R & Db Found

Source: https://www.orbit.com

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Table 3. Patent overview of genipin for several purposes

Title	Publication number	Publication date	Claim	Technology domain	Applicant/Assignee
Pharmaceutical composition comprising genipin and crocin as effective components for prevention or treatment of diseases related to thombosis and health functional food comprising the same	KR20150041698	2013-10-08	Treatment for thrombotic diseases	Pharmaceuticals	Andong National University Korea Bio Medical Science Institute
Application of genipin amino acid derivative as nf-kappa b inhibitor	CN103191106	2013-03-08	Inhibitor of NF-kappa B activity	Food chemistry Pharmaceuticals	University Tianjin Traditional Chinese Medicine
Application of genipin-1-beta-d-gentiobioside to preparation of medicament for treating heart failure disease	CN102000102	2010-11-03	Treatment for heart failure disease	Pharmaceuticals	Nanjing Univerisity of Chinese Medicine Taizhou China Medicine City Traditional Chinese Medicine Research Institute
Stable natural color process, products and use thereof	WO2009120579	2009-03-06	Colorant for beverages, foodstuffs, drugs, dietary supplements, cosmetics, personal care stuffs, and animal feeds.	Basic materials chemistry Food chemistry Organic fine chemistry Transport	Wild Flavors, Inc.
Dyes and the use thereof in compositions, in particular cosmetic compositions	GB0922594	2008-12-31	Make-up to the skin or hair and nails	Basic materials chemistry	LVMH Recherche
Blue colorant derived from *Genipa americana* fruit	WO2010038146	2008-10-03	Extraction of blue colorant to be applied in textile, pharmaceutical, food, cosmetics, and other industries	Basic materials chemistry Pharmaceuticals	Ecoflora S.A.

Source: https://www.orbit.com

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Table 3. Patent overview of genipin for several purposes

Title	Publication number	Publication date	Claim	Technology domain	Applicant/Assignee
Genipin derivatives and uses thereof	WO2004089926	2004-03-31	Treatment of first-phase insulin secretion, non-insulin dependent diabetes mellitus, and ischemia	Pharmaceuticals	Beth Israel Hospital University of Boston
Drug-loaded biological material chemically treated with genipin	WO2004012676	2003-08-01	Cross-linking agent and drug-deliver	Medical technology	GP Medical
Oil body treated by cross-linking agent with increased stability	TW200503633	2003-07-30	Cross-linking agent used in food industry, drug delivery, and biomedical materials	Food chemistry	Challenge Bioproducts

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5. Conclusion

This review presents an overview of the applications of genipin for an array of medical, pharmaceutical and industrial purposes. The possible application as blue colorant for food and cosmetics, as well as its attractive cross-linking and carrier agent characteristics for clinical practices, make genipin a suitable and eco-friendly alternative to synthetic compounds, correspond to the increasing demand for natural products. The major challenge for the industrial use of genipin is its stability, however, due to the advantages of low toxicity, biocompatibility, permeability, and similarity with the extracellular matrix and intrinsic cellular interaction, worth making continuous efforts to overcome this drawback. In addition, genipin-derivatives comply with legal safety barriers regarding the toxicity. In this context, the strategic exploration of novel sources of genipin as well as methodology of extraction and optimization, recovery and purification process, represents an innovation hotspot.

Acknowledgment

We would like to thank “Conselho Nacional de Desenvolvimento Cientifico e Tecnológico – CNPq (grant number 481670/2013-0)”, “Fundação de Amparo a Pesquisa do Estado de São Paulo – FAPESP (grant number 2015/5033-1) and “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES” for financial support.

Conflicts of interest

Authors declare not conflict of interest.

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CAPÍTULO II

DEVELOPMENT AND VALIDATION OF AN UHPLC-DAD METHOD FOR THE SIMULTANEOUS DETERMINATION OF GENIPIN, GENIPOSIDE AND GENIPOSIDIC ACID FROM GENIPAP (*Genipa americana* L. - RUBIACEAE)

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Artigo submetido no Journal of Food Composition and Analysis

Fator de Impacto 2.752 - Qualis A1

Abstract

Genipin is a iridoid blue-based pigment that can be found in unripe fruits of *Genipa americana* L. (genipap) and have been associated with pharmacological effects beyond to be used as an alternative of natural blue colorant in food industry. Thus, a simple, rapid and simultaneous UHPLC-DAD method was carried for separation and determination of three iridoids (geniposidic acid, geniposide and genipin) of mesocarp and endocarp belonging to unripe and ripe genipap fruits. The optimal chromatographic performance was obtained with an AcclaimTM 120 C18 column and a binary mobile phase consisting of methanol and water acidified with 0.1% formic acid. Isolated peaks were detected at 240 nm for the referred iridoids. The method validation assured the compliance of the assay to International Conference on Harmonization standards (ICH) for linearity, LOD, LOQ, inter- and intraday accuracy and precision. The method ensures good selectivity, good accuracy, and adequate detection and quantification limits, which make it suitable for the analysis of target iridoids belonging to Genipa genus in only 30 minutes.

Keywords: iridoid compounds, genipap, natural pigments

Highlights

- Genipin and its glycosylated forms by UHPLC-DAD method and profile of *G. americana* was determined.
- The UHPLC-DAD method was able to separate and determinate genipin, geniposide and geniposidic acid.
- The highest amount of geniposide and genipin were found in ripe endocarp and unripe endocarp, respectively.
- The geniposidic acid it was found only in the ripe endocarp.

Chemical compounds reported in this article

Genipin (PubChem CID: 442424)

Geniposide (PubChem CID: 107848)

Geniposidic acid (PubChem CID: 16020046)

1. Introduction

The rising demand for natural plant-derived alternatives to synthetic and insect-based dyes is associated with the view of the consumer market about effects of food components on physiological functions, health, disease and also their impact on ecological balance. Furthermore, it is a concern of manufactures to satisfy the requirements of regulatory agencies about safety and toxicity of colour additives (Sivakumar et al., 2011; Sahidi et al., 2013; Brauch, 2016). In general, the colorants are added to processed food in order to potentialize existing colours that can be lost either during the manufacture or over the shelf-life, or even to attribute new ones to it (Carocho et al., 2011). They can be artificial/synthetic, nature-identical colorants or synthesized naturally, being that plants, insects/animals and minerals represents the natural sources (Sahidi et al., 2013; Carocho et al., 2015).

A plethora of unknown or underexploited exotic fruits and vegetables can offer a promising variety of novel pigments sources which besides coloring properties, assumes health-promoting potential suggesting their additional use as functional food ingredients

(Brauch, 2016). Although blue colors are scarce in nature and represents a challenge for academic research and industry, the iridoid-based blue pigments (mainly genipin), displays some peculiarities such as stability during thermal treatment, light or pH changes, being more stable than pigments extracted from blue-green algae (Bentes, et al., 2015; Buchweitz, 2016).

Genipin can be found in fruits of *Gardenia jasminoides* J. Ellis (gardenia) or unripe fruits of *Genipa americana* L. (genipap), both species belonging Rubiaceae family. In relation to the genipin availability, the gardenia fruits present a glycosylated form of genipin (geniposide), therefore, it is required a treatment with enzyme β-glucosidase to release the aglycone while genipap fruits possess free genipin (Bentes, et al., 2015; Brauch, 2016; Ramos-de-la-Peña et al., 2016). In terms of yield, *G. jasminoides* and *G. americana* fruits constitute around 0,17% and 1-3% of genipin and 9.6% and 10.7% of geniposide, respectively (Buchweitz, 2016; Ramos-de-la-Peña et al., 2016).

As regards to regulatory aspects, the genipin from *G. jasminoides* is available for food industry in East Asia countries such as Japan and Korea, also being associated with pharmacological effects (antioxidant, anti-inflammatory, antithrombotic, neuroprotective effects, inhibition of lipid peroxidation, etc) while *G. americana* remains underexploited (Kojima et al., 2011; Lee et al., 2014; Chen et al., 2015; Bentes, et al., 2015; Brauch, 2016). For these reasons, we will explore the potential of bioactive compounds from *G. americana* to be employed as additive in food industry or for pharmaceutical purposes.

Genipap occurs naturally in lowlands of Amazon forest and can grow throughout the tropical and partly subtropical areas of Latin America. Since pre-Colombian times ethnic groups of South American Indians uses a dark blue juice from macerated unripe fruit of genipap to paint skin as well as to coloring clothes and pottery (UNCTAD, Nations Conference on Trade and Development, 2005; Brauch, 2016). This juice contains genipin, which reacts with primary amines presents in amino acids and proteins to produce water-soluble dark blue pigments **(Figure 1)** (Buchweitz, 2016). Apart from genipin, genipin-derivatives (geniposide and geniposidic acid) also reacts with other compounds to produce pigments (Wu, 2013). However, the detailed mechanism of pigments formation is unclear (Brauch, 2016; Buchweitz, 2016; Kumar et al., 2016).

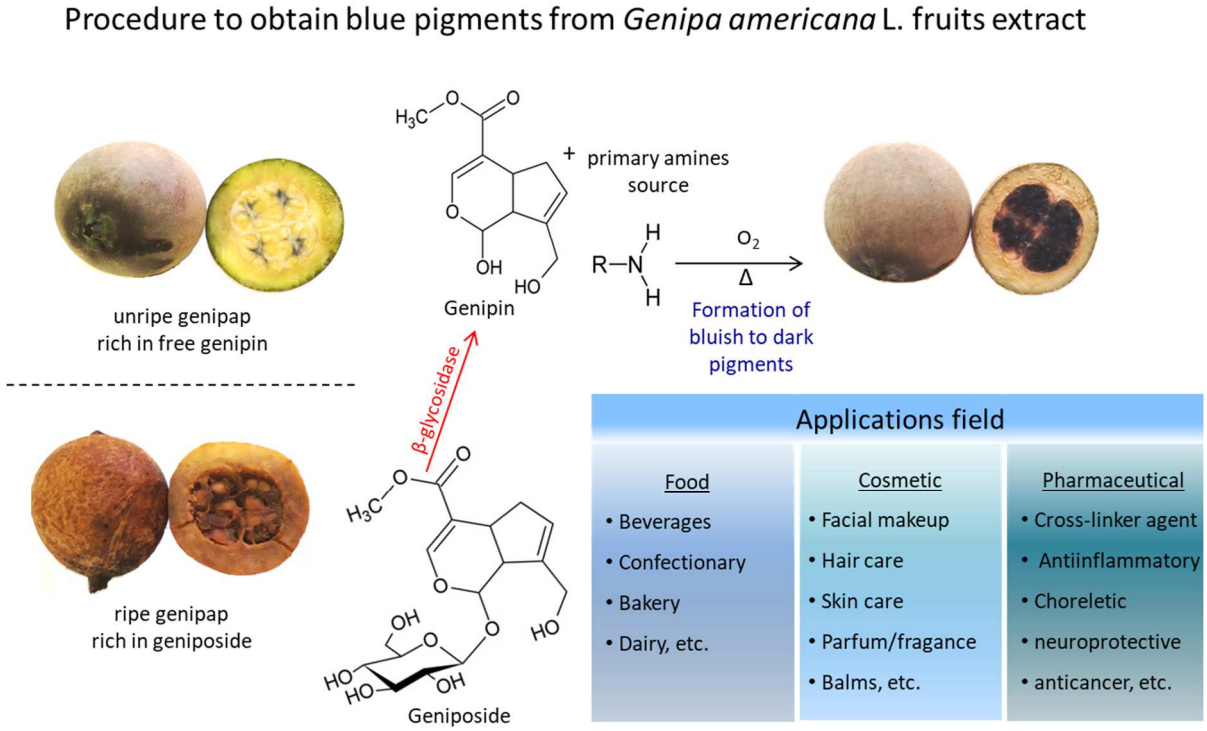

Figure 1. Genipin is a colorless iridoid that is composed of a cyclopentan –[C]-pyran skeleton. The hydroxil group at the C1 position of the genipin pyran ring can be substituted by one or two sugar moieties forming the genipin glycosides genipin-1-O-β-D-glucoside (geniposide) and genipin-1-O-β-D-gentiobioside, respectivelly. Adapted from Brauch, 2016.

With respect to the detection and quantification methods available for genipin and its glucosylated forms, there are scant information about genipin identification while the most of studies have focus on investigation of geniposide. In general, the HPLC in reverse technique using a C_{18} column at isocratic condition as well as the amperometric pulse have been used to detect geniposide. With these techniques is possible to obtaim representative peaks (at 235 – 240 nm) and good reprodutibility and high sensitive with around 90% of sample recovery (Ramos-de-la-Peña et al., 2016).

On other hand, Wang et al (2008) used an electrospray tandem mass spectrometry to quantify geniposide and genipin. Acetronitrile and methanol were used as mobile phase and the gradient establishment time, desolvation temperature and NH_4OAc concentration were studied in order to obtain the better peak intensity. The authors indicate that this

method provide sixfold sensitivity improvement for geniposide and genipin quantificaciton when compared to the sensitivities of isocratic methods.

Farther, Bergonzi et al (2012) identified and quantified genipin and geniposide of *G. jasminoides* Ellis by HPLC-DAD-ESI-MS, using also a C_{18} column by gradient elution with mixtures of methanol and acidified water as mobile phases and detection wavelength was set at 240 nm for iridoid glucosides. Similarly, Bentes et al (2014), tentatively identified 14 iridoids, including genipin and geniposide by HPLC-DAD using using a linear gradient of water and acetonitrile, both containing 0.1% formic acid, showing representative peaks between 200 – 250 nm.

However, some of these methods are time cosuming, expensive or hazardous for the column health. In addition, in the literature there are scant available methods for simultaneous determination of iridoids belonging to *G. americana*. We propose here a rapid and cost-effective quality-control tool for routine quantitative analysis of three iridoids (genipin, geniposide and geniposidic acid) by UHPLC-DAD analysis. This method can be useful for the future studies that will focus on the extraction and purification of iridoids glycoside obtained from genipap as well as another species from Rubiaceae family to be applied as ingredient in food industry or by pharmaceutical supplies.

2. Materials and methods

2.1. Standard chemicals

The geniposidic acid, geniposide and genipin, purity ≥ 98% (HPLC) and sugar standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol was obtained from J.T. Baker (Mexico City, D.F., Mexico), reagent grade formic acid was provided by Merck (Frankfurt, Germany), and water was purified by Milli-Q®$_{plus}$ system (Millipore, Milford, MA, USA). Stock solutions of each standard compound (1mg/mL) were prepared and stored in methanol at 4 ^{o}C. An intermediate solution containing all standard compounds (100µg/mL) was prepared in methanol 50% and this solution was diluted at 9 different levels (10-90µg/mL) for calibration curves and method validation.

2.2. Plant material

The ripe and unripe fruits of genipap were collected at University of Campinas (22° 49' 8.55" S and 47° 04' 24.022" W). The botanical identification was performed in the Department of Vegetal Biology and the exsicate (access number: 110904) was deposited at the Herbarium-UEC of Biology Institute of University of Campinas, State of São Paulo, Brazil.

2.3. Ripeness stage, sugars extraction and HPAEC-PAD analysis

For the ripeness stage determination were selected two batches containing 25 fruits each one, including unripe genipap with of 150 g and 20 cm diameter as well as ripe fruits with 230 g and 25 cm diameter, respectivelly. The ripeness stage of fruits was determined based on instrumental colour parameters, firmness, physicochemical characteristics and sugar profile. All of the fruits used in this study were submitted to firmness and color analysis. Both peel and pulp colour differences were quantified through CIELAB parameters (L*, a*, and b*) using a spectrocolorimeter (Hunter, model ColorQuest XE, Reston, VA, USA), equipped with D65 light source and an observation angle of 10^{o}. The firmness was measured determining the maximum force of penetration (MFP) using a texturometer (Stable MicroSystems, model TA-XT Plus, Surrey, UK) equipped with a P/4N needle probe. The MFP was determined on two opposite faces in the equatorial region of each fruit and the results were expressed in newtons (N). Fruits with MFP values >10 N were considered unripe, and fruits with MFP values <2 N were considered ripe, according described previously by Bentes & Mercadante (2014). For the physicochemical parameters, 20 g of a pool of whole fruit was homogenized with 180 mL of water using a waring blender. The soluble solids (SS) was determined by direct reading of a homogenized whole fruit drop in an Abbe refractometer (WAY, model Abbe Refractometer) at 24 ^{o}C and the results were expressed in Brix. The titratable acidity (TA) of whole fruits was determined by titration with NaOH 0.1N and calculated as percent of citric acid while the ratio was determined by the relation between the soluble solids amount and the titratable acidity (Adolfo Lutz, 2008). Sugar analyses were performed as described by Der Agopian et al. (2008) and Chen et al. (2014), with some modifications as described below:

Fifty grams of each whole genipap fruit (unripe and ripe) and 100 mL of distilled water were homogeneized in a domestic mixer (Black & Decker, Brazil) for 2 minutes and centrifuged (10 min, 3800 gm 10 ^{o}C). The supernatant was removed, filtered through a 0.22 µm filter and 30 µL of each sample were then submitted for the sugars analyses.

The analysis of sugars in unripe and ripe genipap samples was performed by high-performance anion exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD) as detector system (model Dionex ICS - 5000, Thermo Fischer Scientific, Waltham, Ma). It was used a Carbopac PA1 (250 mm × 4 mm, 10 µm particle size) column. The HPAEC–PAD system was equipped with a quaternary pump system, and a gradient elution was used. Two mobile phases were used: solution A (200 mM NaOH), solution B (ultrapure water). The gradient was performed as follows: 0–25 min, 40 - 69% A, 25 - 30 min, 100% A, and 30 - 35 min, 40% A. The (mono- and disaccharides) were identified in sample by comparing the retention times of the standards and the samples. Data acquisition was carried out with the Chromeleon v 7.0 software.

2.4. Iridoids blue-based pigments (IBBP) extraction and iridoids profile by UHPLC-DAD analysis

The unripe and ripe genipap fruits were fractioned in mesocarp and endocarp, totalling 4 samples. All samples were finely minced in a domestic processor and then freeze-dried for 72 hours (Terroni, model LS 3000, São Carlos, Brazil). Subsequently, the freeze-dried samples were powdered using a knife grinder (Marconi, model MA-340, Piracicaba, Brazil) and submitted to extraction procedure in triplicate. An aliquot of 1g powder was extracted eight times with 10 mL of methanol 80% using ultrasonication (40Hz) for 15 minutes followed centrifugation (8000 RPM for 10 min at 5 ^{o}C). All supernatants were combined and rotaevaporated at 60 ^{o}C to dryness (Bücchi Labortechnik AG, model R11 F-108, Flawil, Switzerland). The samples were recovered with 10 mL of water and filtered through 0.20 µm Minisart RC 15 single-use syringe filter (Sartorius, PP-housing, Goettingen, Germany) prior to injection.

The chromatographic analysis was performed according Bergonzi et al (2012) with some modifications. Separation, determination and quantification of compounds in samples

were performed using a Dionex Ultimate 3000 UHPLC system (Sunnyvale, CA, USA) equipped with two quaternary pumps, autosampler, column compartment and photodiode array detector (DAD). The chromatographic separation was performed on an Acclaim^{TM}120 C18 column (5µM 120Å 4.6 x 250 mm) using a binary mobile phase. Solvent A was methanol and solvent B was water acidified with 0.1% formic acid. The gradient elution at 27 ^{o}C was as follows: 0 - 2 min, 10% A and 90% B, 2 - 25 min, 90 to 10% B, 25 - 28 min, 100% A and 0% B, 28 - 30 min, 10% A and 90% B. The autosampler temperature was maintained at 4 ^{o}C and the injection volume was 20µL. The flow-rate was 1.0 mL/min and wavelength of detection was 240 nm. Chromeleon™ software (Sunnyvale, CA, USA) was used for data acquisition.

2.5. Validation method

The method was validated according to the ICH Harmonised Tripartite Guideline (ICH, 2005) over three consecutive days for linearity, limit of detection (LOD), limit of quantification (LOQ), inter- and intraday accuracy and precision.

The solutions of iridoid standards (geniposidic acid, geniposide and genipin) prepared at 9 different concentrations were run in triplicate (intra- and interday) and the calibration curves were constructed by plotting the peak area *versus* analyte standard concentration using the least-squares linear regression method and correlation coefficient (r). LOD and LOQ were calculated according to equations LOD = 3 *s/S* and LOQ = 10 *s/S*, respectively, where *s* is the standard deviation of the blank and *S* is the calibration curve slope. The accuracy was determined dividing the mean concentration for analytical runs (intraday *n = 3*, interday *n = 9*) by the expected concentration, and expressed as percentage. Three independent standards solutions were prepared (10 - 90 µg/mL) and each was injected three times for accuracy evaluation. Accuracy was calculated according to equation Accuracy (%) = 100*Cr/Ct, where Cr is the real concentration and Ct is the theoretical concentration. Accuracy was considered acceptable when 80 - 120% of the theoretical concentration was found. The intra- and interday precision was obtained by calculating the relative standard deviation (RSD) for the mean concentration (*n* = 3 and *n* = 9, respectively). Precision values with RSD less than 5% were considered acceptable.

3. Statistical analysis

All statistical analyses were performed at a significance level of 5% ($p \leq 0.05$) using STATISTICA software (Statsoft, Oklahoma, USA) version 12.0. The data are reported as mean values ± standard deviation of three replicates

4. Results and discussion

4.1. Determination of ripeness stage of the genipap fruit

The ripeness stage of fruits was determined based on instrumental colour parameters, firmness, physicochemical characteristics and HPAEC-PAD sugars analysis as shown in **Table 1**. The weight and diameter of ripe fruit were higher than unripe fruit, while the texture decreased after ripening. The colour of the fruit peel and pulp changed after ripening, mainly the chromaticity coordinate *b** of the pulp and the luminosity of the peel. Genipap fruit changes the peel and pulp colour from green to yellow during the ripening **(see Figure 1)**. These results are in agreement with Bentes & Mercadante (2014), who studied the influence of the stage of ripeness on the composition of iridoids in genipap.

Table 1. Instrumental colour, firmness, physicochemical parameters and sugars profile by HPAEC-PAD analysis of unripe and ripe genipap (*Genipa americana* L.) fruit.

Parameter	Unripe Genipap	Ripe Genipap
Weight (g)	114.24 ± 6.15[b]	225.33 ± 4.73 [a]
Diameter (mm)	18.83 ± 0.76[b]	25.77 ± 1.08 [a]
Texture (N)	123.17 ± 5.67[a]	2.67 ± 0.59 [b]
Peel Colour		
*L**	42.43 ± 2.78[a]	44.68 ± 3.51 [a]
*a**	1.53 ± 0.46[b]	9.28 ± 0.90 [a]
*b**	15.25 ± 2.40[b]	25.08 ± 3.71 [a]
Pulp Colour		
*L**	70.63 ± 2.41[a]	43.44 ± 1.86
*a**	1.22 ± 0.85 [b]	2.91 ± 0.73 [a]
*b**	21.68 ± 3.54 [a]	22.14 ± 2.95 [a]
pH	4.95 ± 0.11 [a]	4.11 ± 0.02[b]
TSS (°Brix)	3.60 ± 0.45 [b]	14.10 ± 1.87 [a]
TTA (g citric acid/100 g dw)	0.79 ± 0.10 [b]	2.40 ± 0.15 [a]
Ratio (TSS/TTA)	4.68 ± 1.08 [b]	5.93 ± 1.18 [a]
Glucose (mg/100 g dw)	0.21 ± 0.01 [b]	10.18 ± 0.12 [a]
Fructose (mg/100 g dw)	0.06 ± 0.01 [b]	9.34 ± 0.06 [a]
Sucrose (mg/100g dw)	0.01 ± 0.00 [b]	0.18 ± 0.01 [a]
Total sugars (mg/100 g dw)	0.28 ± 0.01 [b]	19.70 ± 0.18 [a]

TSS, total soluble solids; **TTA,** total titratable acidity; Luminosity **(*L**)** and the chromaticity coordinates ***a**** and ***b****. Data presented as mean ± standard deviation for the triplicate determination (*n* = 3). Means with different lowercase letters (a, b) within a row were significantly different ($p < 0.05$) by the Student's *t* test.

With regards to the TSS, TTA and total sugars (glucose, fructose and sucrose) we observed a significant increase of these parameters after ripening, whereas the *ratio* showed a slight increase. The unripe and ripe genipap showed different physical and physicochemical properties, which is very important to help researchers and industry in the harvest of these non-climacteric fruits. Similar resultas were reported by Hamacek et al (2013) where ripe genipap showed pH equal to 3.87, TSS (°Brix) of 11.40, TTA equal to 3.33 g of citric acid/100g and TSS/TTA ratio of 3.43.

The TSS content is used as an index of total sugars in fruits and may indicate their ripeness degree. Therefore, its evaluation is importance for both fresh fruits consumption as well as for its processing, as TSS indexes provides better flavour and higher fruit yields in the manufacture of products (Hamacek et al (2013). One of the criteria used for the classification of fruits as flavor is the TTA, which in the case of the ripe genipap was high, which can lead to a reduced acceptance of the fruit in its *in natura* form. According Silva et al (2009) the TTA is also higher in ripe fruits, which may be characteristic of this specie since this parameter for the most fruits instead decreases during ripening process.

4.2. Iridoids profile by UHPLC-DAD-analysis and validation of chromatographic method

For optimization of chromatographic conditions, various linear gradients of methanol and water acidified with 0.1% formic acid were tested at a flow-rate of 1.0 mL/min using the C18 column. Successfully, the gradient system previously mentioned was chosen because all the peaks in the chromatogram were clearly separated, presenting good sharp and symmetrical characteristics. In addition, four UV wavelengths (210, 230, 240 and 260 nm) were tested in order to achieve high sensitivity and little interference, being that the best results were observed at 240 nm. The chromatographic profile of the iridoids at the wavelengths of 240 nm is shown in **Figure 2**.

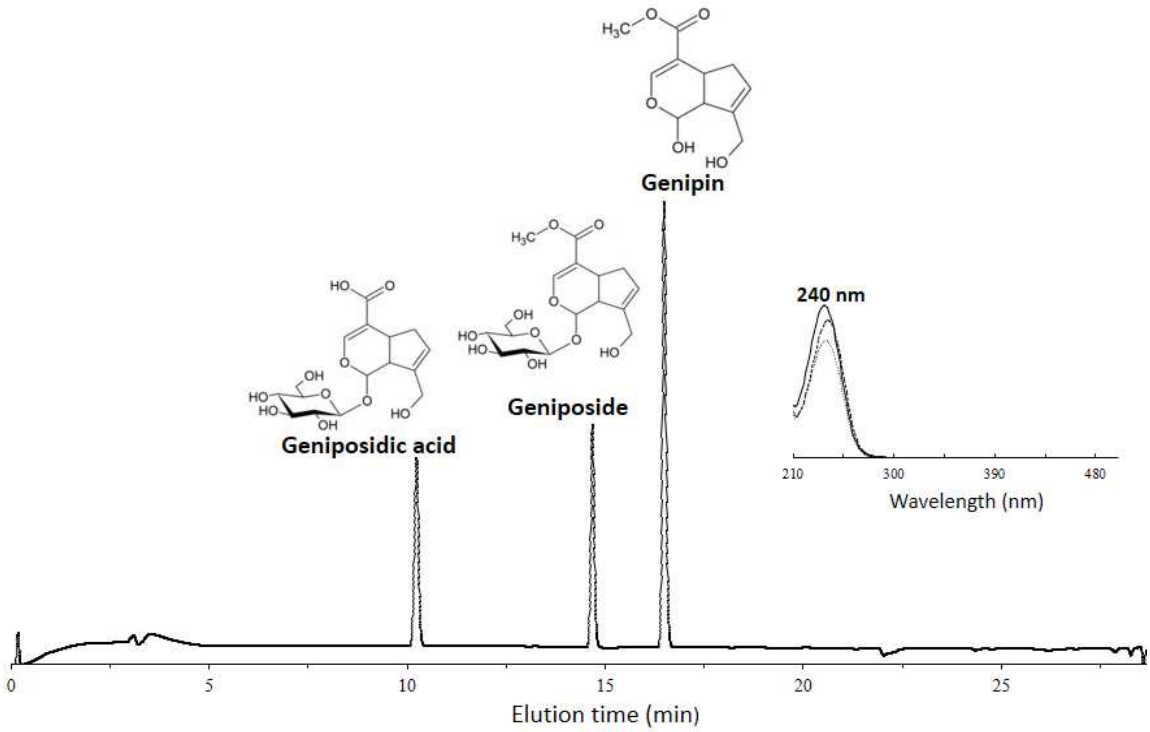

Figure 2. UHPLC-DAD profile at 240 nm of standard solutions of iridoids: geniposidic acid at 10.25min, geniposide at 14.67min and genipin at 16.48 min. The binary mobile phase was methanol and water acidified with 0.1% formic acid. The gradient elution at 27°C was as follows: 0-2 min, 10% A and 90% B, 2-25 min, 90 to 10% B, 25-28 min, 100% A and 0% B, 28-30 min, 10% A and 90% B.

Previously, Bergonzi et al (2012) proposed a method for separation, identification and quantification of constituents of *G. jasminoides,* namely iridoids, caffeoyl quinic acid derivatives and crocins by HPLC-DAD-ESI-MS with around 55 minutes of analysis time. Although it is an efficient method, this running time may be considered disadvantageous for practical applications. Thus, we proposed a modified and validated method to separate geniposide, geniposidic acid and genipin in a shorter time, with only 30 minutes of analysis time.

Concerning the validation method, the linearity was determined with calibration curves over a concentration range of 10 – 90 µg/mL at 9 levels for each iridoid standard (geniposidic acid, geniposide and genipin). The method exhibited very good linearity with correlation coefficient (r) values of 0.9981 to 0.9963, as well as the limits of detection. The LOD, defined with the standard curve parameters, were 0.593 µg/mL, 0.388 µg/mL and 0.708 µg/mL, whereas LOQ were 1.976 µg/mL, 1.293 µg/mL and 2.358 µg/mL, respectively for

geniposidic acid, geniposide and genipin. Validation parameters for the analyzed compounds are presented shown in **Table 2**.

Table 2. Validation parameters (retention time, slope, intercept, linearity (R^2), limit of detection (LOD) and limit of quantification (LOQ)) for the analyzed compounds.

Parameter	Geniposidic Acid	Geniposide	Genipin
Retention time (min)	10.25	14.67	16.48
Linear range (µg/mL)	10 - 90	10 - 90	10 - 90
Slope (SE)	0.6367 (0.00129)	0.6239 (0.00128)	1.3385 (0.00227)
p-value	< 0.0001	< 0.0001	< 0.0001
Intercept (SE)	- 0.1884 (0.07236)	- 0.5792 (0.07231)	- 0.6029 (0.12791)
p-value	0.0180	< 0.0001	0.0002
Correlation coefficient (r)	0.9981	0.9963	0.9977
Linear Regression (Analysis of variance)			
p-value	< 0.0001	< 0.0001	< 0.0001
LOD (µg/mL)	0.593	0.388	0.708
LOQ (µg/mL)	1.976	1.293	2.358

LOD: limit of detection, LOQ: limit of quantification, SE: standard error

In order to evaluate the precision of the method, three replicates at three different concentrations were assessed by through repeatability (intra-day) and reproducibility (inter-day) and expressed by relative standard deviation (RSD) **(Table 3)**. The average percentage was calculated for each level of concentration. Intra- and interday precision and accuracy data for the analyzed compounds are showed in **Table 3** and the RSD values were ≤ 1.25% and ≤ 3.18% for repeatability and reproducibility, respectively. These results show that the proposed method is adequately precise. The accuracy showed values within 93.75-113.60%, indicating excellent accuracy of the proposed method for iridoids quantification.

Table 3. Intraday (repeatability) and interday (reproducibility) precision and accuracy for the analyzed compounds.

Concentration (µg/mL) *(n= 3)*	Geniposidic Acid				Geniposide				Genipin			
	Repeatability		Reproducibility		Repeatability		Reproducibility		Repeatability		Reproducibility	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
10	1.25	103.84	1.23	103.89	0.60	113.07	1.87	113.60	0.30	102.78	3.18	111.60
20	0.31	103.51	0.43	102.10	0.32	104.17	0.70	103.31	0.09	95.79	0.12	100.14
30	0.77	104.33	0.67	103.27	0.13	103.30	0.12	102.36	0.07	100.06	0.48	103.42
40	0.27	101.89	0.34	99.75	0.11	100.43	0.14	99.06	0.10	96.47	0.72	98.91
50	0.54	101.11	0.56	99.61	0.31	100.14	0.49	99.02	0.22	96.82	0.22	99.52
60	0.38	100.20	0.56	96.36	0.19	98.23	0.47	94.64	0.09	93.95	0.18	94.76
70	0.09	97.19	0.51	95.67	0.14	96.80	0.48	95.77	0.09	93.75	0.51	96.62
80	0.25	103.98	0.53	102.47	0.04	100.78	0.76	99.58	0.02	99.38	0.25	102.12
90	0.08	104.04	0.33	101.94	0.09	106.93	0.21	105.17	0.04	100.10	0.29	102.54

RSD: relative standard deviation.

The validated UHPLC-DAD method was then applied to assess the iridoids content in all fractions from genipap (unripe/ripe and mesocarp/endocarp) **(Figure 3)**. The results are summarized in **Table 4**. The highest amount of geniposide and genipin were 128.28 mg/g dry weight (dw) for ripe endocarp and 39.05 mg/g dw of unripe endocarp, respectively. In addition, it was only found geniposidic acid (5.51 mg/g dw) in the ripe endocarp.

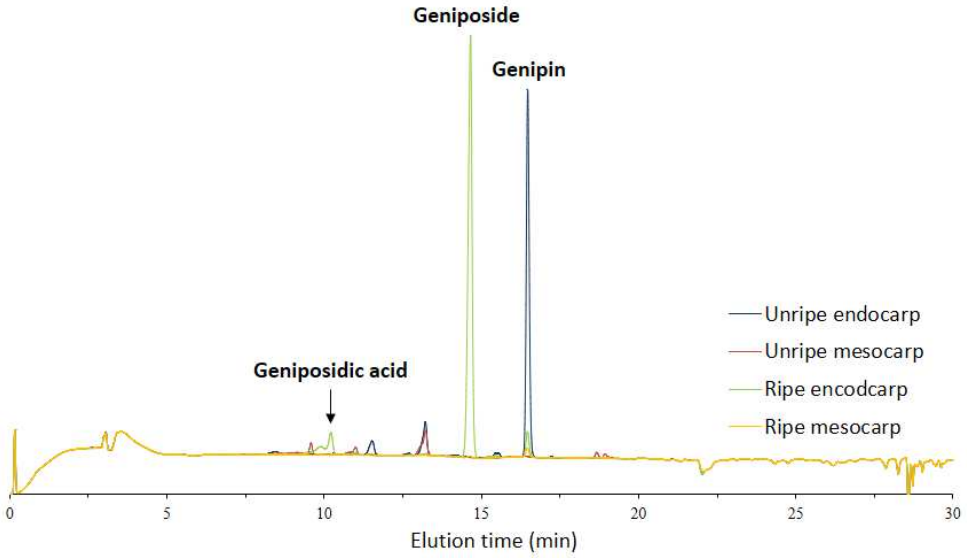

Figure 3. UHPLC-DAD profile at 240 nm of IBBP extracts of *Genipa americana* L.: geniposidic acid at 10.25min, geniposide at 14.67min and genipin at 16.48 min. The binary mobile phase was methanol and water acidified with 0.1% formic acid. The gradient elution at 27oC was as follows: 0-2 min, 10% A and 90% B, 2-25 min, 90 to 10% B, 25-28 min, 100% A and 0% B, 28-30 min, 10% A and 90% B.

Table 4. Concentration of geniposidic acid, geniposide and genipin in endocarp and mesocarp of ripe and unripe genipap (*Genipa americana* L.)

	Unripe		**Ripe**	
Compound	**endocarp**	**mesocarp**	**endocarp**	**mesocarp**
Geniposidic acid	n.d.	n.d.	5.51 ± 0.08*	n.d.
Geniposide	n.d.	1.49 ± 0.09*	128.28 ± 0.28*	n.d.
Genipin	39.05 ± 0.54*	n.d.	3.45 ± 0.03*	1.58 ± 0.03*

*Results expressed in mg/g dry weight, n.d.: not determined (values below LOQ). Data presented as mean ± standard deviation for the triplicate determination (*n= 3*).

Our results are in agreement with Náthia-Neves et al (2017) who also reported that unripe genipap presents a high content of genipin (38 mg/g) in endocarp while mesocarp have high contents of geniposide (20.7 mg/g). Unlike our results, Bentes et al. (2014) reported that highest amount of geniposidic acid, geniposide and genipin were 19.86 ± 0.09 mg/g for unripe endocarp, 117.99 ± 0.93 mg/g for unripe mesocarp and 3.40 ± 0.11 mg/g for unripe endocarp, respectively. We attribute this discrepancy to the fruit batch. Probably this discrepancy its due the *"turning point"* of the biological ripeness indentifiable in the fruit at the moment of extraction since that fruit ripening is associated with important biochemical changes that may significantly influence iridoids contents.

Father, our study indicated important physicochemical changes during fruit ripening levels as well as the decrease of genipin contents genipin at the later stage of ripening and the increase of geniposide contents. In addition, Bentes et al. (2014) reported used only genipin as standard reference to compare iridoids composition while other compounds were indirectly quantified and the results were expressed as genipin equivalent, which can over- or underestimate the quantification.

5. Conclusion

UHPLC-DAD method was validated for linearity, LOD, LOQ, inter- and intraday precision, and accuracy, which means that geniposidic acid, geniposide and genipin can be determined with good separation within only 30 min using this chromatographic system. This method validation was found to be suitable for the routine analysis of iridoids of unripe and ripe genipap fractions of mesocarp and endocarp as well as fruit extracts or commercial samples rich of these iridoids due the simplicity, sensitivity and accuracy for the analyzed compounds.

Acknowledgments

The authors thank the National Council for Scientific and Technological Development (CNPq, grant number 481670/2013-0), the São Paulo Research Foundation (FAPESP, grant number 2015/50333-1], and the Institute of International Education (CAPES)

for financial support and the Rupert Lyons for the language services provided. C. F. F. A. acknowledges FAPESP (studentships 2017/10753-7).

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Publication

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CAPÍTULO III

IRIDOID BLUE-BASED PIGMENTS OF GENIPAP FRUIT (*Genipa americana* L. - RUBIACEAE) EXTRACT: INFLUENCE OF PH AND TEMPERATURE ON COLOR STABILITY AND ANTIOXIDANT CAPACITY DURING *IN VITRO* SIMULATED DIGESTION

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Artigo submetido na Food Chemistry

Fator de Impacto 4.529 - Qualis A1

Abstract

Iridoid blue-based pigments (IBBP) extract of *Genipa americana* L. represents a natural alternative as additive for food applications and also exerts desirable biological effects on human health. In this study the iridoids present in the IBBP extract were identified, the influence of pH and temperature on color change (ΔE) of IBBP was evaluated using a central composite design (CCD) and finally the antioxidant capacity of IBBP was monitored before and after its *in vitro* digestion. Ten glucoside iridoids were detected in the IBBP extract and the main compounds identified were genipin, genipin 1-β-gentiobioside and geniposide. After *in vitro* digestion genipin content significantly decreased while the amount of genipin 1-β-gentiobioside increased 49%, suggesting that this compound was gradually released from IBBP during hydrolysis. Among the conditions tested, the color of IBBP extract was more stable at 12 – 20 ^{o}C and low pH (3.0 - 4.0), suggesting that it is compatible for coloring acidic foods. Finally, the in vitro digestion also increased the antioxidant capacity (ORAC assay) by 39%.

Keywords: iridoid compounds, genipap, natural pigments, genipin, geniposide

Highlights

- Iridoid glucosides are main compounds found in genipap (*G. americana*) fruits
- Natural blue pigments obtained from genipap is an alternative for food applications
- Genipin is the main iridoid blue-based pigment found in unripe genipap
- Iridoid blue-based pigments are more stable at pH 3.0-4.0 and 5-20 ^{o}C
- Genipin bonded forms are bioacessible and can exerts antioxidant potential

Abbreviations

IBBP, iridoid blue-based pigments, L*, lightness/darkness, a*, redness/greenness chroma, b*, yellowness/blueness, ΔE*, color change, CCD, Central Composite Design, ND, non-digested, GP, gastric phase, IP, intestinal phase, not detected, r.t., retention time, ORAC, Oxygen Radical Absorbance Capacity.

Chemical compounds reported in this article

Gardenoside (PubChem CID: 442423)

Genipin (PubChem CID: 442424)

Genipin 1-β-gentiobioside (PubChem CID: 3082301)

Geniposide (PubChem CID: 107848)

Geniposidic acid (PubChem CID: 16020046)

6´-O-p-Coumaroyl-geniposidic acid (PubChem CID: 44255335)

6´-O-p-Feruloyl-geniposidic acid (PubChem CID: not found)

6”-O-p-Coumaroyl-1-β-gentibioside geniposidic acid (PubChem CID: not found)

6”-O-p-Coumaroylgenpin-gentiobioside (PubChem CID: 101408305)

1. Introduction

The fruits of *Genipa americana* are easily obtainable in the lowlands of the Amazon Forest and other parts of Latin America and have long been used by Amazonian indigenous tribes for dyeing artifacts, to paint bodies as well as to treat diseases and also to the feed (Horák, 2014; Brauch, 2016 a; Neri-Numa et al., 2017). The coloring properties of genipap fruit are attributed to the presence of iridoid blue-based pigments, especially genipin and its glycosylated forms (geniposide and geniposidic acid) (Neri-Numa et al., 2017). Genipin is colorless but can react with primary amines in the presence of oxygen to form water-soluble blue pigments with a λ_{max} of 609nm **(see supplementary material, S1)** (Buchweitz, 2016; Brauch et al., 2016a; Neri-Numa et al, 2017). These blue pigments can be also obtained from *Gardenia jasminoides* Ellis, another representative of the Rubiaceae family (Bentes et al., 2015). From a commercial point of view, gardenia blue colorant has been used in food, pharmaceutical and cosmetics industries while genipap is still underexploited and few studies have focused on understanding it´s stability and possible degradation processes (Bentes et al., 2015; He & Huang, 2017).

In general, natural blue colorants are extremely rare in commercial applications. Some exceptions are the blue pigments from *Spirulina* spp, *Gardenia jasminoides*, and *Clitoria ternatea*, besides indigo (*Indigofera tinctoria*) and woad (*Isatis tinctoria*), formerly widely used as blue dyes. One of the main reasons for this scarce availability is related to their instability and to the difficulty of matching the correct hues and intensities of their synthetic counterparts (Jespersen et al., 2005; Lavinia et al., 2015; Brauch et al., 2016 b). In terms of food processing and storage, the color change is considered a relevant parameter and, in the case of natural blue colorants, their hue may change towards purple/violet or fade, resulting in a product with less than optimal visual and aesthetic appeal (Sant´Anna et al., 2013; Myers et al., 2017).

Concerning blue pigments stability of genipin, it is important to mention that their hue is highly affected by pH, temperature, amino acid source and genipin-to-source ratio (Brauch et al, 2016 a). In a previous study, Paik et al (2001) assessed the gardenia blue stability at light, high temperatures (60- 90 ^{o}C) and pH range (5 - 9) using several amino acids (glycine, lysine, or phenylalanine) for blue hues formation. They observed that pigments were more stable in alkaline than acidic or neutral conditions. Farther, the photodegradation were checked incubating blue pigments at pH at 4 ^{o}C in different pH, for 10 hours. As result, amino acid source interfered in a color stability, remaining around 75% and 51% of blue pigments for reactions with glycine and phenylalanine, respectively.

As regards to regulatory aspects, genipin as a natural blue colorant belonging to *G. jasminoides* is available for food and medicinal purposes just in East Asia countries while the blue one obtained from *G. americana* have been used in the United States as a color additive "Fruit juice" (Title 21 CFR, Code of Federal Regulations, § 73.250) despite not being approved for the United States or European Union markets (Brauch, 2016 b; Buchweitz, 2016). Conversely, several companies currently aiming to introduce genipap fruit and its derived products into markets outside Latin America, where they are most likely to be considered as a novel food and food ingredients (Regulation (EC) No 258/97), respectively (Brauch et al 2016 b).

In addition, most of the consumers are more attentive to their health and are avid for healthier food alternatives (Carocho et al., 2015). Therefore, innovative solutions in the field of food additives are being directed by the use of natural and healthier additives (Amchova et al., 2015; Carocho et al., 2015; Rodriguez-Amaya, 2016). Thus, it is essential to quantify the bioaccessible fraction of the ingested bioactive compounds during gastrointestinal digestion and also to determine how much of it is absorbed (bioavailability) (He et al., 2016). For this reason, the parameters affecting the digestive stability and antioxidant activity of bioactive compounds have been assessed preferably through *in vitro* digestion methods (bioaccessibility) before performing *in vivo* trials (Stinco et al., 2012; He et al., 2016).

Although there are a dearth of information exists on genipin toxicological effects, most of the studies about hazard assessment of blue iridoid-based colorants, refer to the Gardenia genus with controversial data about its toxicity (Maillox et a., 2010). However, it was reported that its toxicity was dose and time dependent when supplemented directly to the cartilage culture medium used in tissue-engineering. The lethal dose was at 2200µM, not lethal at 220 µM and beneficial at 22 µM. Short-term exposure at 220 and 22 µM was sufficient to obtain the benefits on the tissue level without the detrimental effects on the cellular level (Lima et al., 2009).

In this context, there is a considerable interest in understanding how the bioactive pigments are released from the food matrix during digestion process, being available for absorption (bioacessibility) and for exerting a biological effect, particularly those related to positive response on human health (Kamiloglu et al., 2015; Schulz et al., 2017).

Thus, the aim of this study was to evaluate the effect of pH and storage temperature on the color stability of iridoid blue-based pigments (IBBP) of genipap fruit extract using a central

composite design (CCD) as well as to evaluate its antioxidant capacity and to identify the iridoid content before and after gastrointestinal *in vitro* digestion.

2. Materials and methods

2.1. Chemicals

Standards of geniposidic acid, geniposide and genipin, purity ≥ 98% (HPLC) and 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate], 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,20-azobis(2-methylamidinopropane)-dihydrochloride (AAPH), sodium fluorescein as well as HPLC grade methanol and formic acid were provided by Merck (Frankfurt, Germany). All other chemicals and solvents in this study were of analytical grade and water was purified by Milli-Q®$_{plus}$ system (Millipore, Milford, MA, USA). The blue commercial colorants (brilliant blue and spirulina) used as color standard were kindly donated by Sensient®.

2.2. Plant material and extraction procedure

Unripe fruits of genipap were collected at the University of Campinas (22°49'8.55"S and 47°04'24.022"W). The botanical identification was performed at the Department of Vegetal Biology and the exsicate (access number: 110904) was deposited at the Herbarium-UEC of Biology Institute of University of Campinas, State of São Paulo, Brazil.

Cano et al. (2016) reported that in *G. americana* fruits, genipin is found mostly in endocarp, while its concentration is lower in the mesocarp. Moreover, free genipin is available only in the unripe fruits, since it becomes glycosylated (geniposide and geniposidic acid) during ripening (Cano et al., 2016). Thus, the extraction procedure used just unripe endocarp of genipap. Sample was freeze-dried for 72 hours (Terroni, model LS 3000, São Carlos, Brazil), powdered using a knife grinder (Marconi, model MA-340, Piracicaba, Brazil) and subsequently submitted to an extraction procedure in triplicate. For this procedure, an aliquot of 10 g of the resulting powder was extracted eight times, each with 100 mL of methanol 80% (v/v) in water, in ultrasonic bath (40Hz) for 15 minutes followed by centrifugation (8000 RPM) for 10 min at 5 °C. All supernatants were combined and evaporated at 60 °C to dryness (Bücchi Labortechnik AG, model R11 F-108, Flawil, Switzerland). The samples were recovered with 10 mL of water and filtered through 0.20 µm regenerated

cellulose Minisart RC 15 single-use syringe filter (this filtrate was called IBBP extract) (Sartorius, PP-housing, Goettingen, Germany) prior to chromatographic analyses.

Prior to the stability evaluation **(section 2.3)**, the amount of IBBP extract added to the solution was standardized, as cited in the sequence. Initially, 150 mg of IBBP extract was dissolved in 30 mL of phosphate-citrate buffer (pH 4.5 at 150 mM). After 30 min of equilibration at room temperature, the absorbances were monitored at visible spectra (500 - 700 nm) using an ultra-fast UV/vis spectrometer (BMG LABTECH GmbH, model SPECTROstar Nano, Ortenberg, Germany). The absorbance curve obtained was compared to solutions containing commercial food colorants (Brilliant blue, Cod. 05649 and Spirulina P-WS (Cod. 409422-0001- Sensient®) (Supplementary material S2A). In the sequence, the iridoid content of IBBP extract was evaluated by UHPLC-DAD analyses, using a method developed and validated in a previous study (Neri-Numa et al., data not published) (Supplementary material S2B). Separation of the iridoids was carried out on a AcclaimTM120 C18 column (5µM 120Å 4.6 x 250 mm) using a binary mobile phase of (A) methanol and (B) water acidified with 0.1% formic acid at the following gradient: 0-2 min, 10% A and 90% B, 2-25 min, 90 to 10% B, 25-28 min, 100% A and 0% B, 28-30 min, 10% A and 90% B. The temperature and flow rates were 27 ^{o}C and 1.0 mL/min and the wavelength of detection was set at 240 nm. The calibration curves of the iridoids were obtained at the range of 10 – 90 µg/mL for genipin (R^2 = 0.9956), geniposide (R^2 = 0.9926) and (R^2 = 0.9962) geniposidic acid. Chromeleon™ software (Sunnyvale, CA, USA) was used for data acquisition.

2.3. Influence of pH and temperature on color stability and genipin contents of IBBP extract

The effect of temperature (in a range of 2.0 to 40 ^{o}C) and pH (in a range of 2.5 to 7.6) on the color stability of IBBP extract was evaluated employing a central composite design with two independents variables (2^2) and three center points **(Table 1)**.

Table 1. Variables and levels evaluated in the central composite design.

Variable	Level				
	-1.41	-1	0	+1	+1.41
pH	2.5	3.2	5	6.8	7.5
Temperature (°C)	2.5	8.0	21.3	34.5	40

These levels were determined based on previous reports on blue pigments stability of *Gardenia sp*. and *Genipa* sp. (Fujikawa et al., 1987; Paik et al., 2001; Renhe et al., 2009). Therefore, IBBP extract standardized was diluted in citrate-phosphate buffer 0.1 M at different pH levels and were then incubated at a given temperature, according to the central composite design matrix. Samples were taken every three days during 30 days to evaluate the kinetics in color change.

In this context, the CIELAB system is related to human color perception in three dimensions or directions of the colour space: the L* axis representing lightness/darkness, a* representing red to green hue, and b* representing the yellow to blue hue. Colour differences (ΔEs in each sample are obtained based on changes in colour coordinates (Δ*L**, Δ*a**, and Δ*b**) (Palacios-Morillo et al, 2016).

Thus, the color (L*, a*, and b*) of the solution was analyzed with a spectrocolorimeter (Hunter, model ColorQuest XE, Reston, VA, USA) using a 1 x 10^{-3} m measuring aperture. and a 3 mL cuvette (1cm path length), equipped with D65 light source and an observation angle of 10°. Reflectance-specular included (RSIN) mode, which measures total reflectance, including diffuse reflectance and specular reflectance, was used. Color difference (ΔE*) was calculated according to Eq. 1

$$\Delta E^* = [(L^* - L^*_{CP0})^2+(a^* - a^*_{CP0})^2+ (b^* - b^*_{CP0})^2]^{1/2} \quad \text{Eq. 1}$$

Where (L*, a*, b*) refers to the color at a given condition and (L^*_{CP0}, a^*_{CP0}, b^*_{CP0}) refers to the average color values for the center points (pH 5.0, 21.3 °C) at time zero, which were 7.74 ± 0.75, -1.38 ± 0.08 and I -2.14 ± 0.37, respectively. For all the other conditions at time zero, L*, a*, b* varied from 8.00 to 8.67, -1.41 to -1.10 and -2.01 to -2.98, respectively.

2.4. In vitro simulated gastrointestinal digestion procedure

The IBBP extract was digested according the method developed by Faller et al (2012) and modified by Sancho et al. (2017). Briefly, 1 mL of sample was mixed with 3.5 mL of saline solution (140 mM NaCl, 5 mM KCl) using a vortex. This mixture was acidified to pH 2.0 with 6 M HCl, and 125 mL of porcine pepsin solution (200 mg pepsin 424 U/mg in 5 mL 0.1 M HCl) was added. The solution was incubated at 37 ^{o}C in a shaking water bath for 1 h under continuous agitation (130 rpm). After gastric phase (GP), the pH was increased to 6.8 with 1M $NaHCO_3$, followed by the addition of 625 µL of pancreatin-bile solution (225 mg of bile extract and 37 mg of pancreatin 4x U.S.P, specifications in 18.7 mL of 0.1 M $NaHCO_3$) and incubation in a shaker at 37 ^{o}C and 130 rpm for 2 h. At the end of intestinal phase (IP), samples were cooled in an ice bath and the digested volume was adjusted to 5.5 mL with saline solution. All fractions were ultrafiltrated using Amicon Ultra Centrifugal Filter Devices (30 kDa, Millipore) to remove proteins and were further stored at -80 ^{o}C for further analysis. A blank sample consisting of saline solution at pH 7.0 was subjected to the digestion procedures to eliminate any interferences coming from the reagents.

2.5. LC-MS analysis

The non-digested IBBP extract (ND) and the digested fractions (GP and IP – see section 2.4) were prepared diluting 50 µL of a 20 mg/mL sample in 950 µL of water resulting in a final concentration of 1 mg of sample per mL. These fractions were analyzed in negative ion mode using an HPLC (Hewlett Packard, Agilent Technologies 1290 series) coupled to Poroshell C18 120 SB-Aq 2.7 µm reversed phase column (2.1x100 mm, Agilent) and a Q-ToF iFunnel 6550 mass spectrometer fitted with an electrospray ionization (ESI) source. Mobile phase A consisted of a 0.1% (v/v) solution of formic acid in water, and mobile phase B was a 0.1% (v/v) of formic acid solution in acetonitrile. The samples were eluted with a flow rate of 0.45 mL/min following linear gradients (v/v) of mobile phases: 0 - 1 min, 5% B, 1 - 10 min, 5% B to 18% B, 10 -13 min 18% B to 70% B, 13 - 15 min, 70% B to 100% B, 15 - 17 min, 100% B and 17 - 20 min post time (5% B). The mass spectrometer parameters used were: VCap 3000 V, fragmentor voltage at 150 V, OCT 1RF Vpp at 750 V, Gas Temperature at 290 °C, Sheath Gas Temperature at 350 ^{o}C, Drying Gas at 12 L/min. Mass spectra were acquired in profile mode and the acquisition range was 100 - 1500 m/z.

2.6. Antioxidant capacity

The antioxidant capacity of ND, GP and IP was determined by ORAC assay according method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004), using a microplate reader (NOVOstar, BMG Labtech®, Offenburg, Germany), accompanied with the MARS Data Analysis Software version 1.3 (BMG Labtech®, Offenburg, Germany). Samples and Trolox standards were prepared with 75 mM phosphate buffer (pH 7.4). In each well containing 20 µL of extract or Trolox standard in different dilutions (to reach a linear response) and 120 µL of fluorescein (70 mM), 60 µl of AAPH solution (12 mM) was added to start the reaction, resulting in a final total volume of 200 µL. The decay of fluorescence was measured every minute for 80 min at 37 ^{o}C, with excitation and emission wavelengths of 485 and 528 nm. A blank experiment (fluorescein + AAPH) with buffer instead of the sample or Trolox was also performed. The results were calculated using the relative area under the curve for samples compared to a Trolox standard curve (25 - 500 µM) prepared under the same experimental conditions. ORAC values were expressed as µM TE/g sample.

3. Statistical analysis

Statistical analyses were conducted using StatSoft, Inc. (2013). STATISTICA software, version 12.0. The results were subjected to ANOVA and differences between means were located using Tukey's multiple comparison test. Significance was determined at $p < 0.05$. All results are presented as means ± standard deviation for triplicates.

The data from the central composite desing were treated using Protimiza software version 2014 (www.http://experimental-design.protimiza.com.br) to model the effect of each variable on color stability. Model fitness was assessed considering a significance level of 5% ($p<0,05$).

4. Results and discussion

4.1. Influence of pH and temperature on color stability and genipin contents of IBBP extract

A Just Notable Difference in color is calculated as a ΔE* of approximately 2.3 (Mokrzycki & Tatol (2011). The kinetic study **(Figure 1)** showed that significant color changes (ΔE* of 2 - 4) were evidenced for days 3 to 12 and even more intense color difference (ΔE* of 3.5 - 7) was noticed for days 15 to 30. In some cases, usually close to the extreme conditions (pH 5.0, 40 ^{o}C, pH 7.6, 21.3 ^{o}C,

pH 6.8, 34.5 ^{o}C, pH 6.8, 8 ^{o}C, pH 2.5, 21.3 ^{o}C, pH 3.2, 8 ^{o}C), a different color ($\Delta E^* > 5$) appeared after day 15, especially after day 18. This was in accordance to the visual analysis: the colors of these samples became brownish **(see supplementary material, S3)**.

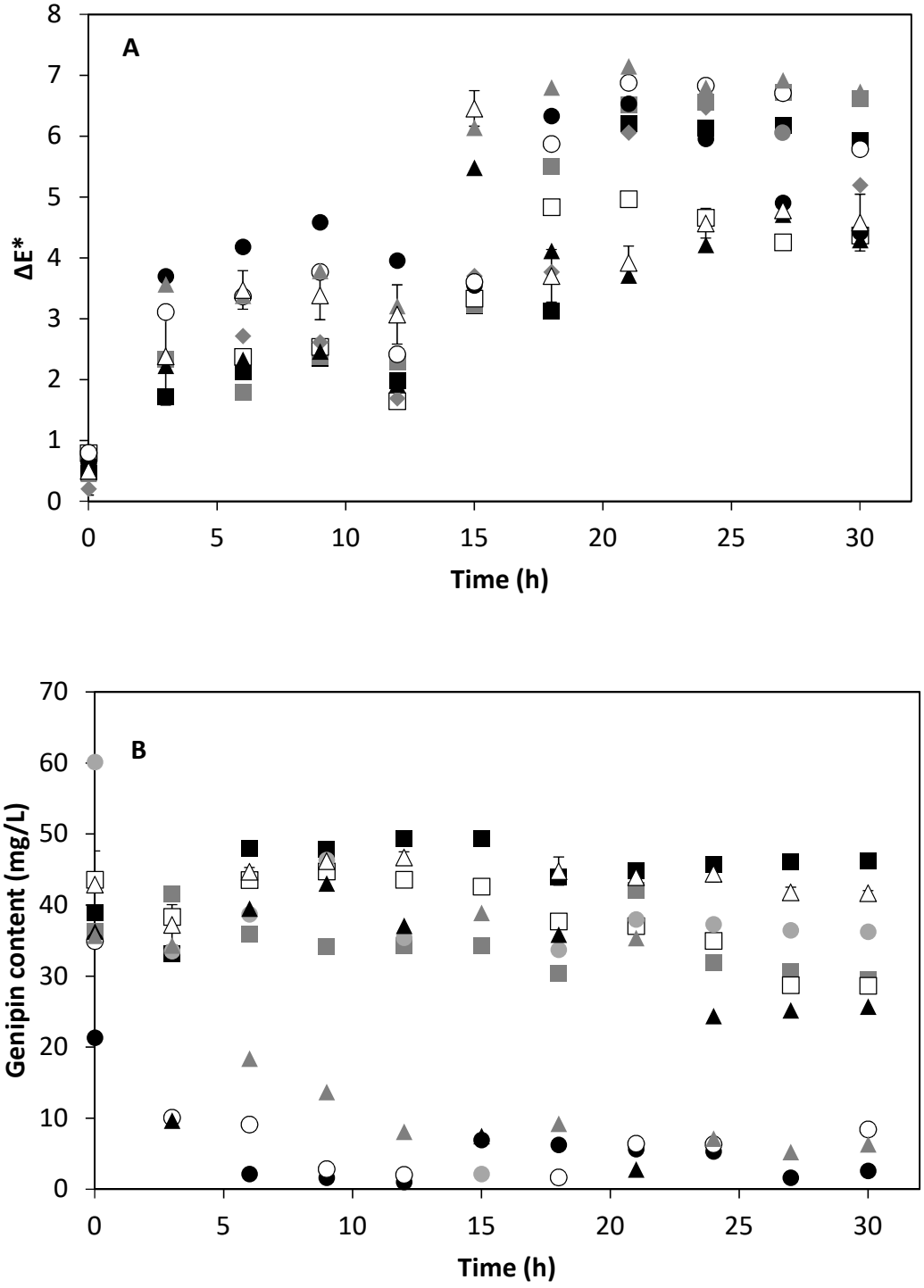

Figure 1. (A) Change in color difference (ΔE*) of IBBP extract or **(B)** genipin content (mg/L) over time at different conditions: pH 3.2, 8 °C (■), pH 6.8, 8 °C (■), pH 3.2, 34.5 °C (□), pH 6.8, 34.5 °C (●), pH 2.5, 21.3 °C (●), pH 7.6, 21.3 °C (○), pH 5.0, 2.5°C (▲), pH 5.0, 40°C (▲), pH 5.0, 21.3 °C (central points) (△).

In general, regardless the conditions **(Table 2)**, the color difference (ΔE*) increased up to the 18th to 21st day and, after that, it stabilized **(Figure 2A)**. Therefore, the color difference was modeled at the 18th day when it is expected to be close to it maximal value **(Table 3)**.

Table 2. 2^2 Central Composite Design matrix and the color difference (ΔE*) after 18 days or genipin content after 9 days of incubation at each condition of pH and temperature (T) (the codified values presented here are found in Table 1).

Trial	pH	T	ΔE*			Genipin content (mg/L)		
			experimental	predicted	Error (%)	experimental	predicted	Error (%)
1)	–1	–1	2.99	3.27	-8.5	47.89	46.40	3.2
2)	+1	–1	5.36	9.95	8.3	34.18	31.50	8.5
3)	–1	+1	4.72	4.87	-3.1	44.76	41.80	7.1
4)	+1	+1	6.20	6.55	-5.3	1.63	<0[a]	-
5)	–1.41	0	3.70	3.42	8.3	46.36	48.39	-4.2
6)	+1.41	0	5.74	5.79	-0.8	2.82	6.65	-57.6
7)	0	–1.41	3.95	4.07	-2.9	43.06	44.89	-4.1
8)	0	+1.41	6.69	6.33	5.7	13.69	17.67	-22.5
9)	0	0	3.24	3.53	-8.2	45.86	46.19	-0.7
10)	0	0	3.48	3.53	-1.4	44.55	46.19	-3.6
11)	0	0	3.87	3.53	9.8	48.17	46.19	4.3

[a]negative values in this model are interpreted as zero.

Table 3. Regression coefficients of the model of color difference after 18 days and genipin content after 9 days of incubation.

Parameters	Regression coefficient		Standard Error		t calculated		P value	
	ΔE*	**Genipin (mg/L)**	**ΔE***	**Genipin (mg/L)**	**ΔE***	**Genipin (mg/L)**	**ΔE***	**Genipin (mg/L)**
Mean	3.53	46.19	0.20	2.27	17.45	20.35	>0.0001	>0.0001
X_1	0.84	-14.80	0.12	1.39	6.80	-10.65	0.0010	0.00013
X_1^2	0.54	-9.39	0.15	1.65	3.69	-5.68	0.0141	0.00236
X_2	0.80	-9.65	0.12	1.39	6.49	-6.95	0.0013	0.00095
X_2^2	0.84	-7.50	0.15	1.65	5.72	-4.53	0.0023	0.00620
$X_1 . X_2$	-0.22	-7.35	0.18	1.97	-1.26	-3.74	0.2620	0.01340

These findings suggest that formation of brownish intermediary pigments arises from genipin ring-opening polymerization or genipin self-reaction to form genipinic acid under pH 5.0 – 7.0 conditions at 21.3 to 40 ^{o}C (Touyama et al., 1994; Wu & Horn 2017).

These data were then treated by the software PROTIMIZA and using only the significant parameters (Mean, X_1, X_1^2, X_2 and X_2^2 in case of ΔE* and all parameters, in case of genipin content), it was possible to verify the validity of the model through an analysis of variance (ANOVA), which is shown on **Table 4**.

Table 4. ANOVA of the quadratic models of color difference and genipin content.

Variation source	SS[a]		df[b]		SM[c]		F value		P value	
	ΔE*	Genipin (mg/L)	ΔE*	Genipin (mg/L)	ΔE*	Genipin (mg/L)	ΔE*	Genipin (mg/L)	ΔE*	Genipin (mg/L)
Regression	15.4	3351.5	4	5	3.9	670.3	28.5	43.4	0.00048	0.00040
Residues	0.8	77.3	6	5	0.1	15.5				
Lack of fit	0.6	70.5	4	3	0.2	23.5	1.5	7.0	0.44	0.13
Pure error	0.2	6.7	2	2	0.1	3.4				
Total	16.2	3428.8	10	10						

[a]SS = Sum of squares, [b]df = degrees of freedom, [c]SM = mean square

R^2 (ΔE*)= 95.01%, R^2 (genipin content)= 97.75%,

$F_{0.95(4,6)} = 4.53$, $F_{0.95(4,2)} = 19.25$, $F_{0.95(5,5)} = 5.05$, $F_{0.95(3,2)} = 19.16$

The ANOVA table shows that the quadratic models adjusted for both responses (color change and genipin content) were satisfactory. Besides a reasonable coefficient of determination and a p value, the F test suggest a good adjust. Consequently, it was possible to obtain the following mathematical models (Eq. 2 and 3) to predict the best conditions in terms of minimal color difference or genipin content of IBBP extract:

$$\Delta E^* = 3.53 + 0.45\ pH + 0.54\ pH^2 + 0.80\ T + 0.84\ T^2 \quad \text{Eq. 2}$$

$$\text{Genipin (mg/L)} = 46.19 - 14.80\ pH - 9.39\ pH^2 - 9.65\ T - 7.50\ T^2 - 7.35\ pH\ T \quad \text{Eq. 3}$$

Where pH and temperature are given in codified values (see Table 1).

The model for color change (Eq. 2), which predicted color difference with deviations always lower than 10% **(Table 2)**, can be expressed as a contour plot shown in **Figure 2A**. This curve indicates that color difference is minimized in acidic conditions (pH 3.0 to 4.0) and low temperatures (12 to 20 ^oC). In such conditions ΔE* would be lower than 3.5, which is less than required to notice a clear difference in color is noticed (Mokrzycki & Tatol, 2011). In terms of food applications, these conditions are seen, for instance, in yoghurts, jams and some acidic beverages. Therefore, these might be target foods to be colored by IBBP extract. However, it is important to note that this model

was not done in a complex mixture, such as foods. It is known, for instance, that proteins react with genipin (Buchweitz, 2016; Brauch et al., 2016 a). Therefore, the results here obtained might not be straight forward extrapolated to real food systems.

These results presented above are in accordance to previous reports on genipin stability. Fujikawa et al (1996) for example, observed that blue pigment from gardenia kept relatively stable after two weeks on low pH (3-6) at 40 ^{o}C. Similarly, Renhe et al (2009) it also reported that optimal pH to maintain the blue color of genipap extract is around 4.0. Cano et al (2014) mentioned that a product formed from the reaction of methionine (MET) with *G. americana* juice submitted to irradiation for 7.46 hours it was twice more stable on pH 3.0. Additionally, some studies mentioned that optimal pH (4.0 ~ 6.0) for pigment production is similar to the optimal pH to keep color stability (Cho et al 2006; Bentes et al., 2015; Wu & Horn, 2017).

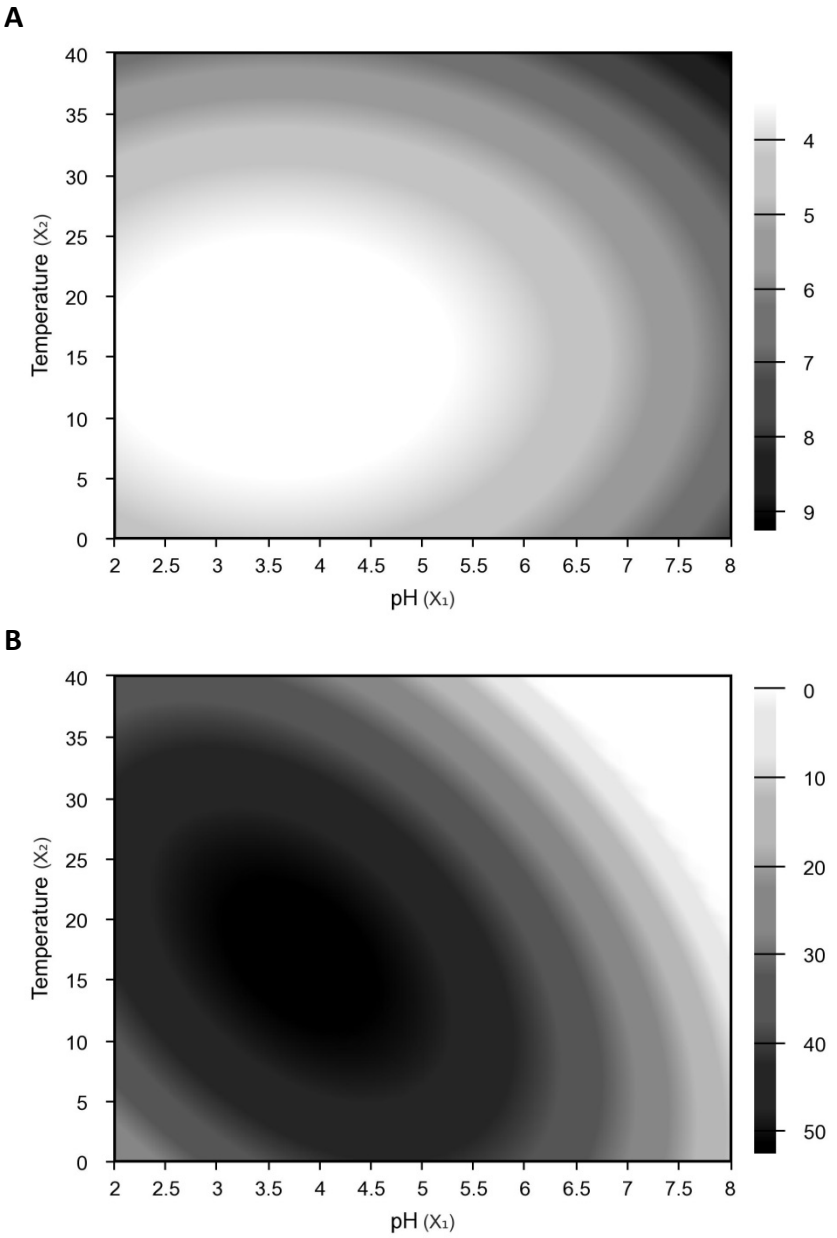

Figure 2. Graphical representation of colour change after 18 days (A) and genipin content (B) after 9 days of incubation, according to Eqs. 2 and 3, respectivelly

As for genipin content, the model (Eq. 3) also presented reasonable deviations, with only higher errors for some axial points **(Table 2)**. The resulting contour plot indicated that genipin stability is maximized in pH values lower than 5 and temperatures lower than 25 ^{o}C, while at pH values higher than 6, specially at higher temperatures, genipin loss is maximized **(Figure 2B)**. This result contradicts a previous report on genipin stability is more stable the alkaline conditions (Paik et al., 2001), but in such study the model was different (e.g. amino acids were added in a purified genipin solution).

4.2. LC-MS analysis and antioxidant profile of IBBP extract after gastrointestinal in vitro digestion

The non-digested (ND) and de digested fractions from IBBP extract of *G. americana* during gastric phase (GP) and intestinal phase (IP) were identified by their masses and the fragmentation pattern **(see supplementary material, S4)**. Ten compounds were separated by UHPLC-ESI-MS/MS analyses, six compounds being present in ND, and seven in both GP and IP fractions. Among them, genipin, genipin 1-β-gentiobioside and geniposide were confirmed with reference standards, while gardenoside, 6’-O-p-Coumaroyl-geniposidic acid and 6'-O-feruloyl-geniposidic acid were tentatively identified considering their masses and fragmentation pattern. It was not possible to identify four compounds **(Table 5)**. Our results are in agreement with Ono et al (2005), pioneers in the isolation and structural elucidation of eleven monoterpenoids from *G. americana.*

Previously, Bentes & Mercadante (2014) reported that geniposide, geniposidic acid, gardenoside and genipin are the major iridoids present in the unripe endocarp fruit while genipin, genipin-1-β-D-gentiobioside, 6’-O-*p*-coumaroyl-geniposidic acid and 6'-O-feruloyl-geniposidic acid were detected as the major components in IBBP extract. This discrepancy could be attributed to the differences in the cultivar, to season and soil conditions, to the “turning point” of the biological ripeness and also to different analytical conditions.

Regarding the importance of knowing the fraction of iridoids that is available for absorption, not only the content was assessed, but the bioaccessibility, as well. Our *in vitro* model of digestion showed a strong decrease in the absolute amount of genipin, while the relative amount of genipin 1-β-gentiobioside increased 49% in digesta.

Table 5. Tentative identification and the amount of the compounds (in terms of absolute or relative area) present in unripe endocarp of *Genipa americana* L. from non-digested (ND) and the digested fractions from IBBP extract during gastric phase (GP) and intestinal phase (IP) using UPLC-ESI(−)-MS/MS.

Tentative assignment	IBBP extract Absolute area (relative area, %)						
	ND	GP	IP	m/z	r.t.	Molecular formula	Ms/Ms
Gardenoside*	$1.58x10^5$ (1.64)	$1.9x10^5$ (1.53)	n.d	403.1247	1.95	$C_{17}H_{24}O_{11}$	241/223/127
Genipin 1-β-gentiobioside*	$4.06x10^6$ (41.98)	$5.5x10^6$ (44.64)	$4.7x10^6$ (86.53)	549.1834	4.03	$C_{23}H_{34}O_{15}$	517/225/123/101
Geniposide*	$1.00x10^5$ (1.04)	$7.4x10^5$ (5.99)	$1.1x10^5$ (2.07)	387.1303	4.55	$C_{17}H_{24}O_{10}$	408/225/123/101
Unknown 1	n.d.	$6.1x10^4$ (0.49)	n.d.	387.1295	4.90	$C_{17}H_{24}O_{10}$	-
Genipin	$4.31x10^6$ (44.66)	$4.7x10^6$ (37.79)	n.d	225.0775	5.12	$C_{11}H_{14}O_5$	225/123/101
6′-O-*p*-coumaroyl-geniposidic acid	$5.83x10^5$ (6.03)	$6.9x10^5$ (5.56)	204767 (3.79)	519.1502	7.88	$C_{25}H_{28}O_{12}$	325/355/163/123/101
6'-O-feruloyl-geniposidic acid	$4.50x10^5$ (4.66)	$4.9x10^5$ (3.99)	$1.1x10^5$ (2.05)	549.1612	8.14	$C_{26}H_{30}O_{13}$	355/193/175/123/101
Unknown 2	n.d.	n.d.	$4.7x10^4$ (0.87)	519.1506	8.26	$C_{25}H_{28}O_{12}$	-
Unknown 3	n.d.	n.d.	$1.4x10^5$ (2.57)	519.1506	10.3	$C_{25}H_{28}O_{12}$	-
Unknown 4	n.d.	n.d.	$1.1x10^5$ (2.1)	549.1612	10.5	$C_{26}H_{30}O_{13}$	-

* detected as formiated form [M+ $HCOO^-$], n.d., not detected, m/z, mass/charge, r.t., retention time.

Considering that the absolute amount (absolute area) of this compound remains almost the same, this relative increase is explained by the disappearance of genipin. This loss in genipin might be explained by the instability of this compound in neutral pH values, such as those found in the intestinal phase of *in vitro* digestion. At pH 5.5 (found in the extract) and room temperature (25 ^{o}C) (conditions found for the non-digested analysis) the genipin content predicted by the model is 39.9 mg/L, while at pH 2.0 and 37 ^{o}C (gastric phase) and pH 7.0 and 37 ^{o}C (intestinal phase) the predicted values are 34.6 mg/L and 0mg/L, respectively **(Figure 2B)**. Some studies reported that genipin is more stable at alkaline pH than in neutral or acidic pHs, remaining stable even after high temperatures (Lee et al., 2003; Wu & Horn, 2017). However, in this study it was clearly demonstrated that this is not a linear behavior. Therefore, depending on the combination of pH and temperature, genipin might be quite unstable.

The *in vitro* digestion also showed that some glucosides (gardenoside and geniposide) and the esters of glucosilated geniposidic acid decreased during the intestinal phase of digestion **(Table 5)**. This is in accordance with previous studies, which mentions the hydrolysis of ester and glycosidc bonds with intestinal enzymes and a decreasing of some phenolic compounds such as flavonols and anthocyanin contents (Sangiovanni et al. 2015; Cassani et al., 2018).

It was possible to observe an increase in the antioxidant capacity during each phase of *in vitro* digestion. After the gastric phase, the antioxidant capacity increased 17% and a further increase of 18% was achieved after the intestinal phase **(Table 6)**.

Table 6. Antioxidant capacity by ORAC assay of IBBP extract from *Genipa americana* L.

IBBP extract	ORAC (µM Trolox equivalents/g)
ND	231.14 ± 13.45 [b]
GP	271.76 ± 6.15 [a,b]
IP	320.39 ± 3.86 [a]

IBBP= iridoid blue-based pigment, **ND=** non digested IBBP extract, **GP=** gastric phase of digested IBBP extract, **IP=** intestinal phase of digested IBBP extract. Data was expressed as mean of triplicate ± standard deviation of triplicate analysis. Different letters in the same column indicate significantly different values ($p < 0.05$).

The increase in antioxidant capacity during *in vitro* digestion was already observed in several polyphenols (Bohn et al. 2015). This change is usually due to the release of the aglycone form, which is generally a more efficient antioxidant (Tagliazucchi et al., 2010; Pino-García). However, an increase in the aglycone forms was not be observed in this study **(Table 4)**, suggesting that the higher antioxidant capacity of IP might be related to the unknown products formed during *in vitro* digestion or to aglycone degradation products. Therefore, these unknown compounds might be good target compounds for further studies involving the relationship between IBP, antioxidant capacity and bioavailability, including experimental design using *in vitro* and *in vivo* models to elucidate their biological effects, considering there is still a lack knowledge on the metabolic pathways of these iridoids.

5. Conclusion

In conclusion, it was observed that the color stability of IBBP extract is compatible with food applications (acidic foods kept under refrigeration temperature). The use of such extract in foods might be interesting not only as an alternative natural blue colorant, but also as a way of increasing the functionality of food products. For instance, it was shown that the antioxidant capacity of IBBP increased after simulated *in vitro* digestion. Therefore, the use of genipin for coloring foods is quite promising.

Acknowledgments

The authors thank the São Paulo Research Foundation (FAPESP, grant number 2015/50333-1], the National Council for Scientific and Technological Development [CNPq, grant number 481670/2013-0] and the Institute of International Education (CAPES) for financial support and the Rupert Lyons for the language services provided. C. F. F. A. acknowledges FAPESP (studentships 2017/10753-7).

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Publication

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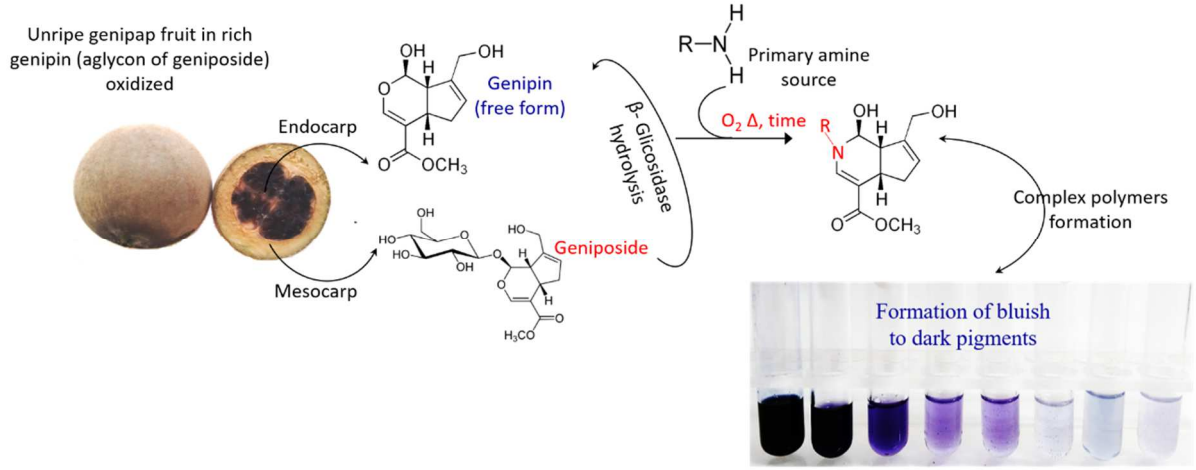
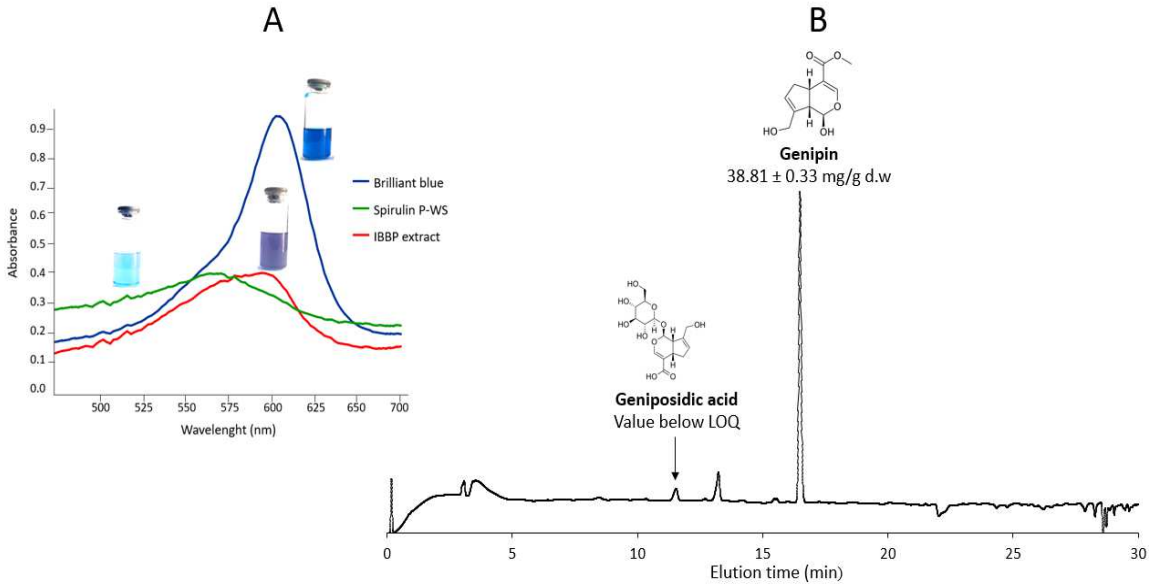
Material Supplementary



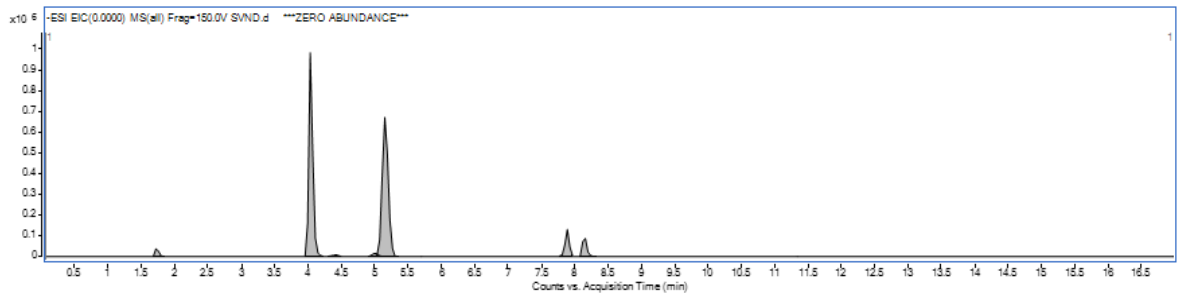
S1. Schematic representation of blue pigments formation from genipin. Genipin is a colorless iridoid that are composed of a cyclopentan –[C]-pyran skeleton. The hydroxil group at the C1 position of the genipin pyran ring can be substituted by one or two sugar moieties forming the genipin glycosides genipin-1-O-β-D-glucoside (geniposide) and genipin-1-O-β-D-gentiobioside, respectivelly. In brief, is possible to obtain bluish/ purplish pigments directly from oxidation of free genipin from endocarp or after the hydrolysis of their precursor geniposide in the mesocarp. Adapted from Cano et al., 2014 and Neri-Numa et al., 2017.



S2. IBBP extract color standardization and genipin content. **(A)** Visible spectra ranging from 500 to 700 nm of IBBP extract and commercial food colorants at 5 mg/ml (w/v) in phosphate-citrate buffer (pH 4.5 at 150mM), **(B)** UHPLC-DAD profile at 240 nm of IBBP extract: geniposidic acid at 10.25 min and genipin at 16.48 min. The binary mobile phase was methanol and water acidified with 0.1% formic acid. The gradient elution at 27 °C was as follows: 0-2 min, 10% A and 90% B, 2-25 min, 90 to 10% B, 25-28 min, 100% A and 0% B, 28-30 min, 10% A and 90% B.

S3. Color change (ΔE*) of IBBP extract over time and under different conditions, from left to right: pH 3.2, 8 °C **(1)**, pH 6.8, 8 °C **(2)**, pH 3.2, 34.5 °C **(3)**, pH 6.8, 34.5 °C **(4)**, pH 2.5, 21.3 °C **(5)**, pH 7.6, 21.3 °C **(6)**, pH 5.0, 2.5 °C **(7)**, pH 5.0, 40 °C **(8)**, pH 5.0, 21.3 °C (**9-11**, center points).

A



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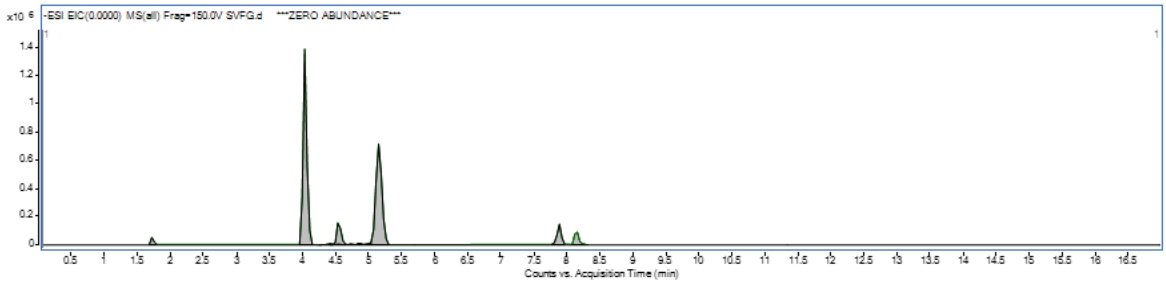
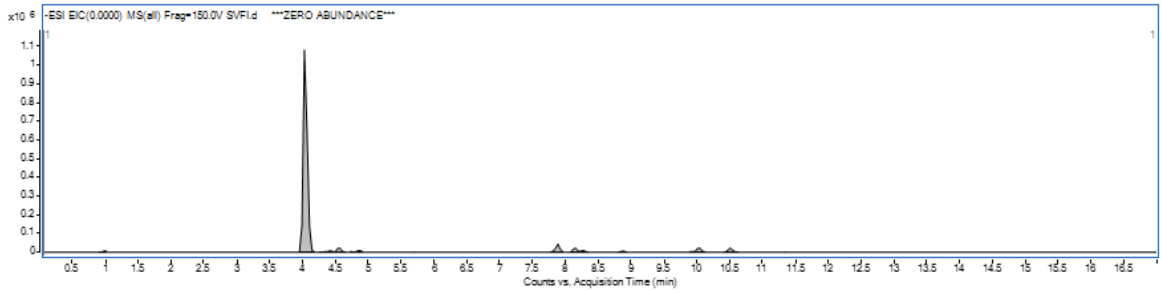


C



S4. Tentative identification and the amount of the compounds (in terms of absolute or relative area) present in **(A)** non-digested IBBP extract (ND) and the **(B - C)** digested fractions (GP and IP, respectively) using UPLC-ESI (−)-MS/MS. These fractions were analysed in negative ion mode using a C18 120 SB-Aq 2.7 µm reversed phase column (2.1x100 mm, Agilent) and a Q-ToF iFunnel 6550 mass spectrometer fitted with an electrospray ionization (ESI) source. Mobile phase A consisted of a 0.1% (v/v) solution of formic acid in water, and mobile phase B was a 0.1% (v/v) of formic acid solution in acetonitrile. The mass spectrometer parameters used were: VCap 3000 V, fragmentor voltage at 150 V, OCT 1RF Vpp at 750 V, Gas Temperature at 290oC, Sheath Gas Temperature at 350 °C, Drying Gas at 12 L/min-1. Mass spectra were acquired in profile mode and the acquisition range was 100 - 1500 m/z.

CAPÍTULO IV

TOXICOLOGICAL EVALUATION OF A NATURAL IRIDOID-BASED BLUE PIGMENT OBTAINED FROM GENIPAP (*Genipa americana* L. - RUBIACEAE)

Artigo submetido na Food and Chemical Toxicology

Fator de Impacto 3.778 - Qualis A1

Iramaia Angélica Neri-Numa, Adriana Della Torre, Celio Fernando Figueiredo Angolini, Bruno Nicolau Paulino, Gustavo Araujo Pereira, Ana Lucia Tasca Gois Ruiz and Glaucia Maria Pastore

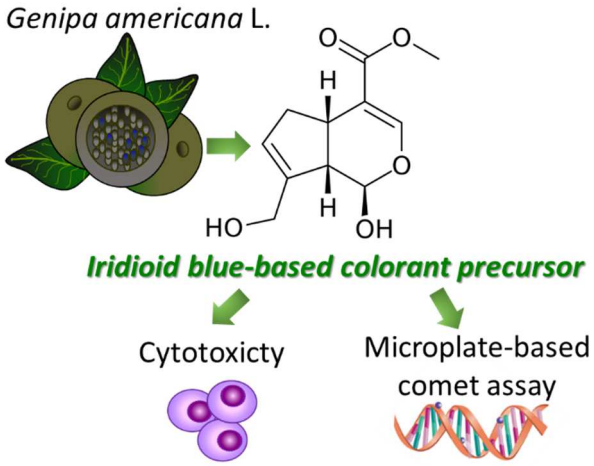
Abstract

Despite the colors properties of *Genipa americana* L. fruits have been known since ancient times, there is a recent growing interest in their blue pigments as a natural alternative to synthetic ones for food and pharmaceutical applications. However, the toxicological studies for the genipap colorant are still incipient, mainly with regard to the possibility to trigger side effects or toxicity after metabolization. Therefore, the goal of our study was to separate and to identify target iridoids compounds both in unripe (UGE) and ripe (RGE) genipap endocarp extracts by exact masses and the fragmentation pattern. Additionally, we evaluate the antiproliferative activity against tumor cells as well as the genotoxicity of genipap extract in the presence or absence of metabolic activation (S9+ / S9-) using CHO-K1 cells by *in vitro* alkaline comet assay. Among all compounds found in UGE and RGE (geniposidic acid, gardenoside, genipin-1-β-gentibioside, geniposide, 6''-O-p-coumaroyl-1-β-gentibioside geniposidic acid, 6''-O-p-coumaroylgenpin-gentibioside, genipin, 6’-O-p-coumaroyl-geniposidic acid, 6’-O-feruloyl-geniposidic acid) one of them is unknown. In addition, both RGE and UGE at concentration studied did not interfere with non-tumor cell lines proliferation besides not being genotoxic. This is the first study in which the genotoxic effects of genipap

extract have been reported. Our results show that genipap extract at 25 µg/mL dosage can be considered a safe option to be exploited as a food colorant with functional appeal.

Keywords: *Genipa americana* L., iridoid glucosides, natural genipap´s blue colorant, genipin, genotoxicity, metabolic activation (S9 + / S9-)

Graphical abstract



Highlights

- Natural blue pigments obtained from genipap (*Genipa americana* L.) it is safe as an alternative for the food industry
- Iridoid glucosides are main compounds found in ripe and unripe genipap fruits
- Genipap extract at 25 µg/mL is not genotoxic
- Genipap extract 25 µg/mL not interfere in non-tumor tumor cell lines proliferation
- Unripe genipap endocarp extract exerts a protective effect on DNA injury

Abbreviations

UGE: unripe genipap endocarp extract, **RGE:** ripe genipap endocarp extract, **DMSO:** dimethilsulfoxide, **SRB:** sulphorhodamine B, **CPA:** cyclophosphamide monohydrate, **MMS:** metylmetanosulfonate, **$S9^-$:** absence of exogenous metabolic activation, **$S9^+$:** presence of exogenous metabolic activation.

Chemical compounds reported in this article

Gardenoside (PubChem CID: 442423)

Genipin (PubChem CID: 442424)

Genipin 1-β-gentiobioside (PubChem CID: 3082301)

Geniposide (PubChem CID: 107848)

Geniposidic acid (PubChem CID: 16020046)

6´-O-p-Coumaroyl-geniposidic acid (PubChem CID: 44255335)

6´-O-p-Feruloyl-geniposidic acid (PubChem CID: not found)

6”-O-p-Coumaroyl-1-β-gentibioside geniposidic acid (PubChem CID: not found)

6”-O-p-Coumaroylgenpin-gentiobioside (PubChem CID: 101408305)

1. Introduction

Since ancient times Amazonian indigenous tribes have used natural colorants obtained from macerated fruits of genipap for dyeing artifacts and painting the body as well as to cure diverse diseases (Bentes & Mercadante, 2014; Horák, 2014; Brauch, 2016a). Nowadays, we know that genipap´s "magical properties" are attributed to the presence of natural pigments blue-based iridoids glycosides, especially geniposide and its bioactive compound genipin (Neri-Numa et al., 2017). Genipin has a peculiar characteristic of being colourless but in the presence of oxygen, it reacts with amine sources producing blue-blackish pigments (Yang et al., 2011). This pigment and its related iridoid glucosides represent a novel and natural alternative for the food, cosmetic and pharmaceutical fields, not only due to its colouring properties or the already known crosslinker behavior. It also has antioxidant, anti-inflammatory and antithrombotic capacities, neuroprotective effects, amongst others (Grecco et al., 2017; Neri-Numa et al., 2017; Sohn et al., 2017).

In addition, the regulatory agencies of herbal drugs and food additives across the globe lay down criteria for the approval of any plant extract which must follow strict standards regarding the main active and/or analytical marker (Bansal et al., 2016; Pérez-Ibarbia et al., 2016; Lehto et al., 2017). Regarding the use of genipin as a natural colorant, the blue one obtained from *G. americana* has been used in the United States as a colour additive "Fruit Juice" (Title 21 CFR, Code of Federal Regulations, § 73.250) despite not being approved for the United States or European Union markets. Conversely, several companies currently aiming to introduce genipap and its derived products into markets outside Latin America, where they are most likely to be considered as a novel food and food ingredients (Regulation [EC] No 258/97), respectively (Brauch et al 2016b).

Nowadays, most of the studies about hazard assessment of blue iridoid-based colorants, refer to the *Gardenia jasminoides* J. Ellis, another representative of the Rubiaceae family and widely used as a traditional Chinese medicine (Sohn et al., 2017). There is scant and controversial data about genipin and its derivatives toxicity. Some studies have reported that genipin from gardenia exhibits hepatotoxicity in rats and causes damage to DNA while other studies have not found any direct cytotoxicity in hepatocellular carcinoma cells or clastogenic

effect on Chinese hamster ovary cells (Ozaki et al., 2002; Tsai et al., 2000; Tan et al., 2016). Recently Minami et al (2017), reported a patient with both ascending colon cancer and mesenteric phlebosclerosis that may have been caused by the long-term use of a Chinese herbs containing gardenia fruits. Similarly, another study has shown that genipin induces ROS production, COX-2 up-regulation, and prostaglandin E_2 production in macrophages (Khanal et al., 2014).

Considering this background and the fact that there are few studies concerning the factors predictive of *G. americana* toxicity, this study proposed to identify a fingerprint profile as well as to investigate the safety of genipap´s blue colorant by *in vitro* assays. Thus, the antiproliferative activity was evaluated against a panel of human cell lines (seven tumor and one non-tumor cell lines) besides the genotoxic evaluation by in vitro comet assay in Chinese hamster ovary (CHO-K1) cells treated in the absence (S9-) or presence (S9+) of exogenous metabolic activation.

2. Material and methods

2.1. Chemicals and cell culture apparatus

Standards of geniposidic acid, geniposide and genipin, purity ≥ 98% (HPLC) and as well as the trichloroacetic acid, sulphorhodamine B, cyclophosphamide monohydrate, metylmetanosulfonate, doxorubicin, S9 from human liver and all reagents used by the comet assay were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (haemoglobin level ≤ 10mg⁄dl) and RPMI 1640 were purchased from Gibco-Invitrogen® (New York, USA). HPLC grade methanol and formic acid were provided by Merck (Frankfurt, Germany). All other chemicals and solvents in this study were of analytical grade and water was purified by Milli-Q®$_{plus}$ system (Millipore, Milford, MA, USA). All tissue culture plates and other plastic wares were from Costar (Corning, New York, USA).

2.2. Plant material, ripeness stage and extraction procedure

The genipap fruits were collected at the University of Campinas (22° 49' 8.55" S and 47° 04' 24.022" W). The botanical identification was performed at the Department of Vegetal Biology and the exsicate (access number: 110904) was deposited at the Herbarium-UEC of Biology Institute of University of Campinas, State of São Paulo, Brazil.

Prior to the extraction procedure, the ripeness stage of fruits was determined based on instrumental colors parameters, firmmes, physicochemical characteristics and sugar analysis by high-performance anion exchange chromatography (HPAEC) coupled to pulsed amperometric detection (PAD) according methodology cited in the **Chapter II**.

For the extraction procedure, both unripe (UGE) and ripe endocarp (RGE) fruits were freeze-dried for 72 hours (Terroni, model LS 3000, São Carlos, Brazil), powdered using a knife grinder (Marconi, model MA-340, Piracicaba, Brazil) and subsequently, submitted to an extraction procedure in triplicate. For this procedure, an aliquot of 10 g of the resulting powder was extracted eight times, each with 100 mL of methanol 80% (v/v) in water, in ultrasonic bath (40Hz) for 15 minutes followed by centrifugation (8000 RPM) for 10 min at 5 ^{o}C. All supernatants were combined and evaporated at 60 ^{o}C to dryness (Bücchi Labortechnik AG, model R11 F-108, Flawil, Switzerland). The samples were recovered with 10 mL of water and filtered through 0.20 µm regenerated cellulose Minisart RC 15 single-use syringe filter (this filtrate was called IBBP extract) (Sartorius, PP-housing, Goettingen, Germany) prior to chromatographic analyses and in vitro assays.

2.3. Chromatographic analysis of plant extract

All extracts were prepared diluting 50 µL of a 20 mg/mL sample in 950 µL of water resulting in a final concentration of 1 mg of sample per mL. These fractions were analyzed in negative ion mode using an HPLC (Hewlett Packard, Agilent Technologies 1290 series) coupled to Poroshell C18 120 SB-Aq 2.7 µm reversed phase column (2.1x100 mm, Agilent) and a Q-ToF iFunnel 6550 mass spectrometer fitted with an electrospray ionization (ESI) source. Mobile phase A consisted of a 0.1% (v/v) solution of formic acid in water, and mobile phase B was a 0.1% (v/v) of formic acid solution in acetonitrile. The samples were eluted with a flow rate of

0.45 mL/min following linear gradients (v/v) of mobile phases: 0 - 1 min, 5% B, 1 - 10 min, 5% B to 18% B, 10 -13 min 18% B to 70% B, 13 - 15 min, 70% B to 100% B, 15 - 17 min, 100% B and 17 - 20 min post time (5% B). The mass spectrometer parameters used were: VCap 3000 V, fragmentor voltage at 150 V, OCT 1RF Vpp at 750 V, Gas Temperature at 290oC, Sheath Gas Temperature at 350 ^{o}C, Drying Gas at 12 L/min. Mass spectra were acquired in profile mode and the acquisition range was 100 - 1500 m/z.

2.4. Cell lines and culture conditions

The tumor cell lines U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (breast expressing phenotype multiple drug resistance), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), HT-29 (colon) and K562 (leukemia) were generously supplied by the National Cancer Institute (NCI, Frederick, MA, USA). The immortalized human keratinocytes (HaCat) and the immortalized Chinese hamster ovary cells (CHO-K1) were kindly donated by Prof. Dr. Ricardo Della Coletta (FOP/UNICAMP) and Prof. Dr. Mário S. Mantovani (UEL), respectively.

For mantaince and experiments, all cell lines were cultivated in complete medium [RPMI 1640 culture medium, supplemented with 5% (v/v) fetal bovine serum (FBS) and penicillin-streptomycin (1000 U/mL: 1000 µg/mL, 10 mL/L RPMI 1640) solution] and incubated at 37°C with 5% CO_2humified atmosphere. Cell lines were used between 4 to 10 passages.

2.5. In vitro antiproliferative activity

The antiproliferative activity of genipap extracts were perfomed according to NCI standard protocol (Monks et al., 1991, Shoemaker, 2006). Samples stock solutions (5 mg) were prepared in DMSO (50 µL) followed by successive dilutions in complete medium to give final concentrations of 0.25, 2.5, 25, and 250 µg/mL. Genipin and geniposide commercial standards, at same final concentrations, were also tested. Doxorubicin was used as positive control at final concentrations of 0.025, 0.25, 2.5, and 25 µg/mL. Then, all tumor and non-tumor cell lines distributed in 96 well plates (100 µl cells/well, cell densities: 3 –7 × 10^4

cells/mL, T1 plates) were incubated for 48 h with each of the four concentrations of sample, doxorubicin, genipin or geniposide solutions (100 µL/well) in triplicate.

A microplate with all the cell lines (T0 control) was also prepared to stablish the cell quantities at the sample addition moment. After 48h, cells at T1 plates were fixed with trichloroacetic acid 50% and cell density was determined by spectrophotometric quantification (540 nm) of cellular protein content, employing sulphorhodamine B assay. Three measurements were obtained at the beginning of sample addition (T0) and 48 h post-incubation without (T1) and with sample (T). The cell proliferation was determined according to the equation $100 \times [(T - T0)/(T1 - T0)]$. A cytostatic effect was observed when $T1 > T \geqslant T0$, while a cytocidal effect occurred when $T < T0$. Therefore, cell proliferation was graphically correlated to sample concentrations and one effective concentration named GI_{50} (sample concentration required to 50% inhibition of cell proliferation) was determined by nonlinear regression analysis using the Origin 8.0 software (OriginLab Corporation, Inc., Northampton, MA, USA).

2.6. Genotoxicity assessment by in vitro alkaline comet assay

Prior to the experiment, 2 x 10^5 CHO-K1 cells/well (2 mL/well) were seeded in a 6-well plate and incubated for 24 hours before the treatments. For the experiment with metabolic activation, a S9 mix solution was prepared by mixing 100 µL S9 fraction into 1.0 mL distilled water, 1.75 mL phosphate buffer (Na2HPO4/ NaH2PO4, pH 7.2), 100 µL NADP (76.5 mg/mL), 25 µL glucose-6-phosphate, 25 µL KCl (1.65 M), 25 µL MgCl2 (0.4 M). Subsequently, this S9 mix (90 µL/well, final concentration of 2%/well) was added to cells together with treatments. In each experiment, cells were treated for 4 hours, in duplicate, with negative control (complete medium), one vehicle control (DMSO 0.25% in complete medium) and one DNA damage inductor (methylethanesulfonate, MMS, 25 µg/mL in S9- experiment, or cyclophosphamide, CPA, 20 µg/ml, in S9+ experiment) besides the samples UGE and RGE (25 µg/ml, based on the antiproliferative assay).

2.7. Preparation of comet slides

After treatment, the cell suspensions were prepared and processed using the standard alkaline comet assay methodology described by Singh et al. (1988) with minor modifications. Each cell suspension (100 µL) were mixed with warmed (40 ^{o}C) 0.5% low melting point agarose (100 µL) and quickly dropped onto slides (2 slides/cell suspension) previously coated with 1.5% normal melting agarose. Then, the slides were covered with coverslips, manteined for 5 min at 4 ^{o}C and submitted to lysis by immersion in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 Mm Tris, pH 10, with 1% Triton X-100 and 10% DMSO) overnight at 4 ^{o}C, without coverslips. Subsequently, the slides were incubated in an electrophoresis solution (0.5% EDTA and 3% NaOH, pH 13) at 4 ^{o}C for 30 min. Electrophoresis was done at 25 V and 300 mA (0.90 V/cm) for 25 min at 4 ^{o}C, in darkness. Lastly, slides were neutralized in Tris buffer (0.4 M Tris/HCl pH 7.5) for 15 min at 4 °C, fixed with 100% ethanol, dryed at room temperature and stained with of an ethidium bromide aqueous solution (20 mg/mL, 50 µl/slide). The slides were analysed using a fluorescence microscope and imaging system (Carl Ziess Axio Vision SE64, 400x, excitation filter, λ 516–560 nm, barrier filter λ 590 nm- Carl Ziess, Göttingen, Germany) supplied with the Comet Imager 2.2 Software (MetaSystems, Altlussheim, Germany). At least, fifty nucleoids were examined per slide (one hundred per treatment) and DNA damage was expressed as DNA amount on tail (%DNA, a direct parameter) and as Olive Tail Moment (OTM), a mathematical relation which results from % DNA in tail multiplied by Tail length × 0.01 (constant factor related to the microscope).

3. Statistical analysis

GraphPad Prism 5.0 program (La Jolla, CA, USA) was used for data analysis. Statistically significant differences between groups were calculated by the application of the one-way analysis of variance (ANOVA) for unpaired observations between controls and experimental samples. Tukey test was used for multiple comparisons, p values of 0.05 or less were considered statistically significant. All tests were performed in triplicate.

4. Results and discussion

4.1. Chromatographic analysis of plant extract

The target iridoid compounds from *G. americana* were identified by their exact masses and the fragmentation pattern. Nine compounds were separated by UHPLC-ESI-MS/MS analysis, five compounds being present in UGE and six RGE extract **(Figure 1)**. Among them, genipin 1-β-gentiobioside, geniposide, and geniposidic acid were confirmed with reference standards, the gardenoside was tentatively identified considering their mass fragments, while six compounds have not been identified **(Table 1)**.

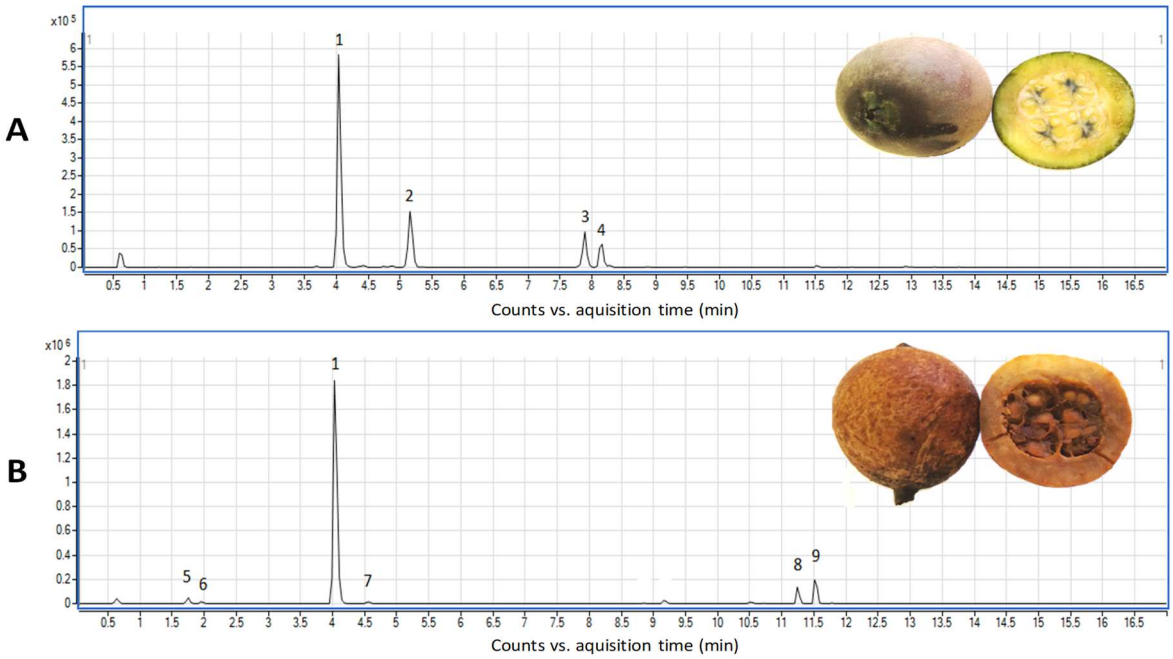
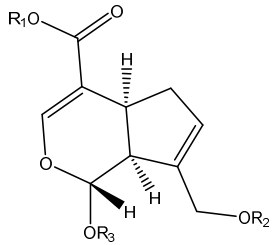


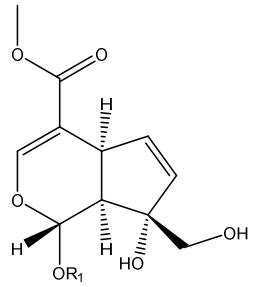
Figure 1. LC-ESI (-) q-ToF base peak chromatogram of detected compounds from genipap extracts. **(A)** UGE, **1=** genipin 1-β-gentiobioside, **2=** Genipin, **3=** 6'-O-p-Coumaroyl-geniposide acid, and **4=** 6'-O-feruloyl-geniposide acid, and **(B) 1=** genipin 1-β-gentiobioside, **5=** geniposidic acid, **6=** gardenoside, **7=** geniposide, **8=** 6'-O-p-Coumaroylgenipin-gentiobioside geniposidid acid and **9=** 6'-O-p-Coumaroylgenipin-gentibioside. **UGE,** urnipe genipap extract and **RGE,** ripe genipap extract.

Table 1. Compounds tentatively identified in unripe (UGE) and ripe (RGE) *Genipa america L.* endocarp extracts using UPLC-ESI(−)-MS/MS.

Tentative assignment	Absolute area		m/z	r.t.	Molecular formula	Error (ppm)	MS^2
	UGE	RGE					
Geniposidic acid (5)	-	2.45×10^5	373.1144	1.75	$C_{16}H_{22}O_{10}$	-0.9	-
Gardenoside* (6)	-	9.06×10^5	403.1247	1.95	$C_{17}H_{24}O_{11}$	-0.42	241/223/127
Genipin 1-β-gentiobioside* (1)	4.02×10^6	1.35×10^7	549.1834	4.03	$C_{23}H_{34}O_{15}$	-2.61	517/225/123/101
Geniposide (7)*	-	3.95×10^5	387.1303	4.55	$C_{17}H_{24}O_{10}$	-0.48	408/225/123/101
6''-O-p-Coumaroyl-1-β-gentiobioside geniposidic acid (8)	-	7.17×10^5	681.2036	11.24	$C_{31}H_{38}O_{17}$	0.45	517/313/163/101
6''-O-p-Coumaroylgenipin-gentiobioside (9)	-	1.25×10^6	695.2207	11.52	$C_{32}H_{40}O_{17}$	-1.96	469/225/163/123/101
Unknown (2)	8.00×10^5	-	504.0727	5.15	-	-	-
Genipin (2)	1.09×10^6	-	475.1198	5.15	-	-	-
6'-O-p-Coumaroyl-geniposidic acid (3)	5.92×10^5	-	519.1502	7.88	$C_{25}H_{28}O_{12}$	0.85	325/355/163/123/101
6'-O-feruloyl-geniposidic acid (4)	4.50×10^5	-	549.1612	8.14	$C_{26}H_{30}O_{13}$	0.45	355/193/175/123/101



1 $R_1 = CH_3, R_2 = H, R_3 =$ β-gentiobioside
3 $R_1 = H, R_2 = H, R_3 =$ β-D-glucosyl-6'-O-*p*-coumaroyl
4 $R_1 = H, R_2 = H, R_3 =$ β-D-glucosyl-6'-O-feruloyl
5 $R_1 = H, R_2 = H, R_3 =$ β-D-glucosyl
7 $R_1 = CH_3, R_2 = H, R_3 =$ β-D-glucosyl
8 $R_1 = H, R_2 = H, R_3 =$ β-gentiobioside-6''-O-p-coumaroyl
9 $R_1 = CH_3, R_2 = H, R_3 =$ β-gentiobioside-6''-O-p-coumaroyl



6 $R_1 =$ β-D-glucosyl

*detected as formiated form [M+ $HCOO^-$]

Our results are in agreement with Ono et al (2005), pioneers in the isolation and structural elucidation of eleven monoterpenoids from *G. americana.* In addition, we also observed that the genipin-1-β-D-gentiobioside amount in the ripe endocarp extract was 3,4 times more than in unripe endocarp extract. On the other hand, geniposide and geniposidic acid were found just in ripe endocarp extract. Previously, Bentes & Mercadante (2014) reported that geniposide and geniposidic acid are the major iridoids present in the unripe endocarp fruit while the genipin-1-β-D-gentiobioside was the major iridoid in the ripe endocarp and was not detected in unripe genipap fruits. We attribute this discrepancy to the fruit batch. Probably this discrepancy its due the "turning point" of the biological ripeness identifiable in the fruit at the moment of extraction.

4.2. In vitro antiproliferative activity

The antiproliferative profiles of unriped and riped genipap extracts, genipin and geniposide standards as well as the positive control doxorubicin are shown in **Figure 2 (A-E)**. This activity was expressed as the sample concentration required to inhibit in 50% the cell growth after 48h-exposition, named GI_{50} **(Table 2)**.

Table 2. *In vitro* antiproliferative activity evaluation genipin, geniposide, unripe and ripe genipap extracts.

	GI_{50} (μg/mL)								
	2	**m**	**a**	**7**	**4**	**p**	**h**	**k**	**q**
Doxorubicin	< 0.025	< 0.025	0.41	0.028	< 0.025	0.23	0.19	0.036	< 0.025
Genipin	21.3	7.5	15.7	22.7	29.0	14.4	26.1	14.5	17.3
Geniposide	*	*	*	*	*	*	*	*	*
UGE	29.7	29.6	45.7	62.8	*	73.1	142.5	85.3	69.4
RGE	*	*	*	*	*	*	*	*	*

Human tumor cell lines: **2=** U251 (glioma, CNS), **m=** MCF-7 (breast), **a=** NCI-ADR/RES (breast expressing phenotype multiple drug resistance), **7=** 786-0 (renal), **4=** NCI-H460 (lung, non-small cells), **p=** PC-3 (prostate), **h=** HT-29 (colon), **k=** K562 (leukemia), and **q=** immortalized keratinocyte (non-tumoral cell). **UGE,** unripe genipap endocarp, **RGE,** ripe genipap endocarp. * GI_{50} > 250 μg/mL (growth inhibition).

Both geniposide and ripe genipap extract (RGE) were inactive (GI_{50} > 250 μg/ml) in the experimental conditions. Moreover, genipin inhibited all cell lines evaluated with GI_{50} values form 7.5 (breast, MCF 7) to 29.0 μg/ml (NCI-H460), while unripe genipap extract showed a cytostatic effect against U251 (glioma, GI_{50} = 29.7 μg/ml) and MCF 7 (breast, GI_{50} = 29.6 μg/ml) cell lines.

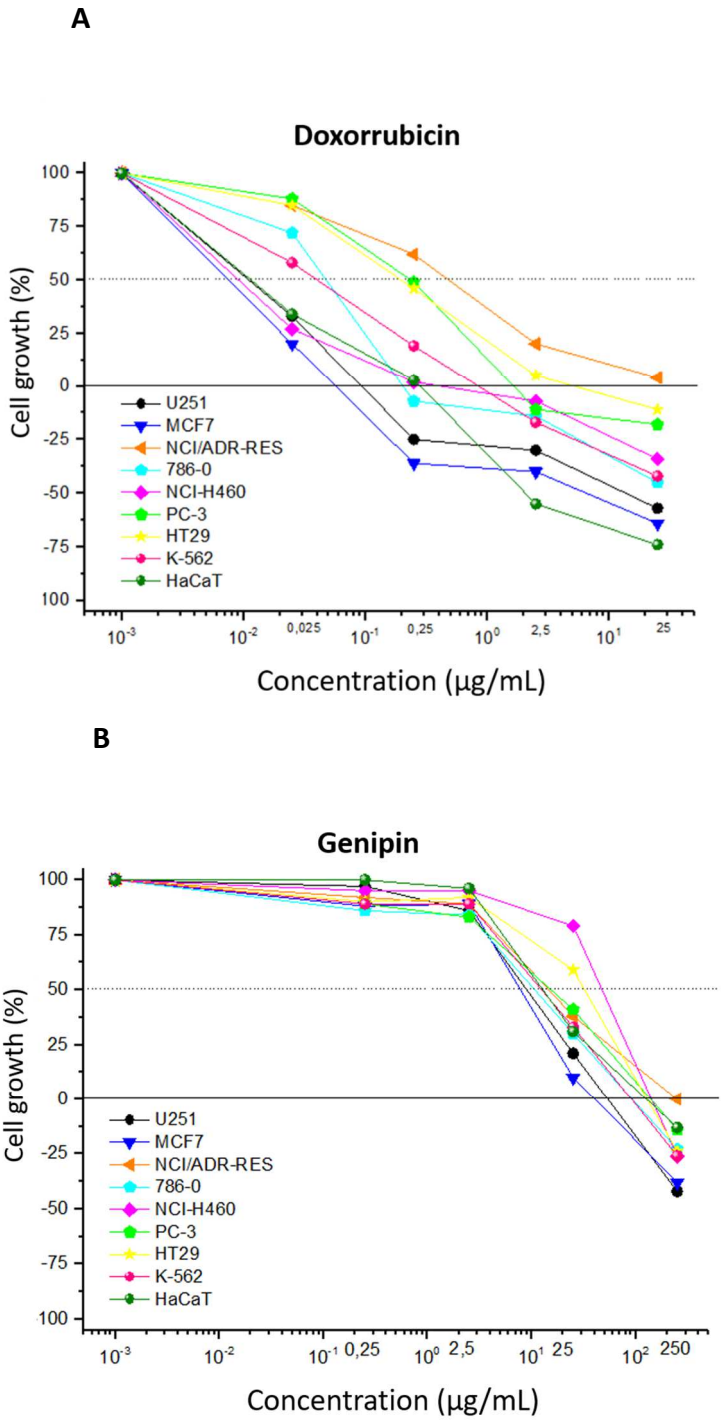


Figure 2. *In vitro* antiproliferativ activity of doxorrubicin **(A)** and genipin **(B)** against seven human tumor cell lines and a non-tumoral human cell: **2=** U251 (glioma, CNS), **m=** MCF-7 (breast), **a=** NCI-ADR/RES (breast expressing phenotype multiple drug resistance), **7=** 786-0 (renal), **4=** NCI-H460 (lung, non-small cells), **p** = PC-3 (prostate), **h=** HT-29 (colon), **k=** K562 (leukemia) and **q=** immortalized keratinocyte (non-tumoral cell). * GI_{50} > 250 µg/mL (growth inhibition).

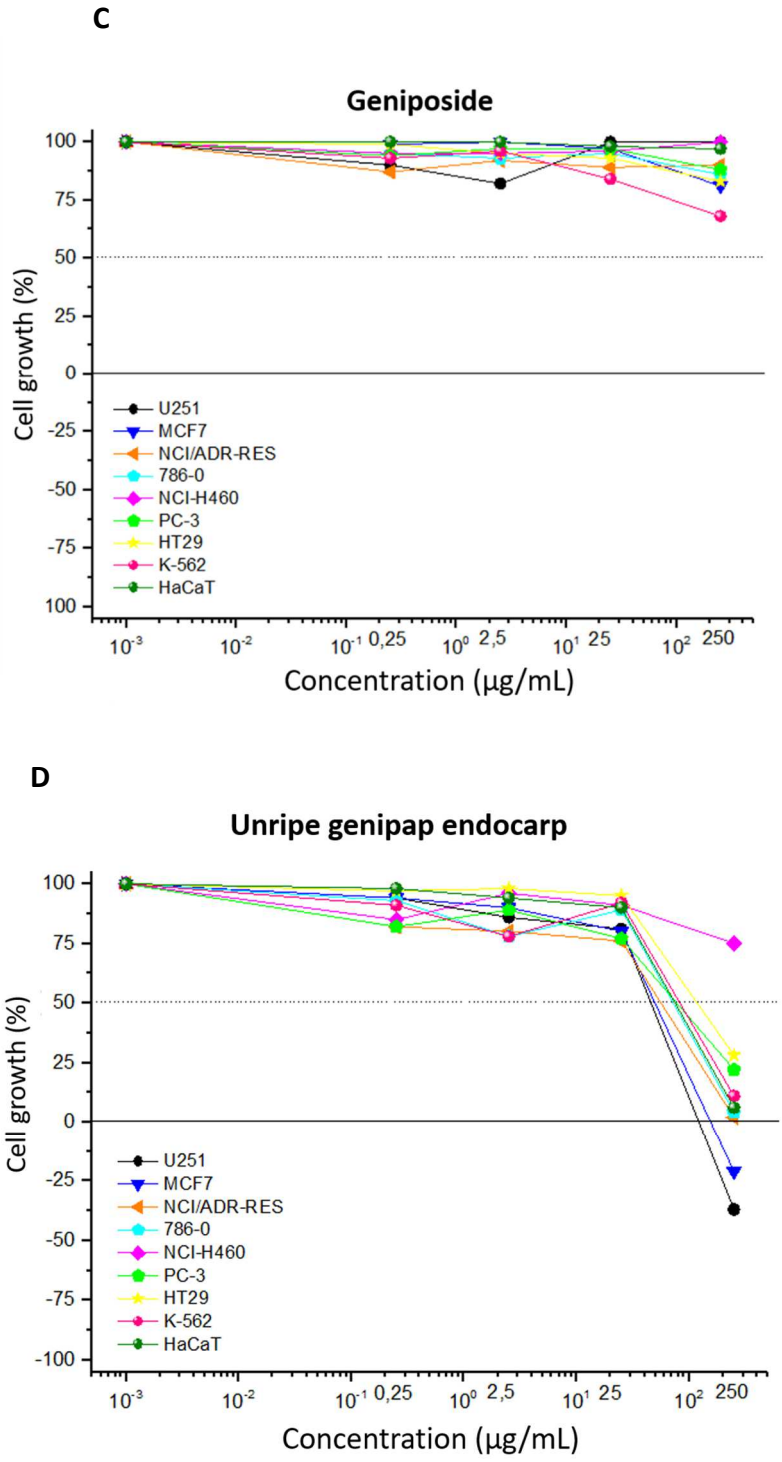


Figure 2. *In vitro* antiproliferativ activity of geniposide **(C)** and unripe genipap endocarp **(D)** against seven human tumor cell lines and a non-tumoral human cell: **2=** U251 (glioma, CNS), **m=** MCF-7 (breast), **a=** NCI-ADR/RES (breast expressing phenotype multiple drug resistance), **7=** 786-0 (renal), **4=** NCI-H460 (lung, non-small cells), **p=** PC-3 (prostate), **h=** HT-29 (colon), **k=** K562 (leukemia) and **q=** immortalized keratinocyte (non-tumoral cell). * GI_{50} > 250 µg/mL (growth inhibition).

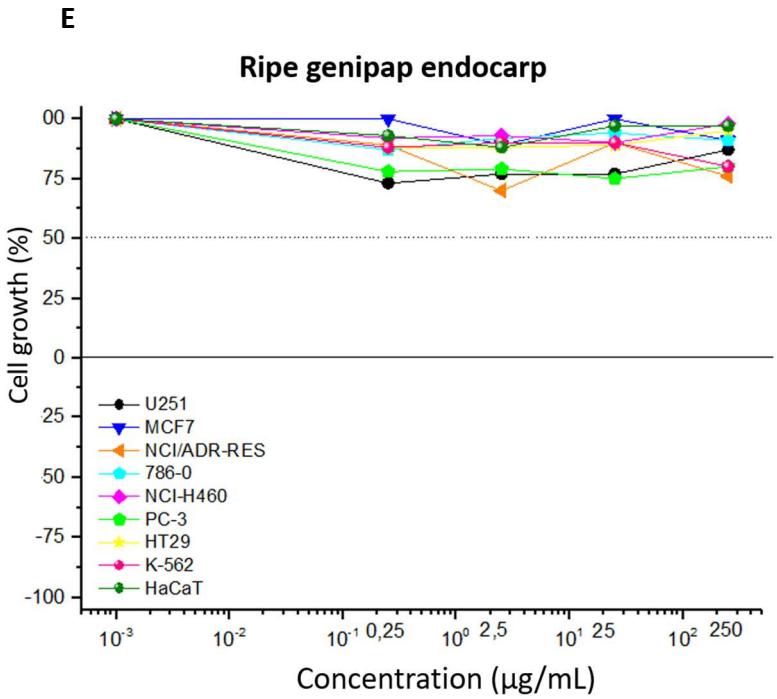


Figure 2. *In vitro* antiproliferativ activity of ripe genipap endocarp **(E)** against seven human tumor cell lines and a non-tumoral human cell: **2=** U251 (glioma, CNS), **m=** MCF-7 (breast), **a=** NCI-ADR/RES (breast expressing phenotype multiple drug resistance), **7=** 786-0 (renal), **4=** NCI-H460 (lung, non-small cells), **p=** PC-3 (prostate), **h=** HT-29 (colon), **k=** K562 (leukemia) and **q=** immortalized keratinocyte (non-tumoral cell). * GI_{50} > 250 µg/mL (growth inhibition).

A previous study showed that a gardenia herbal formulation containing 1.03% genipin gentiobioside, 5.90% gardenoside, 1.26% crocin 1 and 0.17% crocin 2, at 400 µg/mL, inhibited in 80.2% the viability of HepG2 cells (Chen et al., 2017). More, the antiproliferative effects of genipin has been described against different cell lines such as MDA-MB-231 (breast, IC_{50} = 327 µM) (Kim et al., 2012), steroidogenic human granulosa-like tumor cells (IC_{50} upto 100 µM) (Zuo et al (2017) and AGS cells (human gastric carcinoma, at 100 µM, apoptosis induction via p53-independent Egr1/p21 signalling pathway) (Ko et al, 2015). Also, geniposide (50-100 µM) can enhances the doxorubicin efficacy against MG63/DOX cancer cell-derived in xenographic model indicating that this iridoid may be employed in combination with conventional anti-neoplastic drugs to prevent multidrug resistance (Huang et al., 2017).

4.3. Genotoxicity assessment by in vitro alkaline comet assay

The responses of CHO-K1 cells to genipap extracts, CPA and MMS for the comet assay parameters Olive tail moment (OTM) and percent DNA in tail (% tail DNA) are shown in **Figures 3** and **Table 3 (see also supplementary material, S1 and S2)**. No DNA damage was detected by comet assay on CHO-K1 after 4h of treatment with both UGE and RGE at 25µg/mL in comparison to untreated cells, both with or without metabolic activation (S9 + / S9-). Therefore, being considered not genotoxic at concentration of 25µg/mL. It was also observed a protective activity of UGE against DNA damage in CHO-K1 cells. However, there is a lack of studies investigating the antimutagenic/antigenotoxic substances of plants from the Rubiaceae family, especially those iridoids belonging to the genus Genipa. To our knowledge, this is the first study in which the genotoxic effects of genipap extract have been demonstrated *in vitro* by comet assay.

Table 3. Genotoxic effects of unripe (UGE) and ripe (RGE) genipap endocarp extracts on CHO-K1 cells by Comet assay evaluation.

	Tail moment Olive [a]		**% Tail DNA [b]**	
Treatment	**S9^{-} [c]**	**S9^{+} [d]**	**S9^{-}**	**S9^{+}**
CHO-K1	3.45 ± 0.30	3.37 ± 0.31	6.84 ± 0.59	6.39 ± 0.58
DMSO	3.14 ± 0.31	3.55 ± 0.30	6.17 ± 0.62	6.28 ± 0.53
MMS	80.55 ± 2.95 ***	n.t.	70.75 ± 1.11 ***	n.t.
CPA	n.t.	11.92 ± 0.63 ***	n.t.	20.52 ± 1.04 ***
UGE	7.51 ± 0.42	5.78 ± 0.33 ***	14.73 ± 0.79 ***	11.13 ± 0.66 ***
RGE	6.92 ± 0.40	7.13 ± 0.36 ***	13.37 ± 0.80 ***	14.11 ± 0.71 ***

Results expressed as mean ± standard error of at least 100 nucleoids per treatment, **a)** Tail moment Olive: mathematic relation between the tail DNA content (%) and tail length, **b)** %Tail DNA: tail DNA content expressed as percentage, **c)** experiment without S9 mix adition, d) experiment with S9 mix adition, Treatments: **CHO-K1=** untreated Chinese hamster ovary cells, **DMSO=** dimethyl sulfoxide at 0.25% (vehicle), **MMS=** methylethanesulfonate at 25 µg/ml, **CPA=** cyclophosphamide at 20 µg/ml, **RGE=** ripe genipap endocarp extract at 25 µg/ml, **UGE=** unripe genipap endocarp extract at 25 µg/ml, n.t.: not tested. Data analysed by ANOVA followed by Tukey test, ***$p < 0.001$.

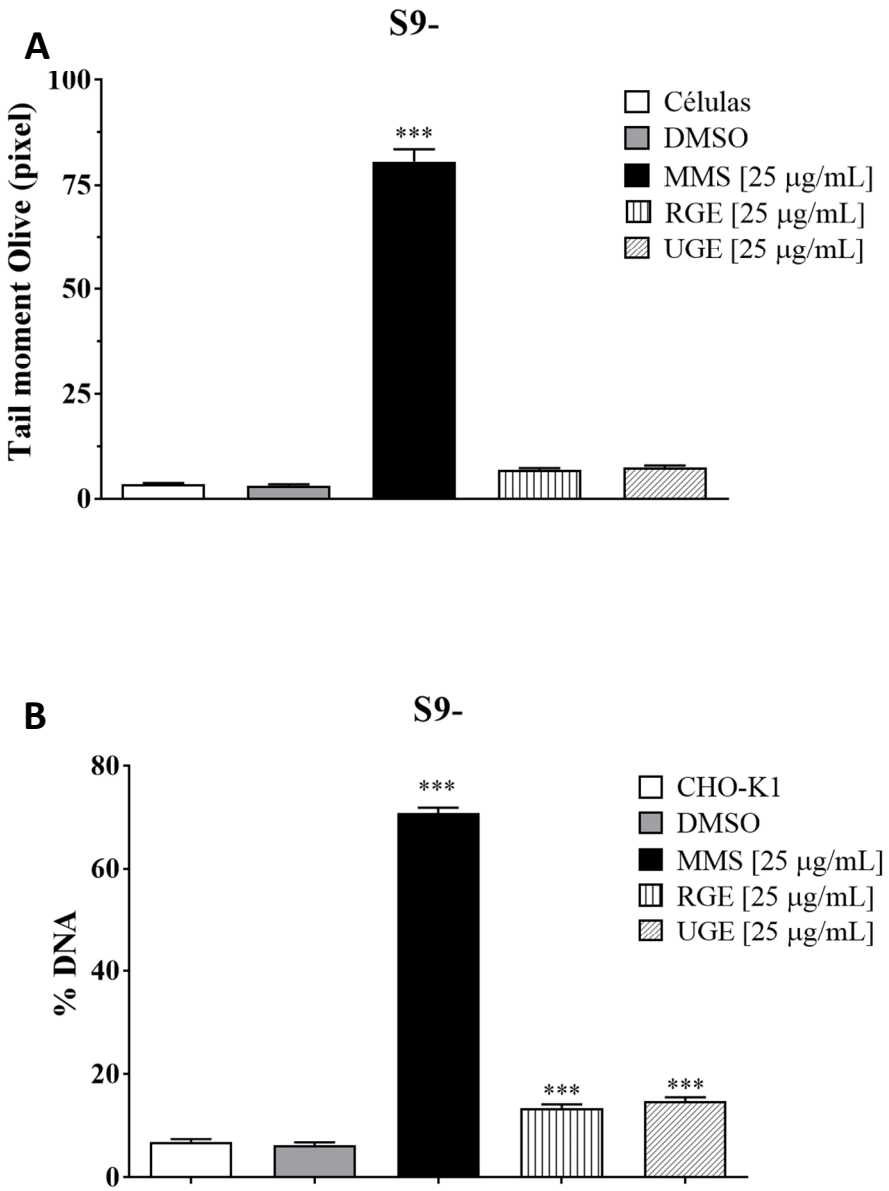


Figure 3. Graphical representation of effects of genipap extracts and their respective positive (MMS) and negative (DMSO) controls, in absence of metabolic activation ($S9^-$) on DNA strand breaks. The results were expressed as Mean ± SD of Olive tail moment **(A)** and % DNA damage **(B)**. Significant differences were determined by ANOVA, Tukey's test. *** *p < 0.0001.* **CHOK-K1 cells=** Chinese hamster ovary cells, **DMSO=** dimethyl sulfoxide, **UGE=** unripe genipap endocarp, **RGE=** ripe genipap endocarp, **MMS=** methylethanosulfonate, **CPA=** cyclophosphamide

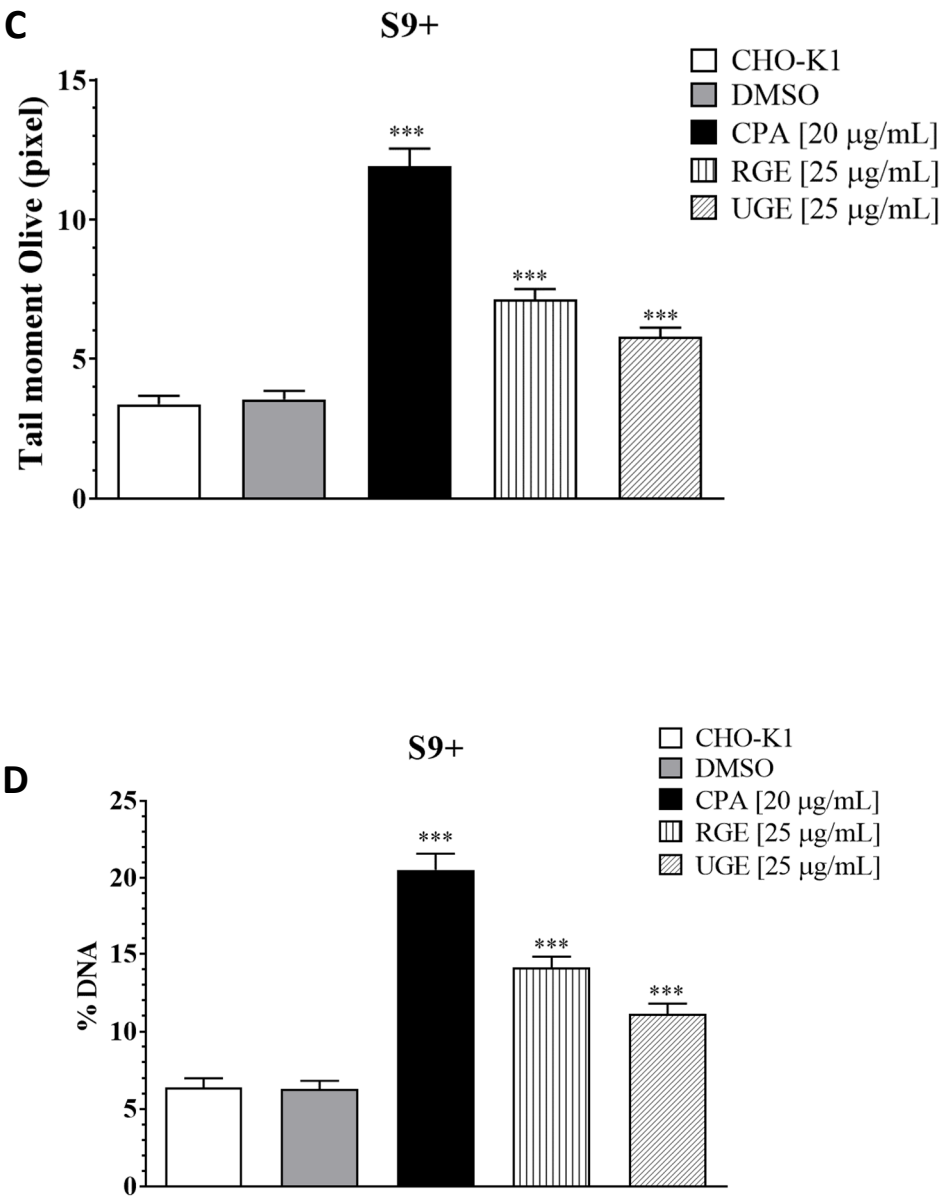


Figure 3. Graphical representation of effects of genipap extracts and their respective positive (CPA) and negative (DMSO) controls, in absence of metabolic activation ($S9^-$) on DNA strand breaks. The results were expressed as Mean ± SD of Olive tail moment **(C)** and % DNA damage **(D)**. Significant differences were determined by ANOVA, Tukey's test. *** *p < 0.0001.* **CHOK-K1 cells=** Chinese hamster ovary cells, **DMSO=** dimethyl sulfoxide, **UGE=** unripe genipap endocarp, **RGE=** ripe genipap endocarp, **MMS=** methylethanosulfonate, **CPA=** cyclophosphamide

4. Conclusion

In this study we identified a presence of iridoid glucosides (e.g. genipin) as main substances in the UGE and RGE extracts. The results genipap extract at 25 μg/mL is not genotoxic and did not interfere with non-tumor cell lines proliferation (CHO-K1 and HaCaT). In addition, the iridoids belonging to UGE extract seems to exert protective effect on DNA injury. Therefore, the UGE (at 25 μg/mL dosage) can be considered a safe option to be exploited as a food colorant with functional appeal. We also emphasize that metabolism of blue-based iridoids from genipap is not completely understood being necessary comprehensive and comparatively studies. Thus, next steps of our work will focus on *in vivo* assays to assess the metabolism of iridoids from genipap as well as their distribution and safety for food and pharmaceutical purposes.

Acknowledgments

The authors thank the São Paulo Research Foundation (FAPESP, grant number 2015/50333-1], the National Council for Scientific and Technological Development [CNPq, grant number 481670/2013-0] and the Institute of International Education (CAPES) for financial support and the Rupert Lyons for the language services provided. C. F. F. A. acknowledges FAPESP (studentships 2017/10753-7).

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Publication

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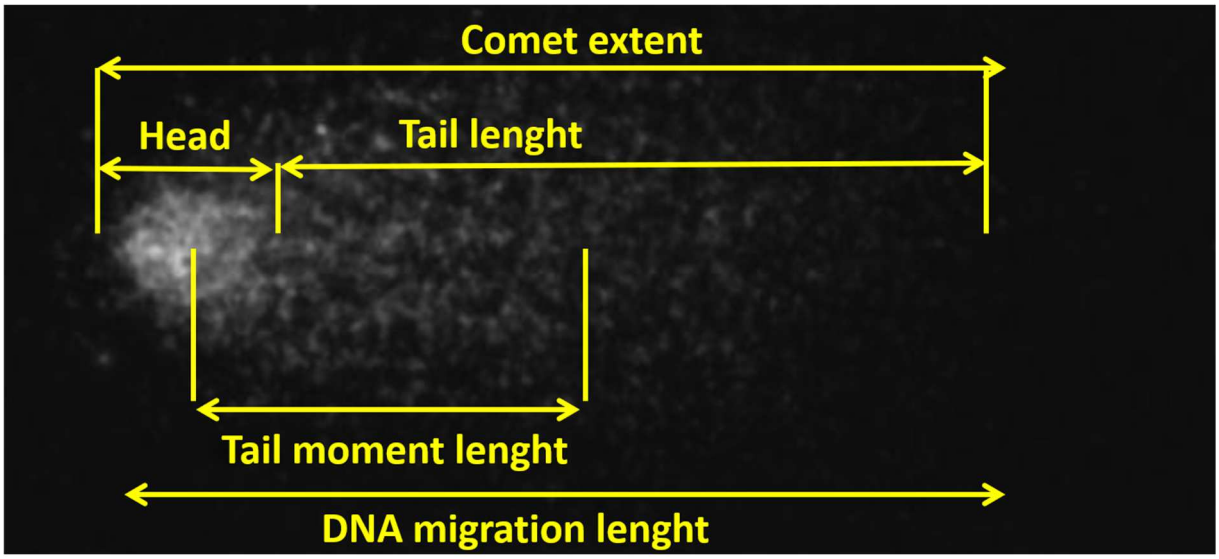
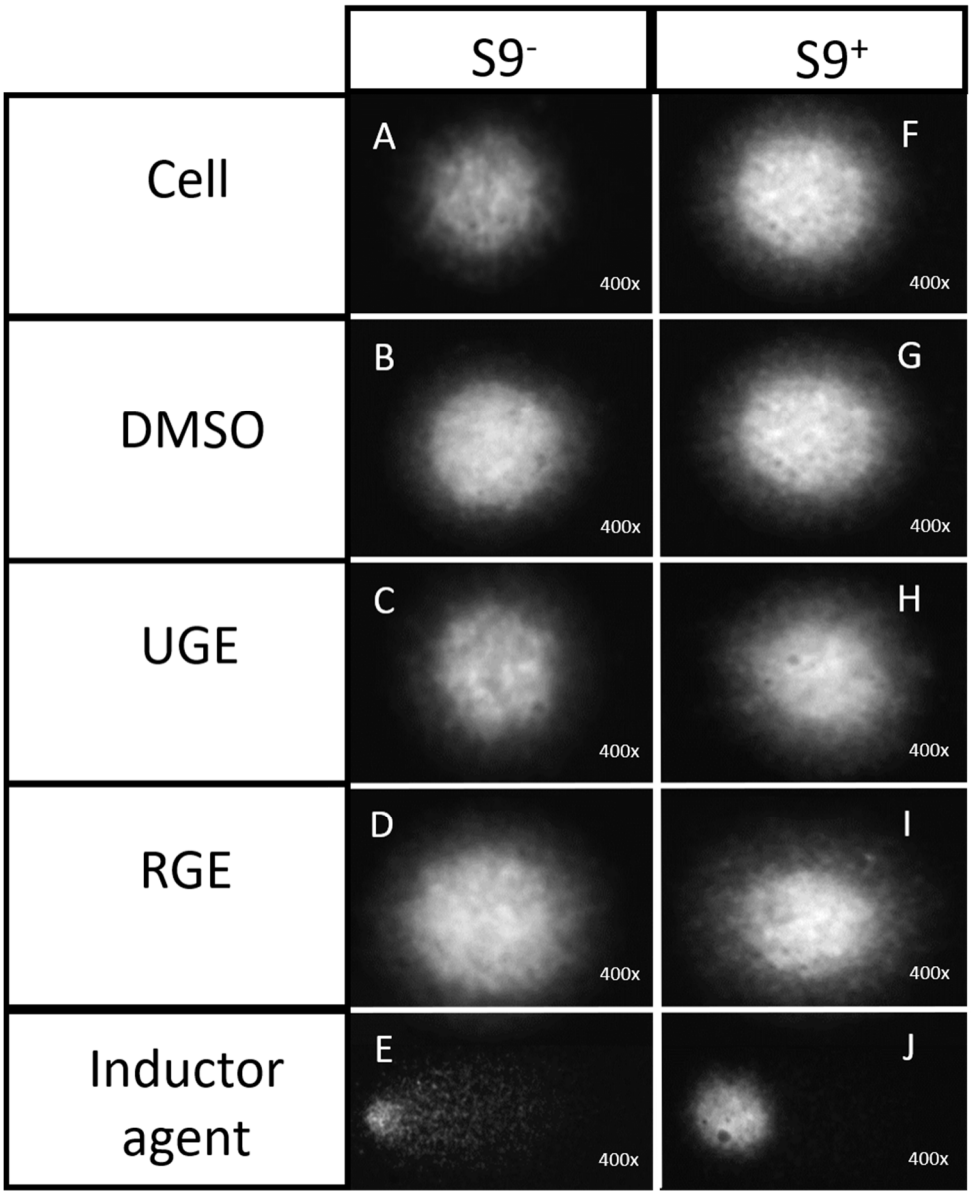
Material Supplementary



S1. Schematic representation of comet areas including % DNA and comet tail olive moment calculation. **Comet extent:** the distance from the leading edge to the trailing edge, **Tail Length:** the distance of DNA migration from the body of the nuclear core, **% DNA:** amount of migrating DNA, **Olive Tail Moment:** Tail DNA % x Tail moment length (Tail moment length is measured from the center of the head to the center of the tail).



S2. Representative microphotographs showing DNA damage induced in CHO-K1 cells. Images were captured with a Fluorescence microscope (excitation filter, λ 516–560 nm, barrier filter λ 590 nm) and olive tail moments were measured using the Comet Imager 2.2 Software. **A-E=** exposition to $S9^-$ and **F-J=** exposition to $S9^+$. **Treatments**: untreated CHOK-K1 cells **(A and F),** treated with DMSO at 0.25% (vehicle) **(B and G)**, treated with UGE at 25 µg/mL **(C and H)**, treated with RGE at 25 µg/mL **(D and I)**, treated with MMS at 25 µg/ml **(E)** and CPA at 20 µg/mL **(J)**, respectivelly. **CHOK-K1 cells=** Chinese hamster ovary cells, **DMSO=** dimethyl sulfoxide, **UGE=** unripe genipap endocarp, **RGE=** ripe genipap endocarp, **MMS=** methylethanosulfonate, **CPA=** cyclophosphamide

CAPÍTULO V

IRIDOID BLUE-BASED PIGMENTS OF *Genipa ameriana* L. (RUBIACEAE) EXTRACT: MICROENCAPSULATION, INFLUENCE OF SIMULATED DIGESTION ON ANTIOXIDANT CAPACITY AND APOPTOSIS INDUCTION

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Artigo submetido na Food Research International

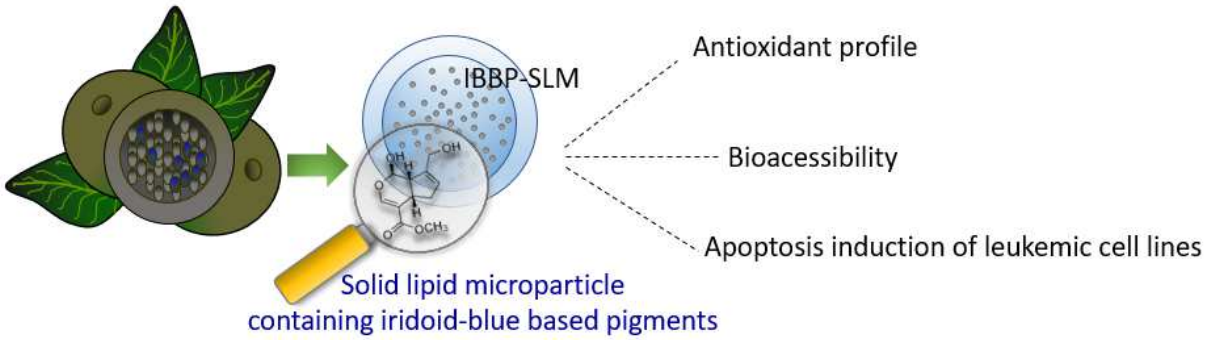
Fator de Impacto 3.086 - Qualis A1

Abstract

Iridoid blue-based pigments (IBBP) extract of *Genipa americana* L. represents a natural alternative for food applications and also exerts desirable biological effects on human health. In this study we produced and characterized the IBBP extract in solid lipid microparticles (SLM) using a spray chilling technique as well as identified the iridoids content, assessing the antioxidant capacity before and after its *in vitro* digestion and finally desirable biological effects on human health. Eleven glucoside iridoids were detected and the main compounds identified were genipin, and genipin 1-β-gentiobioside. The results obtained indicate that the spray chilling technique for the production of SLM containing IBBP extract have a weak performance in protecting the IBBP from degradation. However, IBBP-SLM seems to exert a desirable apoptosis induction (at 25 μg/mL dosage). Thus, the next steps for our work will focus on the improvement of the encapsulation efficiency, varying the proportion of the carrier to the active material as well as the use of other types of carriers.

Keywords: Iridoids blue-based pigments, *Genipa americana,* natural pigments, solid lipid microparticle, PS exposure

Graphical abstract



Highlights

- Iridoid glucosides are the main compounds found in genipap (*G. americana*) fruits
- Iridoid contents changes during *in vitro* digestion
- Stearic acid as wall material for iridoids solid microparticles showed some disadvantages
- stearic acid shown strong antiproliferative activity against cancer cell lines
- Iridoid microparticles induced phosphatidylserine exposure in leukemia

Abbreviations

IBBP, iridoid blue-based pigments, ND, non-digested, GP, gastric phase, IP, intestinal phase, m/z, mass/charge, r.t, retention time, MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide, PS, phosphatidylserine exposure.

Chemical compounds reported in this article

Gardenoside (PubChem CID: 442423)

Genipin (PubChem CID: 442424)

Genipin 1-β-gentiobioside (PubChem CID: 3082301)

Geniposide (PubChem CID: 107848)

Geniposidic acid (PubChem CID: 16020046)

6´-O-p-Coumaroyl-geniposidic acid (PubChem CID: 44255335)

6´-O-p-Feruloyl-geniposidic acid (PubChem CID: not found)

6”-O-p-Coumaroyl-1-β-gentibioside geniposidic acid (PubChem CID: not found)

6”-O-p-Coumaroylgenpin-gentiobioside (PubChem CID: 101408305)

1. Introduction

The *Genipa americana* L. is a tropical fruit belonging from Rubiaceae family rich in iridoids, mainly genipin, followed by genipinic acid, genipin-gentiobioside, geniposide and geniposidic acid (Wu & Horn, 2017). Genipin is a iridoid ester that reacts with amino groups in the presence of oxygen producing water-soluble bluish-violet pigments. In addition, it can be hydrolyzed to generate genipinic acid which also can react with different compounds to generate red and brown colorants **(Figure 1)** (Neri-Numa et al., 2017; Wu & Horn, 2017). As it is a precursor of natural blue pigment, the genipin has being aroused interest to replace synthetic colorants in food applications (Neri-Numa et al., 2017). Specially, into markets outside of Latin America exploiting them as novel food and food ingredients (Regulation [EC] N, 258/97), respectively (Brauch et al., 2016).

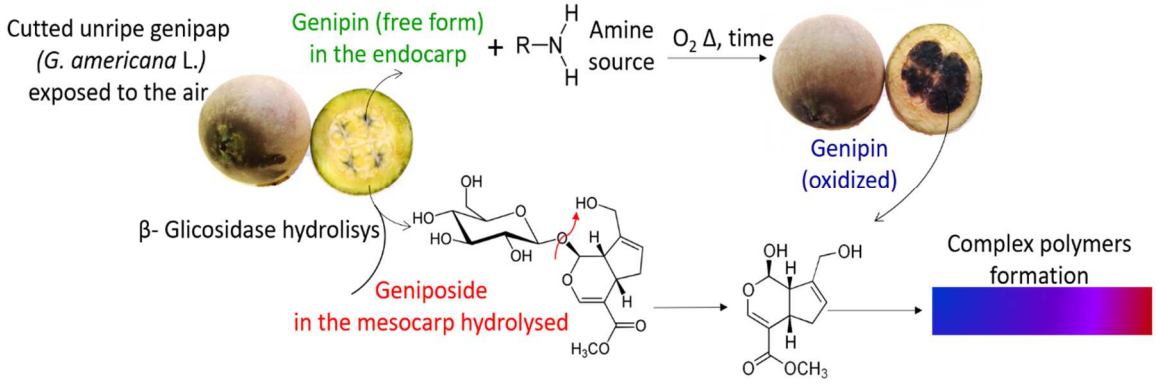


Figure 1. Schematic representation of complex polymers formation with bluish-violet hues from genipin. In brief, it is possible to obtain bluish/ purplish pigments directly from oxidation of free genipin from endocarp or after the hydrolysis of their precursor geniposide in the mesocarp. Adapted from Cano et al., 2014 and Neri-Numa et al., 2017.

Moreover, iridoids or iridoid-rich plants are linked with a wide range of biological activities (Katerova et al., 2017). Both genipin and geniposide have been reported as promising option for pharmacological applications not only due its crosslinking properties but also by suppression of *Helicobacter pylori* infections, effects of antioxidation, anti-inflammation, anti-atherosclerosis, antihypertension, antiplatelet aggregation, and so on (Kim et al., 2016; Li et al., 2016; Chang et al., 2017; Neri-Numa et al., 2017; Zhang et al., 2017).

Studies involving human cell lines revealed that genipin and its glucoside form exert an apoptotic effect on cancer cells (human non-small cell lung and MDA-MB-231 human breast, respectively), fibroblast-like synoviocytes in arthritis as well as on pancreatic β-cells from high glucose-mediated injury (Cao et al., 2010; Kim et al., 2012; Yang et al., 2013; Li et al., 2016; Liu et al., 2017).

The apoptosis is a hallmark of cancer involving a complex multistep process genetically encoded to induce the programmed cell death (Tomasetti et al., 2017, WHO, 2017). Thus, the plant derived compounds have been coming forward as a plausible approach for cancer management through apoptosis induction playing a role as potential drug target (Farazuddin et al., 2012; Ediriweera et al., 2017). However, if ingested high doses, many phytochemicals may not exert their benefits due its toxicity apart causing undesirable effects such as fast elimination, non-target accumulation or poor solubility (Farazuddin et al., 2012).

In addition, rich extract of phytochemicals can have low solubility or be chemically unstable, degrading rapidly when exposed to the external environment (O_2, temperature, light, pH, etc) hampering its use in food or pharmaceutical applications (Lee & Wong, 2014). For this reason, the encapsulation technologies have been proposed to overcome the referred drawbacks (Onwulata, 2013; Xiao et al., 2017).

Among microencapsulation techniques, spray-chilling results in a formation of a stable and free flowing powder. This technique uses lipids as carriers, being the most commonly used fatty acids, alcohols, triacylglycerols and waxes, but shall present solid at room temperature to ensure the formation of the microparticles. The active of interest must be dispersed or to form an emulsion (o/w) with the lipid carrier, which is molten, then is atomized in a refrigerated chamber where the solidification and formation of the particles occurs (Okuro et al., 2013).

Spray chilling stands out in the pharmaceutical and food fields because it is a low-cost continuous process, and it is easy to scale. In addition, some studies reported that technique promotes a suitable phytochemical-delivery system, improving their pharmacokinetic properties as well as its uptake by cancer cells and thereby increasing the efficacy of the entrapped bioactive substance (Okuro et al., 2013; Kwak, 2014; Paulo & Santos, 2017).

Due to the gastrointestinal tract complexity, the *in vitro* static digestion models have been used to assess the microparticles behavior, as well as the release rates and bioavailability of bioactive compounds were performed using in vitro static digestion models (De Souza Simões et al., 2017).

Thus, considering there is no information available regarding the cell growth inhibition and apoptosis induction by iridoid blue-based pigments (IBBP) of *G. americana* L. extract, the aim of this study it was to compare IBBP extract *versus* solid microparticles containing IBBP extract (SLM-IBBP) through its antioxidant profile before and after gastrointestinal *in vitro* digestion as well as the antiproliferative activity and apoptosis induction assay using cancer cell lines.

2. Materials and methods

2.1. Chemical and cell culture apparatus

Standards of geniposidic acid, geniposide and genipin, purity ≥ 98% (HPLC) and as well as the trichloroacetic acid, sulphorhodamine B, cyclophosphamide monohydrate, doxorubicin, vincristin and all reagents used by antiproliferative and apoptosis induction assays were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum and RPMI 1640 were purchased from Gibco-Invitrogen® (New York, USA). APC Annexin V was purchased from BD Bioscienses (San Jose, California, USA). HPLC grade methanol and formic acid were provided by Merck (Frankfurt, Germany). Polyglycerol polyricinoleate (GRINSTED® PGPR90) was kindly donated by Danisco Brasil Ltda (Cotia, SP, Brazil) and 8.2% Stearic acid was determined by AOCS method. All other chemicals and solvents in this study were of analytical grade and water was purified by Milli-Q®$_{plus}$ system (Millipore, Milford, MA, USA). All tissue culture plates and other plastic wares were from Costar (Corning, New York, USA).

2.2. Plant material and extraction procedure

Unripe fruits of genipap were collected at the University of Campinas (22° 49' 8.55" S and 47° 04' 24.022" W). The botanical identification was performed at the Department of Vegetal Biology and the exsicate (access number: 110904) was deposited at the Herbarium-UEC of Biology Institute of University of Campinas, State of São Paulo, Brazil.

Cano et al. (2016) reported that in *G. americana* fruits, genipin is found mostly in endocarp, while its concentration is lower in the mesocarp. Moreover, free genipin is available only in the unripe fruits, since it becomes glycosylated (geniposide and geniposidic acid) during ripening (Cano et al., 2016). Thus, the extraction procedure used just unripe endocarp of genipap. Sample was freeze-dried for 72 hours (Terroni, model LS 3000, São Carlos, Brazil), powdered using a knife grinder (Marconi, model MA-340, Piracicaba, Brazil) and subsequently submitted to the extraction procedure in triplicate. For this procedure, an aliquot of 10 g of the resulting powder was extracted eight times, each with 100 mL of methanol 80% (v/v) in water, in ultrasonic bath (40Hz) for 15 minutes followed by centrifugation (8000 RPM) for 10 min at 5 ^{o}C. All supernatants were combined and evaporated under vaccum at 60 ^{o}C to dryness (Bücchi Labortechnik AG, model R11 F-108, Flawil, Switzerland). The samples were recovered with 10 mL of water and filtered through 0.20 µm regenerated cellulose Minisart RC 15 single-

use syringe filter (this filtrate was called IBBP extract) (Sartorius, PP-housing, Goettingen, Germany) prior to chromatographic analyses.

2.3. *Preparation of solid lipid microparticles containing IBBP extract*

Solid lipid microparticles (SLM) were prepared using a Buchi B-290 spray dryer set to the spray chiller mode (Büchi, Flawil, Switzerland), according Sartori et al. (2015) and Consoli et al. (2016) with slight modifications. Briefly, it was used stearic acid (90% $w/w_{dispersion}$) as carrier agent and IBBP extract (10% $v/w_{dispersion}$) as active substance. The dispersion of IBBP extract in the lipid matrix was performed using PGPR (polyglycerol polyricinoleate at concentration of 5% ($w/w_{stearic\ acid}$), Sartori et al. (2015) as emulsifier to formation of a water-in-oil (w/o) emulsion. The aqueous phase was dispersed gradually into the lipid phase (heating to 85 °C to maintained stearic acid in a liquid phase until the end of the process). After the solution was emulsified using high-speed blender (24.000 rpm by 5 min) (IKA, Ultra Turrax T18, Germany) for 1 min, and then submitted to the spray chilling process. The emulsion was fed into a heated (85 ^{o}C) double fluid atomizer with a nozzle diameter of 2.0 mm using a peristaltic pump. SLM were formed within a cooled chamber where the inlet air temperature was 7 ^{o}C. Atomizing air and cooling air flow rates were 1052 L/h and 35,000 L/h, respectively. The resulting SLM were called IBBP-SLM, collected and stored in sealed flasks at 5 ^{o}C, for further analyses.

2.4. Characterization of IBBP-SLM by morphology and thermal properties

The scanning Electron Microscopy (SEM) was applied to evaluate the morphology of the IBBP-SLM. The sample was put on a circular aluminium stubs with double carbon sticky tape and coated with 200 Å of gold on the Sputter Coater (EMITECH, model K450, Kent, United Kingdom). The micrographs were obtained using a scanning electron microscope (SEM) with energy dispersive X-ray detection (Leo 440i, model 6070, Cambridge, England), accelerating potential of 15 kV, and current of 50 pA. Micrographs were obtained with amplitude of 500 x and 5000 x.

Regarding thermal analysis was performed using a differential scanning calorimetry (DSC) (DSC1, Mettler Toledo, Schwerzenbach, Switzerland) equipped with a

cooling system. IBBP-SLM was weighed (~5mg) in aluminium pans and sealed hermetically. Analysis conditions were: heating to 80 ^{o}C maintained for 5 min, cooled to - 40 ^{o}C (10 ^{o}C /min) for 30 min, and then heated to 80 ^{o}C at 5 ^{o}C/min. A sealed empty aluminium pan was used as reference for all the DSC runs. The thermal characteristics were obtained from the thermograms of the samples using the software Universal Analysis 3.9A. The DSC analyses were performed in triplicate, and the results are presented as mean values.

2.5. Total encapsulation efficiency (TE)

The TE of the resulting SLM was defined as the ratio between the total amount of IBBP (IBBPt) present in the sample and the initial amount of IBBP present on SLM (IBBPi). Thus, iridoids content of IBBP-SLM was determined by UHPLC-DAD analysis, using a Dionex Ultimate 3000 UHPLC system (Sunnyvale, CA, USA) controlled by Chromeleon™ software. An AcclaimTM120 C18 column (5µM 120Å 4.6 x 250 mm) was used to separate samples. Owing the hydrophilic nature of genipin, the extraction of outer IBBP was performed suspending 100 mg of SLM in 10 mL of methanol 50% (v/v) in water while entrapped iridoids were extracted using a mixture (10 mL) of chloroform: methanol 50% (2:1, v/v), in ultrasonic bath (40Hz) for 15 minutes followed by centrifugation (10.000 RPM) for 10 min at 25 °C. Both aliquots from the superficial fraction and the aqueous phase of entrapped fraction were filtered through 0.20 µm Chromafil® Xtra PVDF-20/25 single-use syringe filter. Then, IBBP quantification were determined from peak area using a standard calibration curve for geniposidic acid (R^2=0.9962), geniposide (R^2=0.9926), and genipin (R^2=0.9956). The TE and EE were further calculated by the following equation:

$$TE\ (\%) = (IBBP_i / IBBP) \times 100 \qquad \text{Eq. 1}$$

Where, IBBP is the extract iridoids amount, $IBBP_i$ is the iridoid content on the SLM and IBBPi is the iridoid amount on the particle surface.

2.6. In vitro simulated gastrointestinal digestion procedure

The IBBP extract was digested according the method developed by Faller et al (2012) and modified by Sancho et al. (2017). Briefly, 1 mL of sample was mixed with 3.5 mL of saline solution (140 mM NaCl, 5 mM KCl) in order to achieve the final concentration of 5.5 mg/mL. After vortex agitation, this mixture was acidified to pH 2.0 with 6 M HCl, and 125 mL of porcine pepsin solution (200 mg pepsin at 3,260 U/mg protein in 5 mL 0.1 M HCl) was added. The solution was incubated at 37 °C in a shaking water bath for 1 h under continuous agitation (130 rpm). After gastric phase (GP), the pH was increased to 6.8 with 1M $NaHCO_3$, followed by the addition of 625 µL of pancreatin-bile solution (225 mg of bile extract at 3,260 U/mg protein and 37 mg of pancreatin at 4 x USP specifications in 18.7 mL of 0.1 M $NaHCO_3$) and incubation in a shaker at 37 ^oC and 130 rpm for 2 h. At the end of intestinal phase (IP), samples were cooled in an ice bath and the digested volume was adjusted to 5.5 mL with saline solution. All fractions were ultrafiltrated using Amicon Ultra Centrifugal Filter Devices (30 kDa, Millipore) to remove proteins and were further stored at -80 ^oC for further analysis. A blank sample consisting of saline solution at pH 7.0 was subjected to the digestion procedures to eliminate any interferences coming from the reagents.

2.7. LC-MS analysis

The non-digested of both IBBP extract and IBBP-SLM (ND) and the digested fractions (GP and IP) were prepared diluting 50 µL of at 20 mg/mL sample in 950 µL of water resulting in a final concentration of 1 mg of sample per mL. These fractions were analyzed in negative ion mode using an HPLC (Hewlett Packard, Agilent Technologies 1290 series) coupled to Poroshell C18 120 SB-Aq 2.7 µm reversed phase column (2.1x100 mm, Agilent) and a Q-ToF iFunnel 6550 mass spectrometer fitted with an electrospray ionization (ESI) source. Mobile phase A consisted of a 0.1% (v/v) solution of formic acid in water, and mobile phase B was a 0.1% (v/v) of formic acid solution in acetonitrile. The samples were eluted with a flow rate of 0.45 mL/min following linear gradients (v/v) of mobile phases: 0 - 1 min, 5% B, 1 - 10 min, 5% B to 18% B, 10 -13 min 18% B to 70% B, 13 - 15 min, 70% B to 100% B, 15 - 17 min, 100% B and 17 - 20 min post time (5% B). The mass spectrometer parameters used were: VCap 3000 V, fragmentor voltage at 150 V, OCT 1RF Vpp at 750 V, Gas Temperature

at 290ºC, Sheath Gas Temperature at 350 ºC, Drying Gas at 12 L/min. Mass spectra were acquired in profile mode and the acquisition range was 100 - 1500 m/z.

2.8. Antioxidant capacity

The antioxidant capacity of the undigested and digested IBBP extract and IBBP-SLM were performed by ORAC assay according method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004), using a microplate reader (NOVOstar, BMG Labtech®, Offenburg, Germany), accompanied with the MARS Data Analysis Software version 1.3 (BMG Labtech®, Offenburg, Germany). Samples and Trolox standards were prepared with 75 mM phosphate buffer (pH 7.4). In each well containing 20 µL of extract or Trolox standard in different dilutions (to reach a linear response) and 120 µL of fluorescein (70 mM), 60 µl of AAPH solution (12 mM) was added to start the reaction, resulting in a final total volume of 200 µL. The decay of fluorescence was measured every minute for 80 min at 37 ºC, with excitation and emission wavelengths of 485 and 528 nm. A blank experiment (fluorescein + AAPH) with buffer instead of the sample or Trolox was also performed. The results were calculated using the relative area under the curve for samples compared to a Trolox standard curve (25 - 500 µM) prepared under the same experimental conditions. ORAC values were expressed as µM TE/g sample.

2.9. Cell lines and culture conditions

The tumor cell lines U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (breast expressing phenotype multiple drug resistance), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), HT-29 (colon) and K562 (leukemia) were generously supplied by the National Cancer Institute (NCI, Frederick, MA, USA). The immortalized human keratinocytes (HaCat) and the immortalized Chinese hamster ovary cells (CHO-K1) were kindly donated by Prof. Dr. Ricardo Della Coletta (FOP/UNICAMP) and Prof. Dr. Mário S. Mantovani (UEL), respectively.

Besides these cell lines, the human leukemic cells K562 (chronic myelogenous human leukemia), HL60 and NB4 (human acute promyelocytic leukemia), RAJI (human Burkitt lymphoma), JURKAT (human lymphoid T leukemia), REH and NALM6 (human acute

lymphoblastic leukemia) were generously supplied by the Gustave Roussy Institute (Villejuif Cedex, France).

For maintenance and experiments, all tumor cell lines were cultivated in complete medium [RPMI 1640 culture medium, supplemented with 5% (v/v) fetal bovine serum (FBS) and penicillin-streptomycin (1000 U/mL: 1000 µg/mL, 10 mL/L RPMI 1640) solution] and incubated at 37 ^{o}C with 5% CO_2 humified atmosphere. Cell lines were used between 4 to 10 passages. As for the leukemic cell lines, cells were cultivated in Dubelcco´s modified Eagle´s medium [DMEM, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 1% (v/v) non-essential amino acids, 2 mM L-gutamine, 1% antibiotic PSN (5 mg/mL penicillin, 5 mg/mL streotimycin and 10 mg/mL neomycin) solution and pyruvate] and incubated at 37 ^{o}C with 5% CO_2 humified atmosphere. Before the experiments, cell viability was determined by trypan blue dye exclusion method and was consistently greater than 98%.

2.10. In vitro antiproliferative activity

The antiproliferative activity of IBBP extract and IBBP-SLM as well as encapsulating material (stearic acid) were perfomed according to NCI standard protocol (Monks et al., 1991, Shoemaker, 2006). Samples stock solutions (5 mg) were prepared in DMSO (50 µL) followed by successive dilutions in complete medium to give final concentrations of 0.25, 2.5, 25, and 250 µg/mL. Doxorubicin was used as positive control at final concentrations of 0.025, 0.25, 2.5, and 25 µg/mL. Then, all tumor and non-tumor cell lines distributed in 96 well plates (100 µL cells/well, cell densities: 3 –7 × 10^4 cells/ml, T1 plates) were incubated for 48 h with each of the four concentrations of sample, doxorubicin, genipin or geniposide solutions (100 µL/well) in triplicate.

A microplate with all the cell lines (T0 control) was also prepared to stablish the cell quantities at the sample addition moment. After 48h, cells at T1 plates were fixed with trichloroacetic acid 50% and cell density was determined by spectrophotometric quantification (540 nm) of cellular protein content, employing sulphorhodamine B assay. Three measurements were obtained at the beginning of sample addition (T0) and 48 h post-incubation without (T1) and with sample (T). The cell proliferation was determined according

to the equation 100 × [(T − T0)/(T1 − T0)]. A cytostatic effect was observed when T1 > T ⩾ T0, while a cytocidal effect occurred when T < T0. Therefore, cell proliferation was graphically correlated to sample concentrations and one effective concentration named GI_{50} (sample concentration required to 50% inhibition of cell proliferation) was determined by nonlinear regression analysis using the Origin 8.0 software (OriginLab Corporation, Inc., Northampton, MA, USA).

2.11. Cell viability by MTT assay

Cell viability was assessed by performing 3-(4,5-dimethylthiazol-2yl)-2,5-diphenil tetrazolium bromide (MTT) (Mosmann, 1983) assay to determine the IC_{50} concentrations of the IBBP-SLM. For this assay, the human leukemic cells were plated (1×10^4 cells per well) in a 96-well plate, cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% antibiotic PSN (penicillin, streptomycin and neomycin) solution at 37 °C with 5% CO_2 humified atmosphere. Then was added in the cell suspension 180 µL of supplemented DMEM containing 20 µL of IBBP-SLM (final concentrations of 0.25, 2.5, 25, and 250 µg/mL). DMSO was used for solubilization of the tested samples at a maximun concentration of 0.1% well/treatment (a concentration which there is no cytotoxicity to the cells). Commercial standard genipin and vincristin, at the same concentrations tested, were used as an iridoid and positive controls, respectivelly. After 48 hours of incubation, 20 µL of MTT (0.5 mg/mL, pH 7.4) solution was added in culture medium and incubated for 4 h. Formazan precipitate was dissolved in 100 µL DMSO/well, and the concentration leading to 50% inhibition of viability (IC_{50}) after 48h was determined by measuring the absorbance at wavelength of 570 nm as an indicator of MTT reductase activity (Mosmann, 1983), using a Synergy ELISA microplate reader (BioTek Instruments, Highland Park, Winooski, USA). The viability of the treated cells was expressed as a percentage relative to the viability of untreated cells (incubated with growth medium only). The experiment was performed in triplicate and results were expressed as mean ± standard deviation values.

2.12. Detection of apoptosis based on phosphatidylserine exposure

The effect of IBBP-SLM on early apoptosis was evaluated by flow cytometry using an APC Annexin V kit (BD Biosciences Europe, Oxford, UK). The procedure was based on the well-known phenomenon of phosphatidylserine (PS) externalization to the cells outer surface membrane that occurs at early stages of apoptosis (Engeland et al., 1998). Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including APC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, APC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation (BD Pharmingen™).

Briefly, leukemic cells (HL60, NB4, NALM6 and JURKAT) were cultured as conditions previously described. The experiments were carried out using 25 µg/mL of IBBP-SLM, genipin commercial standard (as iridoid control) and vincristin (based on the antiproliferative and MTT assays) through a kinetic study for 48 h with reading each 6 h. After treatment, the cells were washed with cold PBS, transferred 100 L of the solution (1 x 10^5 cell) to a culture tube and incubated for 15 min with APC Annexin-V solution according to the manufacturer's protocol. After incubation, 400 µL of 1 x binding buffer was added and analysed on a BD FACS Canto™ flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with 488 nm argon ion lase (for scatter determination, 7-AAD, and PI excitation) and a 635 nm red diode laser (for APC-annexin V excitation). Data were acquired using BD FACSDiva™ v6.1.3 software (BD Biosciences, Heidelberg, Germany). The percentagem of annexin V and PI-positive (propidium iodide) cells was determined based on the dot plots in relation to its size, FSC (forward scatter) and cytoplasmic complexity, SSC (site scatter), delimiting a region with the cells of interest (granulocytes), named P1. Thereafter, data acquisition (for each sample, at least 50,000 events form each cell population) was performed using the percentages of viable, apoptosis and necrosis cell populations were calculated corresponding to the distribution in the set quadrants.

3. Statistical analysis

Statistical analyses were conducted using StatSoft, Inc. (2013) STATISTICA software, version 12.0. The results were subjected to ANOVA and differences between means were located using Tukey's multiple comparison test. Significance was determined at $p < 0.05$. All results are presented as means ± standard deviation for triplicates.

4. Results and discussion

4.1. Characterization of IBBP-SLM by morphology and thermal properties

The morphological analysis and DSC profiles are represented by **Figure 1** and **2**, respectivelly. SEM micrographs of IBBP-SLM showed spherical shapes with different diameters presenting a smooth surface with some crystal formation on the microparticle surface. It was also observed that particle agglomerations are consequence of partially melted particles and the presence of cracked particles suggested weak core material protection and also reduced functionality. Our results are in agreement with Consoli et al (2016) who also mentioned some imperfection and wrinkles on the surface of gallic acid microparticles produced by fully hydrogenated soybean oil as well as the occurrence of crystals and particle agglomeration as a result of incompletely solidification.

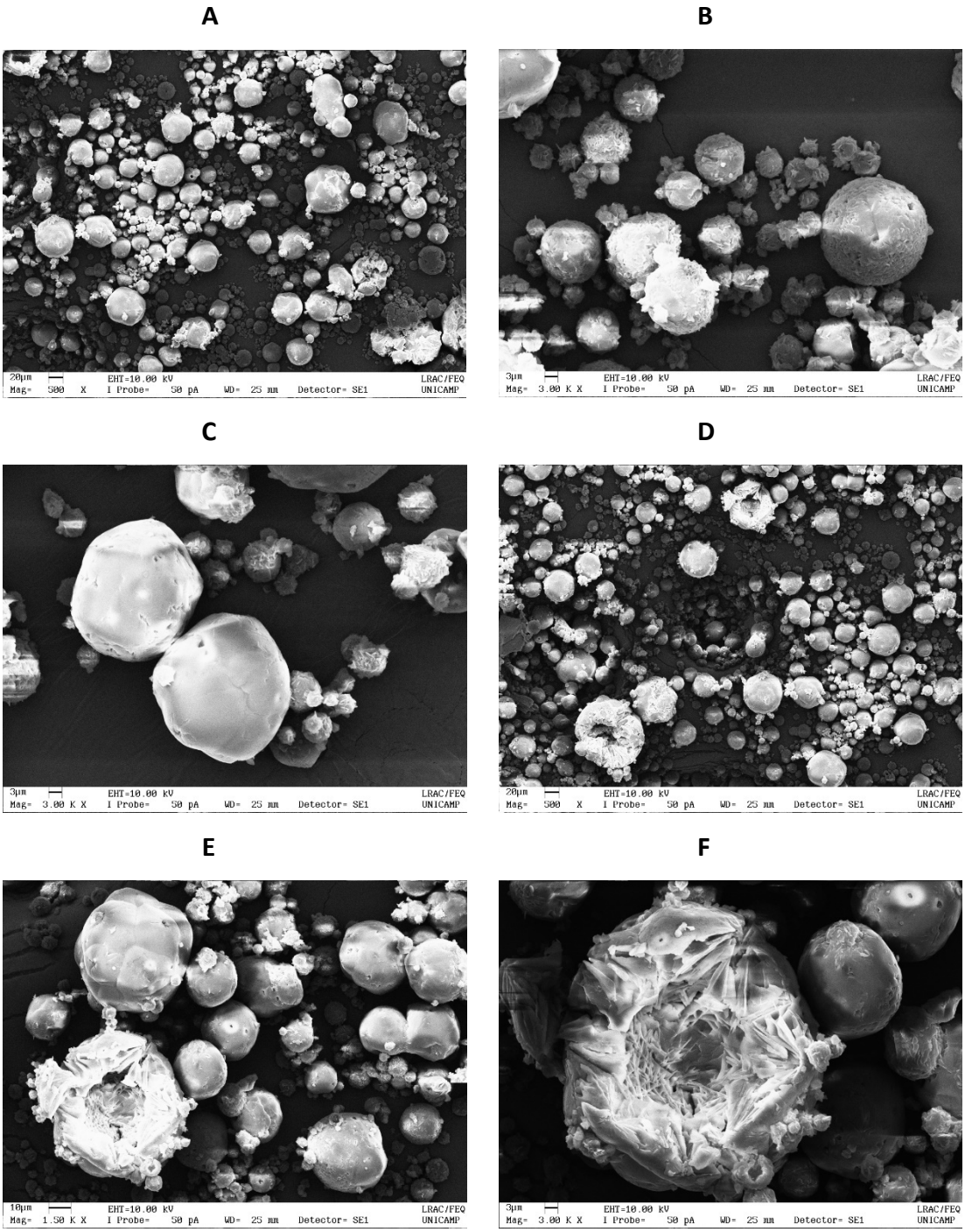
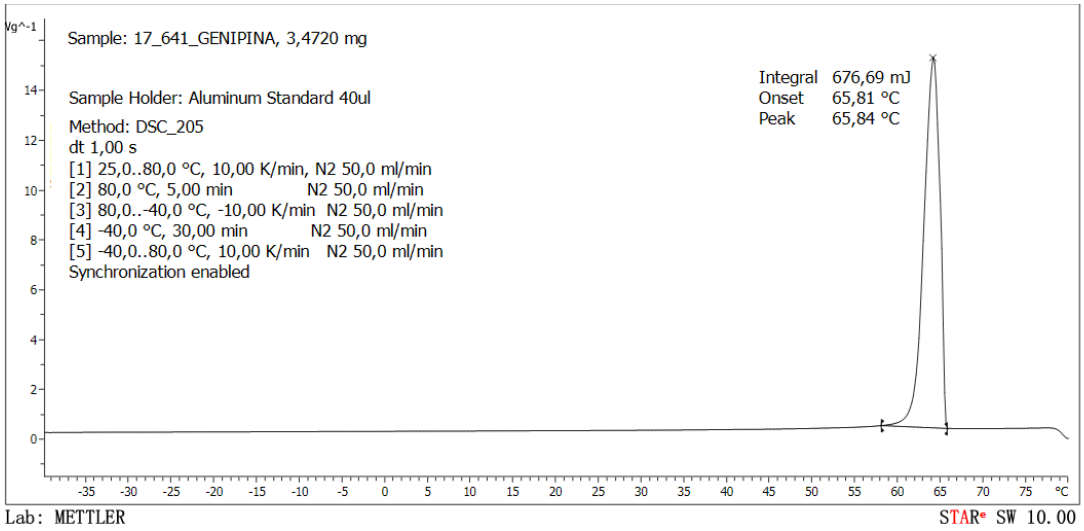


Figure 1. SEM micrographs of solid lipid microparticles loaded with Iridoid blue-based pigments of *Genipa americana* L. (Rubiaceae) extract produced by spray chilling**. (A)** microparticle agglomeration profile (500 x), **(B)** crystal on the surface (3000 x), **(C)** smooth surface (500 x), **(D)** microparticle agglomeration with merging particle profile and with some cracks (500 x), **(E)** microparticle cracks (1500 x), **(F)** microparticle cracks (3000 x).

The DSC curve of IBBP-SLM presented two clearly defined peaks: an endothermic peak at 66 °C (676.69 mJ) and an exothermic peak at 70 °C (677.02 mJ), corresponding to the melting and crystallization points, respectivelly **(Figure 2)**.

A



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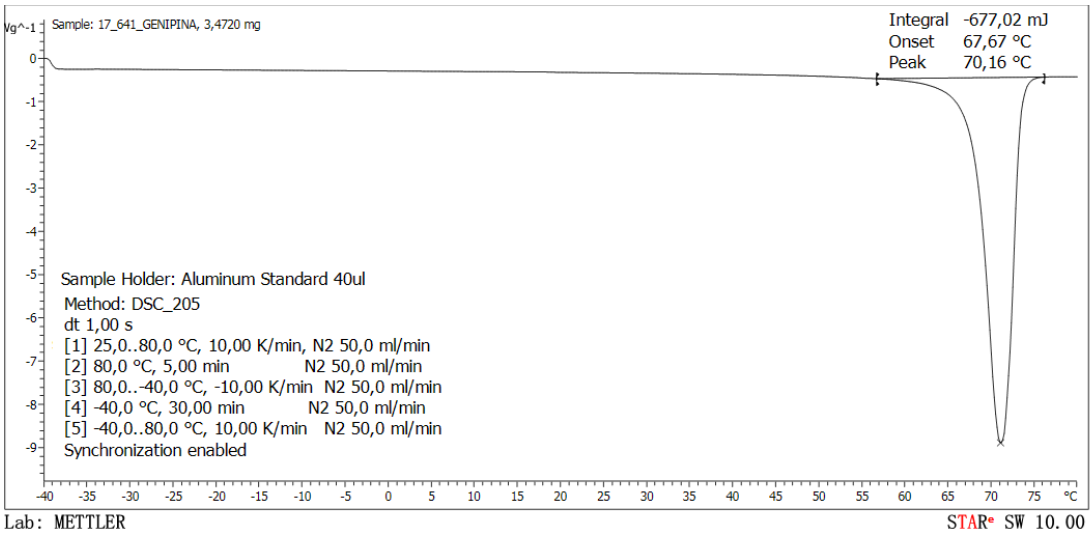

Figure 2. DSC termogram of IBBP-SLM. (A) Endothermic peak of melting point at 66 °C, (B) Exothermic peak of crystallization point at 70 °C.

These results suggest that SLM melting point can be a trigger for food applications. Since depending of the application type, the IBBP can be released with temperatures above 66 °C. This may be a positive point because the active will only have contact with the food if there is an increase in temperature. Our results are in agreement with Feldman et al. (1989) who mentioned that fatt acids as capric, lauric, palmitic and stearic acids are good candidates for latent heat thermal energy storage in heating applications.

4.2. *Total encapsulation effieciency*

Table 1 summarizes the results of TE indicating the IBBP content in both superficial and overall microparticle **(see also supplementary material S1)**.

Table 1. Total and superficial IBBP content in SLM produced by spray chilling

Compound	TE (%)	IBBP-SLM$_S$ (%)
Geniposidic acid	15.46 ± 0.85	0
Geniposide	16.18 ± 0.61	16.18 ± 0.61
Genipin	19.06 ± 1.15	7.52 ± 0.31

*Results expressed in mg/g dry weight, n.d.: not determined (values below LOQ). Data presented as mean ± standard deviation for the triplicate determination (*n= 3*). **IBBP=** iridoid blue=based pigment, **SLM=** solid lipid micropartilce, **TE=** total encapsulation efficiency, **IBBP-SLM$_S$=** iridoids content present in surface

The TE values showed that, in general, the microencapsulation process resulted in similar value for all compounds analysed, with approximately 15% on the incorporated of SLM (geniposidic acid, geniposide and genipin). Geniposide retention was expressed as all content on surface of SLM. This could be due to wall material (stearic acid) composition that not has affinity to this compound, resulting in expulsion through the lipid matrix. Therefore, improving the lipid matrix composition may be an alternative to better affinity for this iridoid. In contrast, the geniposid acid demonstrated more affinity to lipid matrix because no concentration of this compound was quantified on SLM surface. Thus, for further studies involving the relationship

between IBBP-SLM and biological effects it is required a re-evaluation on conditions process, concentration of iridioids and the type of carrier employed during SLM production.

4.3. *LC-MS analysis and antioxidant activity of IBBP extract and IBBP-SLM after gastrointestinal in vitro digestion*

Eleven compounds belonging to non-digested (ND) and digested fractions during the gastric phase (GP) and the intestinal phase (IP) from both IBBP extract and IBBP-SLM of *G. americana* were separated by UHPLC-ESI-MS/MS analyses **(see supplementary material, S2)**. Among them, genipin, genipin 1-β-gentiobioside and geniposide were confirmed with reference standards, while gardenoside derivative, gardenoside, 6’-O-p-Coumaroyl-geniposidic acid, 6'-O-feruloyl-geniposidic acid and 6'-O-p-Coumaroylgenipin gentibioside were tentatively identified considering their exact masses and fragmentation patterns in comparison to the literature data. Whilst, two compounds are unknown **(Table 2)**. It was not possible to identify two of the compounds. Similar results were reported by Ono et al (2005) who previously isolated and elucidated structurally several monoterpenoids from *G. americana*.

With respect to the digestion effects on samples, it was observed that most of the iridoid contents changed during the digestion process. In particular, our findings showed that iridoids of IBBP-SLM degraded during *in vitro* digestion, leaving just genipin 1-β-gentibioside remaining after IP **(Table 2)**. This loss in IBBP content might be explained by the instability of these compounds in neutral or alkaline pH values, or the hydrolysis of ester and glycosidc bonds with intestinal enzymes which has been observed by several authors who evaluated the stability of genipin and its glucoside forms during the digestive process (Lee et al., 2003; Wu & Horn, 2017).

Table 1. Abundance of the compounds in unripe endocarp of *Genipa americana* L. from non-digested (ND) and the digested fractions from IBBP extract and IBBP-SLM during gastric phase (GP) and intestinal phase (IP) using UPLC-ESI(−)-MS/MS.

	Absolute area (relative area)									
	IBBP extract			IBBP-SLM						
Tentative assignment	**ND**	**GP**	**IP**	**ND**	**GP**	**IP**	**m/z**	**r.t.**	**Molecular formula**	**MS/MS**
Gardenoside derivative*(1)	1.92×10^5 (0.9)	3.35×10^5 (0.79)	2.19×10^5 (1.15)	n.d.	n.d.	n.d.	391.12	1.35	$C_{16}H_{24}O_{11}$	-
Gardenoside (3)	3.17×10^5 (1.49)	6.10×10^5 (1.44)	3.2×10^5 (1.68)	n.d.	n.d.	n.d.	403.12	1.95	$C_{17}H_{24}O_{11}$	241/223/127
Geniposidic acid (2)	3.35×10^5 (1.57)	5.78×10^5 (1.36)	3.62×10^5 (1.90)	n.d.	n.d.	n.d.	373.11	1.75	$C_{16}H_{22}O_{10}$	-
Genipin 1-β-gentibioside* (4)	1.45×10^7 (68.2)	2.61×10^7 (61.48)	1.62×10^7 (85.33)	1.02×10^4 (2.56)	3.81×10^4 (5.76)	3.07×10^4 (100)	549.18	4.03	$C_{23}H_{24}O_{10}$	517/225/123/101
Geniposide* (5)	2.90×10^5 (1.38)	7.35×10^5 (1.73)	3.72×10^5 (1.96)	n.d.	n.d.	n.d.	387.13	4.55	$C_{17}H_{24}O_{10}$	408/225/123/101
Unknown (6)	7.53×10^5 (3.53)	2.04 x 106 (4.80)	n.d.	n.d.	n.d.	n.d.	387.12	4.90	$C_{17}H_{24}O_{10}$	-

* detected as formiated form [M+ $HCOO^-$], n.d., not detected, m/z, mass/charge, r.t., retention time

Continue

Table 1. Abundance of the compounds in unripe endocarp of *Genipa americana* L. from non-digested (ND) and the digested fractions from IBBP extract and IBBP-SLM during gastric phase (GP) and intestinal phase (IP) using UPLC-ESI(−)-MS/MS.

	Absolute area (relative area)									
	IBBP extract			IBBP-SLM						
Tentative assignment	**ND**	**GP**	**IP**	**ND**	**GP**	**IP**	**m/z**	**r.t.**	**Molecular formula**	**MS/MS**
Genipin (7)	3.83 x 10^6 (17.95)	9.88 x 10^6 (23.26)	7.00 x 10^5 (3.67)	3.88 x 10^5 (97.44)	6.02 x 10^5 (90.81)	n.d.	225.08	5.12	C11H1$_4$O5	-
6’-O-p-Coumaroyl-geniposidic acid (8)	5.91 x 10^5 (2.77)	1.21 x 10^6 (2.86)	4.31 x 10^5 (2.26)	n.d.	2.27 x 10^4 (3.43)	n.d.	519.15	7.88	$C_{25}H_{28}O_{12}$	241/223/127
6'-O-Feruloyl-geniposidic acid (9)	3.77 x 10^5 (1.77)	7.96 x 10^5 (1.87)	2.63 x 10^5 (1.38)	n.d.	n.d.	n.d.	549.16	8.14	$C_{26}H30O_{13}$	325/355/163/123/101
6’-O-p-Coumaroylgenipin gentibioside (11)	9.73 x 10^4 (0.46)	1.73 x 10^5 (0.41)	n.d.	n.d.	n.d.	n.d.	695.22	11.52	$C_{32}H_{40}O_{17}$	469/225/163/123/101
Unknown (10)	n.d.	n.d.	1.29 (0.68)	n.d.	n.d.	n.d.	519.15	9.94	$C_{25}H_{28}O_{12}$	-

* detected as formiated form [M+ $HCOO^-$], n.d., not detected, m/z, mass/charge, r.t., retention time

The antioxidant capacity of digested and non-digested samples (IBBP extract and IBBP-SLM) were assessed. Results shown **(Figure 3)** that microencapsulation process has influence on antioxidant capacity of IBBP-SLM. In spite of antioxidant capacities of both the IBBP extract and the IBBP-SLM were significantly higher ($p<0.05$) after digestion meaning (IP= 46% and 100%, respectivelly), it can be observed that release of iridoids presente in the IBBP extract (ND and IP with ORAC values of 843.47 and 817.54 µM TE/g, respectivelly) were higher than IBBP-SLM fractions.

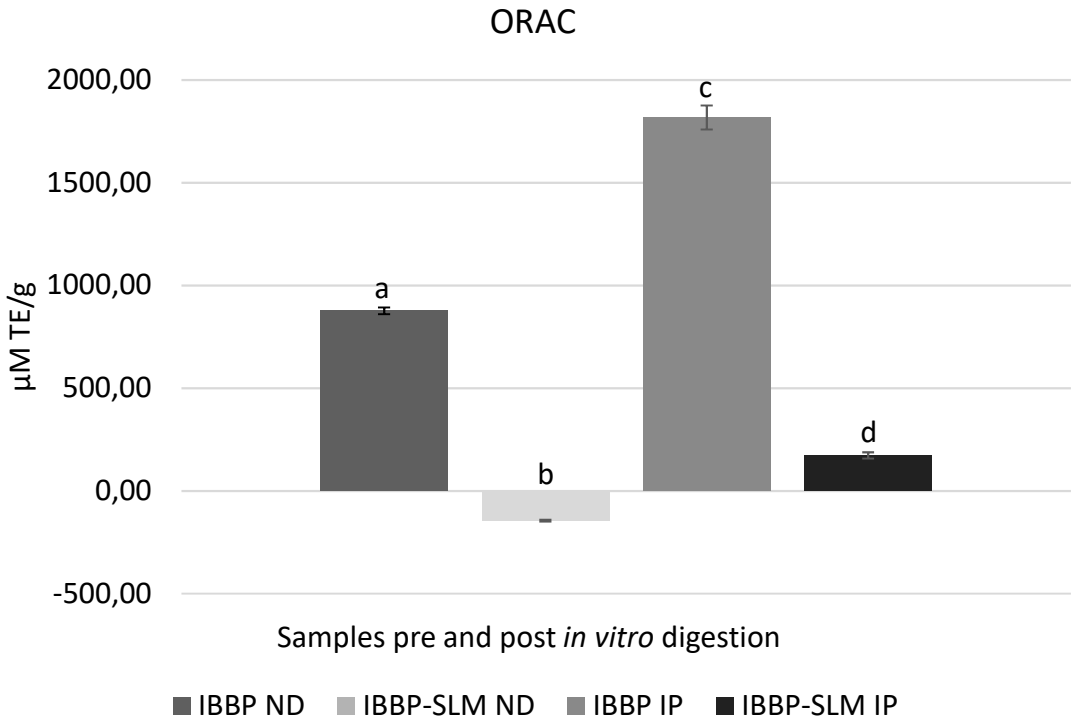

Figure 3. The ORAC value of non-digested and digested IBBP extract and IBBP-SLM. **IBBP=** iridoid blue-based pigments, **ND=** non digested, **IP=** intestinal phase of digested samples, **SLM=** solid lipid microparticle. Data was expressed as mean ± standard deviation. Different letters indicate significantly different values ($p< 0.05$) of triplicate analysis.

Thus, values obtained are related with the microparticle characteristics (e.g. size, concentration of iridoids found in the formulation, type of carrier) and test conditions (e.g. aqueous solution and temperature), where iridoids entrapped into stearic acid microparticles

was not available to react with oxidizing agents before digestion process (e.g. ND fraction without antioxidant capacity). On other hand, digestion process favoured the release of iridoid aglycone forms (e.g. genipin 1-β-gentibioside) from IBBP-SLM after intestinal phase (ORAC value = 173.05 μM TE/g).

In addition, the antioxidant capacity increase of IP can be related to the unknown products formed during *in vitro* digestion or to aglycone degradation products. Therefore, these unknown compounds might be good target compounds for further studies involving the relationship between IBBP-SLM and bioavailability, including experimental design using *in vivo* models to elucidate their biological effects, considering there is still a lack knowledge on the metabolic pathways of these iridoids.

4.4. *In vitro antiproliferative activity*

The antiproliferative profiles of IBBP extract, IBBP-SLM and stearic acid (wall material) as well as the positive control doxorubicin are shown in **Figure 4 (A-E**). This activity was expressed as the sample concentration required to inhibit in 50% the cell growth after 48h-exposition, named GI_{50} **(Table 2)**. In the experimental conditions the IBBP extract was inactive or presented a weak activity against (GI_{50} > 250 μg/mL) most of cell lines while the IBBP-SLM inhibited all cell lines (breast to colon= 0.97 μg/mL to leukemia= 43.5 μg/mL) evaluated with GI_{50}, with exception of U251 (glioma) and 786-O (renal) cell lines which IBBP-SLM was inactive.

On other hand, the stearic acid (wall material) showed a strong antiproliferative against all cell lines (glioma= 1.4 μg/mL to lung= 26.2 μg/mL) at low concentrations. Thus, these results added to morphology analysis of SLM, TE and *in vitro* digestion data suggest that kind of the wall material (stearic acid) had effect on the quality of the experiment, since the high rigidy of microparticle surface did not allow the complete dissolution of IBBP-SLM and nor to release the total entrapped iridoids under analysis conditions.

Table 2. *In vitro* antiproliferative activity evaluation of IBBP extract, IBBP-SLM and stearic acid (encapsulating material)

	TGI_{50} (µg/mL)								
	2	**m**	**a**	**7**	**4**	**o**	**h**	**k**	**q**
Doxorubicin	0.81	0.57	> 25	1.1	> 25	1.8	> 25	1.2	0.58
IBBP extract	99.4	108.8	*	*	*	*	*	141.2	140.0
IBBP-SLM	**	19.2	0.97	**	3.5	91.4	0.97	43.5	1.4
Stearic acid	1.4	10.1	4.8	9.8	26.2	13.3	20.7	16.0	11.0

Human tumor cell lines: **2** = U251 (glioma, CNS), **m** = MCF-7 (breast), **a** = NCI-ADR/RES (breast expressing phenotype multiple drug resistance), **7** = 786-0 (renal), **4** = NCI-H460 (lung, non-small cells), **o** = OVCAR-03 (ovary), **h** = HT-29 (colon), **k** = K562 (leukemia), and **q** = immortalized keratinocyte (non-tumoral cell). **IBBP** = iridoid blue-based pigments, **SLM** = solid lipid microparticle. * TGI_{50} > 250 µg/mL and ** TGI_{50} < 0.25 µg/mL (total growth inhibition).

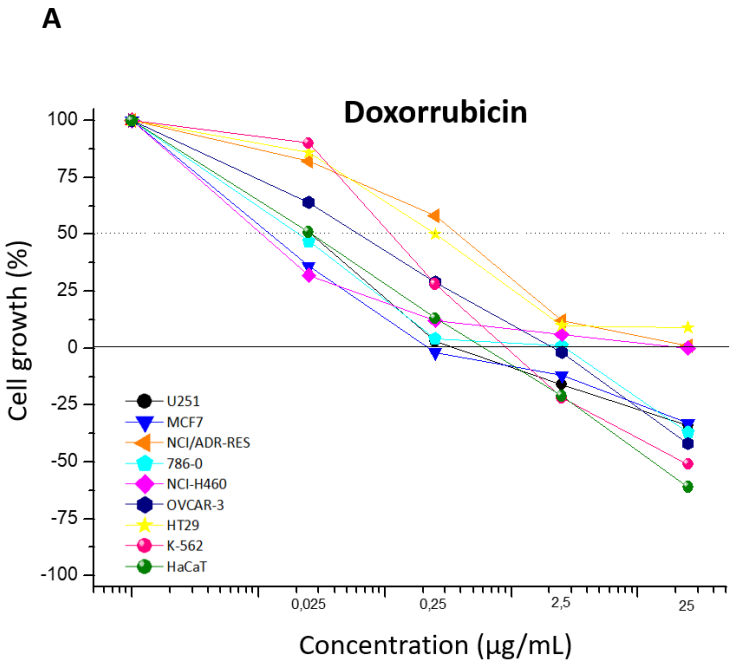

Figure 4. *In vitro* antiproliferative activity evaluation of Doxorrubicin **(A)** against eight human tumor cell lines and a non-tumor human cell: **U251** (glioma, CNS), **MCF-7** (breast), NCI-ADR/RES (breast expressing phenotype multiple drug resistance), **786-0** (renal), NCI-H460 (lung, non-small cells), **OVCAR-03** (ovary), HT-29 (colon), **K562** (leukemia), and **HaCat** immortalized keratinocyte (non-tumor cell). **IBBP=** iridoid blue-based pigments, **SLM=** solid lipid microparticle.

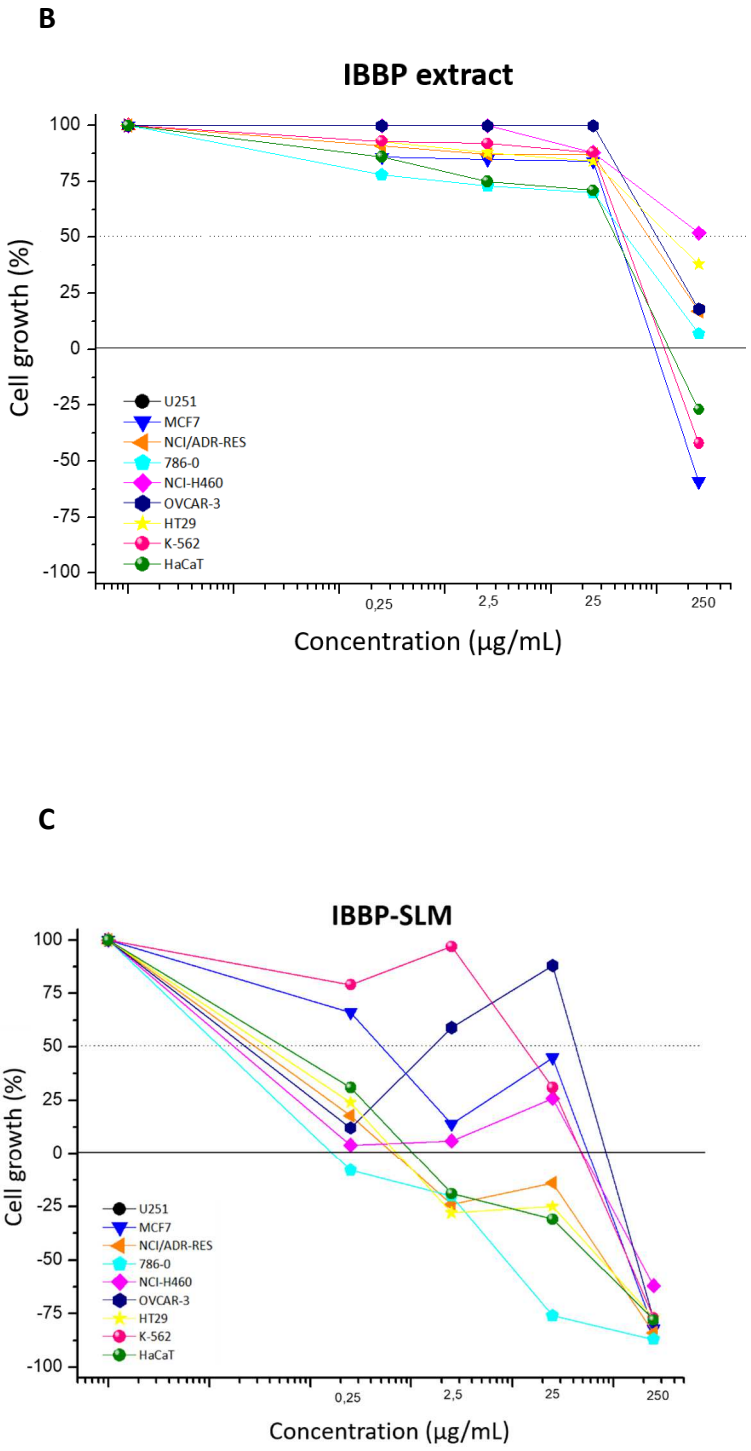

Figure 4. *In vitro* antiproliferative activity evaluation of IBBP extract **(B)** and IBBP-SLM **(C)** against eight human tumor cell lines and a non-tumor human cell: **U251** (glioma, CNS), **MCF-7** (breast), **NCI-ADR/RES** (breast expressing phenotype multiple drug resistance), **786-0** (renal), **NCI-H460** (lung, non-small cells), **OVCAR-03** (ovary), **HT-29** (colon), **K562** (leukemia), and **HaCat** immortalized keratinocyte (non-tumor cell). **IBBP=** iridoid blue-based pigments, **SLM=** solid lipid microparticle.

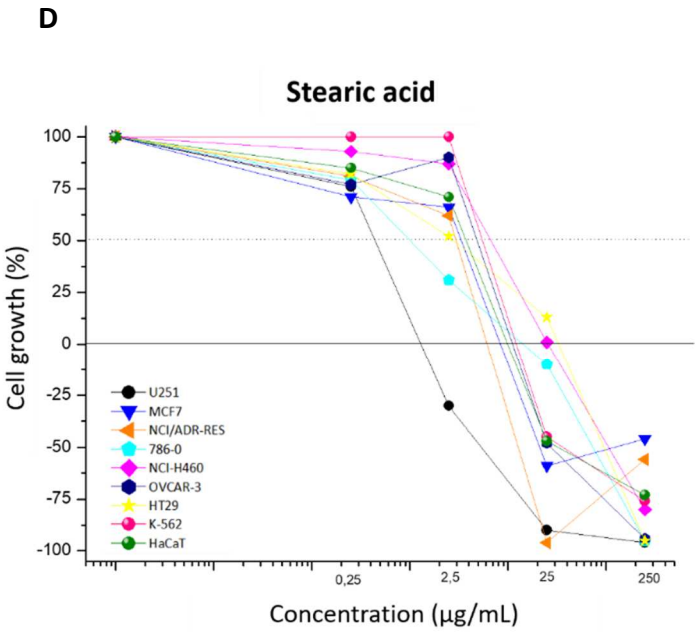

Figure 4. *In vitro* antiproliferative activity evaluation of Steraric acid **(D)** against eight human tumor cell lines and a non-tumor human cell: **U251** (glioma, CNS), **MCF-7** (breast), **NCI-ADR/RES** (breast expressing phenotype multiple drug resistance), **786-0** (renal), **NCI-H460** (lung, non-small cells), **OVCAR-03** (ovary), **HT-29** (colon), **K562** (leukemia), and HaCat immortalized keratinocyte (non-tumor cell). **IBBP=** iridoid blue-based pigments, **SLM=** solid lipid microparticle.

Some studies report that release of active material from the SLM can be influenced by the type of lipid matrix, production parameters as well as the solubility of the bioactive compound in lipid and partition coefficient (Wissing et al., 2004, Hu et al. 2005). Regarding the ability of IBBP extract inhibit cancer cell growth, both geniposide and genipin have been shown active against several cancer cell types (Habtemariam & Lentini, 2018). In a previous study, Feng et al (2011) reported that genipin can inhibits the proliferation of K562 cells in a IC_{50} value of 250 µmol/L. In the same way, Yang et al (2013) observed that IC_{50} of antiproliferative activity of genipin against H1299 cell line (lung, non-small cells) was 351.5 µM. On other hand, Habtemariam & Lentini (2018) draw attention about geniposide and genipin values as anticancer lead compounds since the effective dose of them is far higher than 100 - 200 µM and hence should be considered weak. Therefore, future studies are required to confirm the pharmacological properties using structural design and *in vivo* assays.

4.5. *Cell viability by MTT assay and detection of apoptosis based on phosphatidylserine exposure*

The effect of IBBP-SLM, genipin commercial standard (iridoid control) and vincristin (positive control) on cell viability of leukemica cell lines after 48h-exposition were assessed by MTT assay and results are shown in **Table 3.** According to the IC_{50} results, the IBBP-SLM showed a weak cytotoxic effect against JURKAT, REH and NALM6 (IC_{50} = 249.4, 224.0 and 225.3µg/mL. Thus, IBBP-SLM at concentration of IC_{50} was used in the subsequent study. After analysing the data obtained from MTT assay, when comparing the effect of IBBP-SLM with iridoid control (genipin standard) and vincristin, four cells lines (HL60, Jurkat, Nalm6 and NB4) were selected to evaluate the apoptosis induction by PS exposure. Thus, annexin V/PI binding was assessed each 6 h during 48h-exposure and results are shown in **Figure 5 to 8 (see also supplementary material S3).**

Table 3. Effect on the viability of leukemic cells treated with IBBP-SLM, genipin commercial standard (iridoid control) and vincristin

	IC_{50} (µg/mL)						
	K562	**HL60**	**NB4**	**RAJI**	**JURKAT**	**REH**	**NALM6**
IBBP extract	>250	>250	>250	>250	249.4 ± 32.4	224.0 ± 14.4	225.3 ± 13.5
Genipin standard	>250	57.3 ± 26.3	81.1 ± 46.8	152.8 ± 20.6	96.4 ± 7.4	65.9 ± 3.3	80.3 ± 5.0
Vincristin	>250	0.048 ± 0.008	0.009 ± 0.01	0.5 ± 0.261	0.031 ± 0.016	0.008 ± 0.001	0.013 ± 0.004

IBBP= iridoid blue-based pigments, **SLM=** solid lipid microparticle, **K562** (chronic myelogenous human leukemia, **HL60** and **NB4=** human acute promyelocytic leukemia, **RAJI=** human Burkitt lymphoma, **JURKAT=** human lymphoid T leukemia, **REH** and **NALM6=** human acute lymphoblastic leukemia. Data was expressed as mean of triplicate ± standard deviation of triplicate analysis.

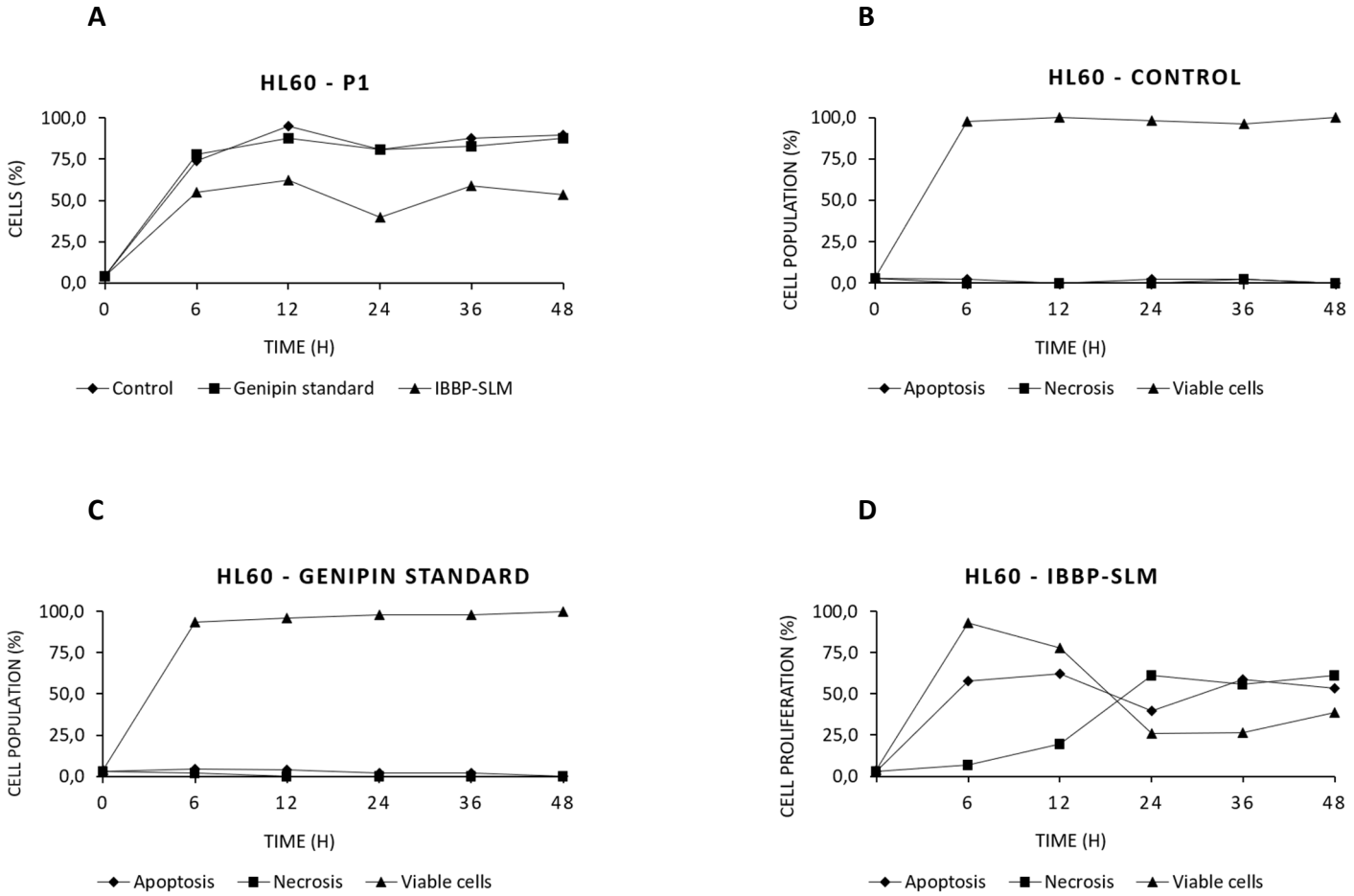

Figure 6. Graphic representation of kinetic analysis of cell death induction based on phosphatidylserine exposure in leukemia cell lines evidencing the populations of viable, apoptotic and necrotic cells from P1 region. **IBBP=** iridoid blue-based pigments, **SLM=** solid lipid microparticle, **P1=** cells of interest, **HL60 =** human acute promyelocytic leukemia. Results were obtained from a single experiment.

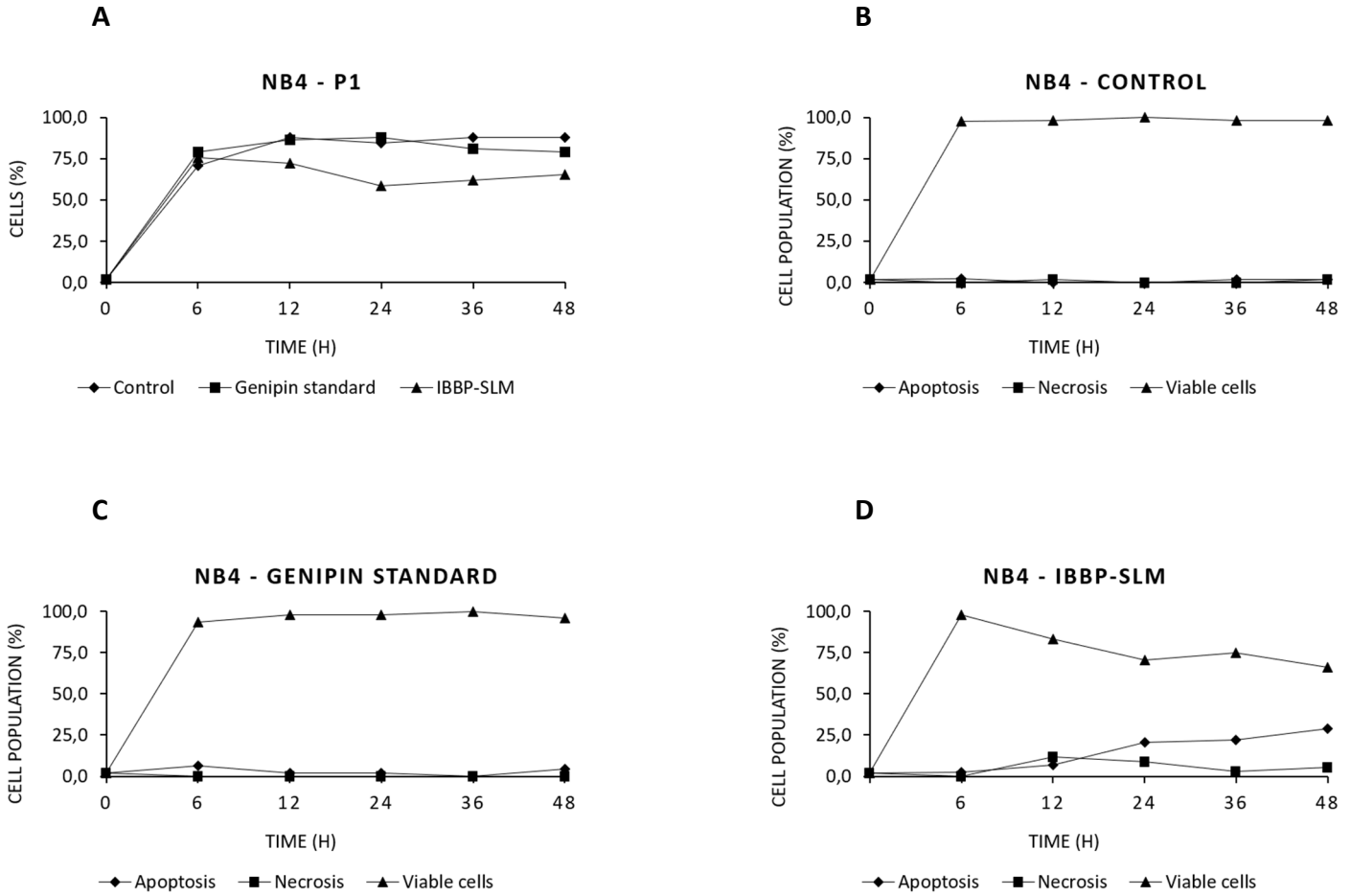

Figure 7. Graphic representation of kinetic analysis of cell death induction based on phosphatidylserine exposure in leukemia cell lines evidencing the populations of viable, apoptotic and necrotic cells from P1 region. **IBBP=** iridoid blue-based pigments, **SLM=** solid lipid microparticle, **P1=** cells of interest, **NB4=** human acute promyelocytic leukemia. Results were obtained from a single experiment.

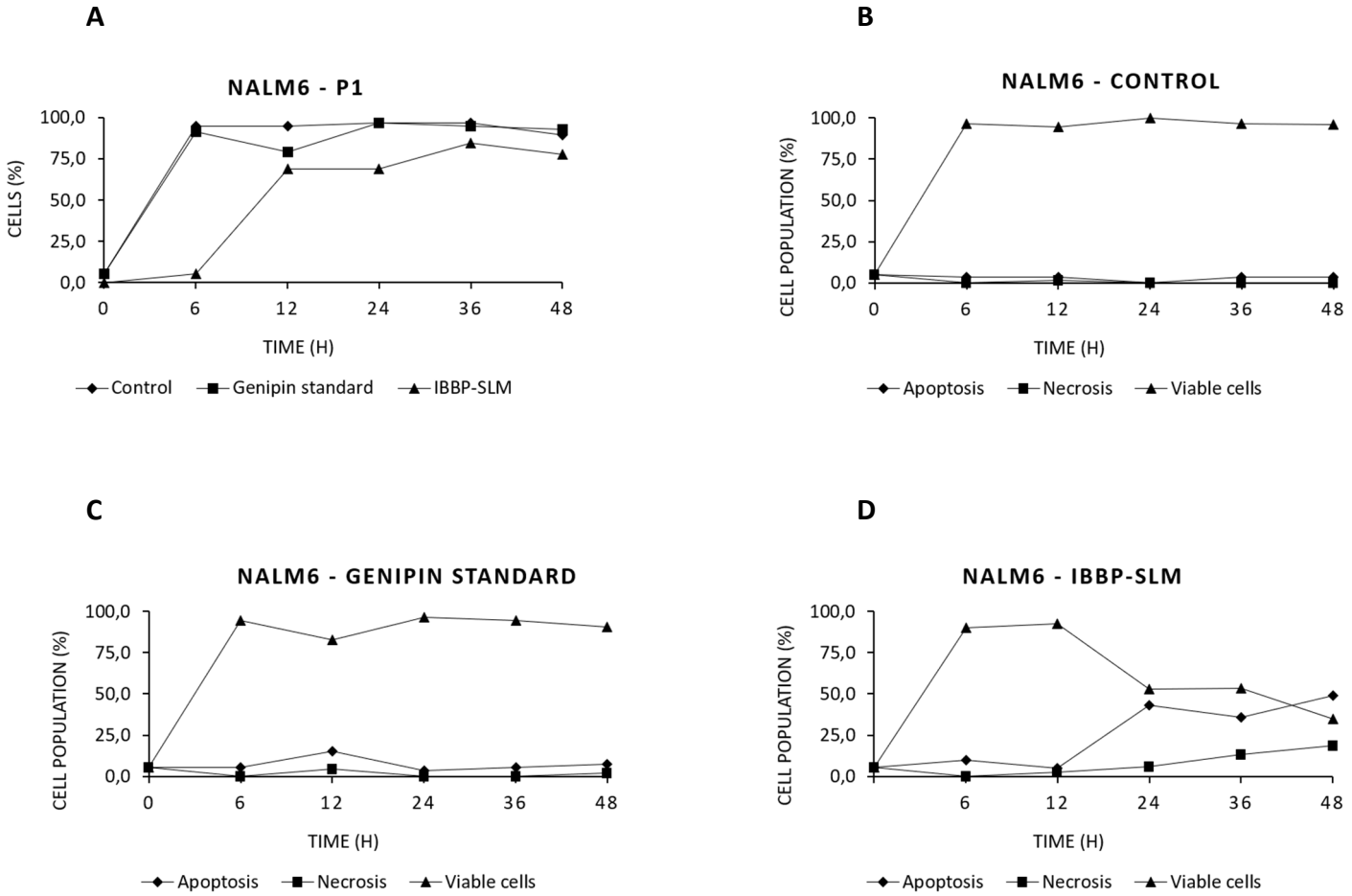

Figure 8. Graphic representation of kinetic analysis of cell death induction based on phosphatidylserine exposure in leukemia cell lines evidencing the populations of viable, apoptotic and necrotic cells from P1 region. **IBBP=** iridoid blue-based pigments, **SLM=** solid lipid microparticle, **P1=** cells of interest, **NALM6=** human acute lymphoblastic leukemia Results were obtained from a single experiment.

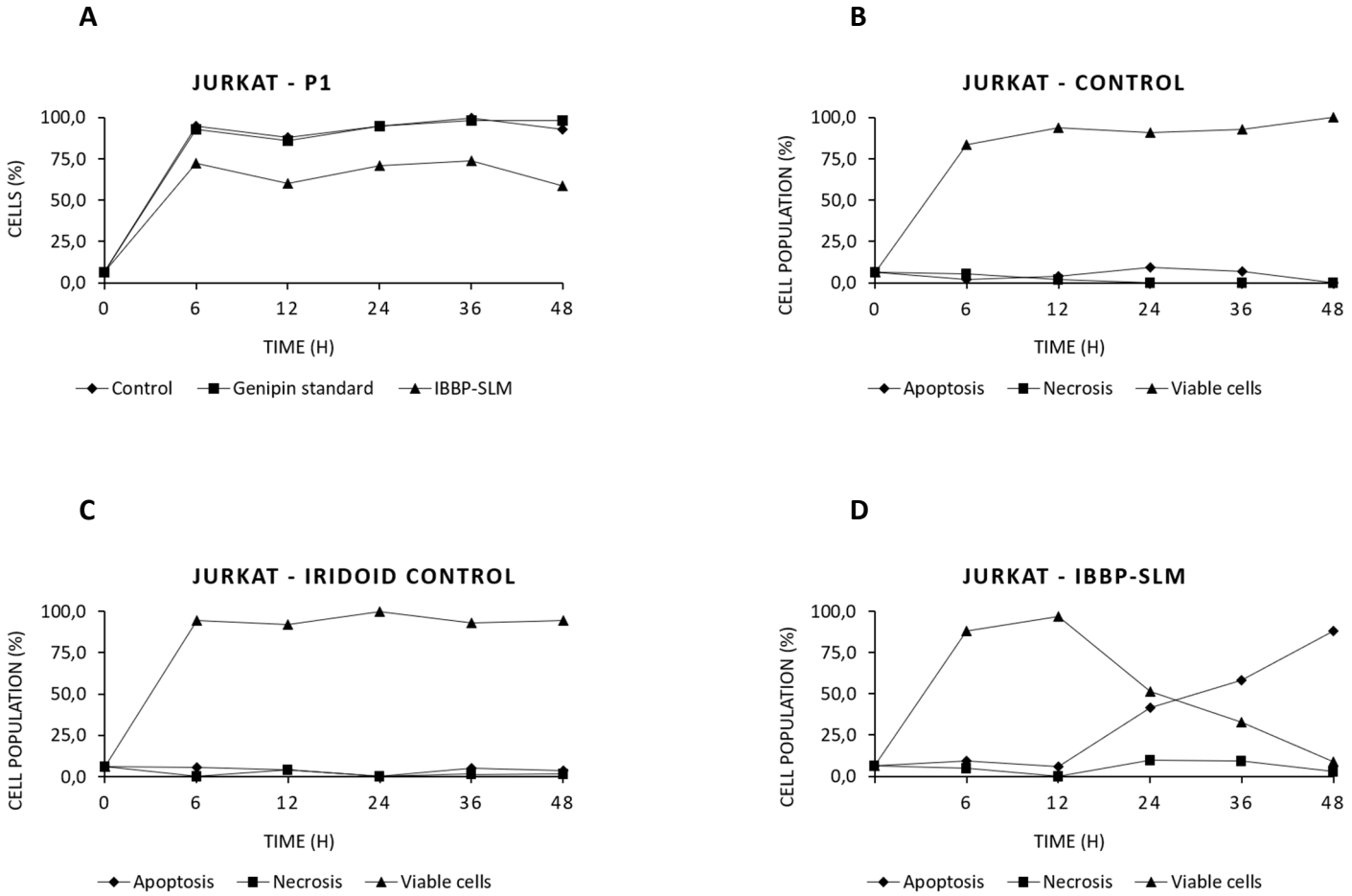

Figure 9. Graphic representation of kinetic analysis of cell death induction based on phosphatidylserine exposure in leukemia cell lines evidencing the populations of viable, apoptotic and necrotic cells from P1 region. IBBP= iridoid blue-based pigments, **SLM=** solid lipid microparticle, **P1=** cells of interest, **K562** (chronic myelogenous human leukemia, **JURKAT=** human lymphoid T leukemia. Results were obtained from a single experiment.

It was observed that IBBP-SLM (25 μg/mL) reached the maximum PS exposure at 12 h for HL60 (62.1% of Annexin V/PI positive cells) while the IBBP-SLM induced 42.9 and 41.5% of PS exposition on the NALM6 and JURKAT cell lines in 24 h and 28.9%o NB4 cell lines in 48 h, when compared to untreated cells (0%), respectivelly.

Previously, Feng et al (2010) reported that genipin-treated K562 cells for 24h at 200 – 400 μmol/L induced apoptosis in 17.9 – 27.6% (of PI positive cells), respectivelly. In addition, it was suggested that genipin induces apopstosis throught the activation of JNK and induction of the Fas ligand. Similarly, Cao et al (2010) reported that genipin-treated HeLa cells for 24h at 135 μg/mL for 24h resulted in a significant increase in the apoptosis percentage (3.7 - 35.1%) by activation of the c-Jun NH_2- terminal kinase and p53 protein in HeLa cells.

Recently, Li et al (2018) reported that genipin promotes apoptosis in bladder cancer cells (T24 and 5637 cell lines) at 60 μM for 48h via inactivation of PI3k/Akt signaling, representing an increase in the percentage of 4.5±0.9 to 34.6±2.1% in T24 cells and from 3.9 ± 0.8 to 28.5 ± 2.4% in 5637 cells (of Annexin V-positive apoptotic cells). However, there are scant studies focusing on the protective effects of IBBP on leukemic cells and no studies about encapsulated iridoids from *G. americana*. Thus, for further studies is still necessary to test another lipid matrix aiming to improve the microparticle production as well as a comprehensive and comparative studies.

5. Conclusion

The results obtained indicate that the spray chilling technique for the production of lipid microparticles containing IBBP extract have some disadvantages when stearic acid is used. The SEM micrographs confirmed that SLM had relatively spherical profiles and the DSC indicates a good thermal stability in spite of the weak performance in protecting the IBBP from degradation. However, IBBP-SLM seems to exert desirable biological effects on human health especially on cell viability as well as on apoptosis induction (at 25 μg/mL dosage). We also emphasize that the metabolism of iridoid blue-based pigments from genipap is not completely understood, needing comprehensive and comparative studies. Thus, the next steps for our work will focus on the improvement of the encapsulation efficiency, varying the proportion of

the carrier to the active material as well as the use of other types of carriers. An evaluation of the metabolism and distribution of microparticles and their safety for food and pharmaceutical purposes will also be required. In addition, to the best of our knowledge, this report describes the first time that IBBP-SLM extract from genipap has been encapsulated and submitted to *in vitro* digestion and biological assays.

Acknowledgments

The authors thank the São Paulo Research Foundation (FAPESP, grant number 2015/50333-1), National Council for Scientific and Technological Development (CNPq, grant number 481670/2013-0) and Institute of International Education (CAPES) for financial support and the Rupert Lyons for the language services provided. C. F. F. A. acknowledges FAPESP (studentships 2017/10753-7)

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Publication

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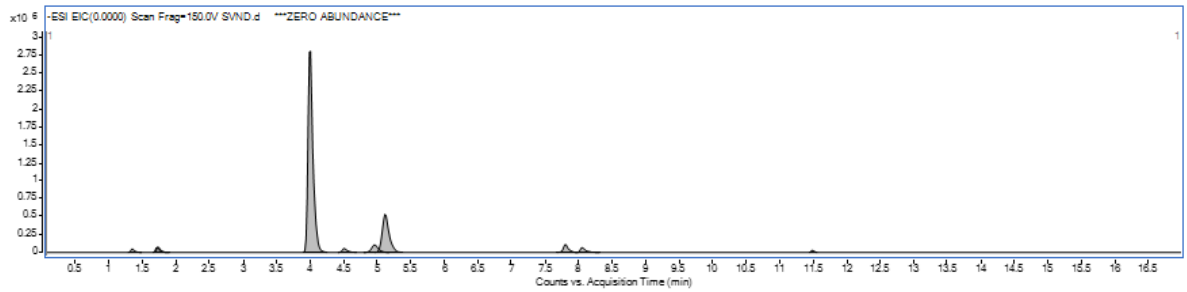
Material Supplementary

S1. Concentration of geniposidic acid, geniposide and genipin present in IBBP extract and superficial and entrapped iridoids from microparticle

Compound	IBBP extract	IBBP-SLM$_s$	IBBP-SLM$_e$	IBBP-SLM$_{s+e}$
Geniposidic acid	251.66 ± 7.49	n.d	38.87 ± 1.49	38.87 ± 1.49
Geniposide	88.62 ± 2.78	14.33 ± 0.10	n.d	14.33 ± 0.10
Genipin	3188.13 ± 107.47	239.58 ± 3.52	367.31 ± 15.66	606.89 ± 19.19

*Results expressed in mg/g dry weight, n.d.: not determined (values below LOQ). Data presented as mean ± standard deviation for the triplicate determination (*n= 3*). **IBBP-SLM$_s$=** iridoids content present in surface, **IBBP-SLM$_e$=** iridoids content entrapped, **IBBP-SLM$_{s+e}$=** total iridoids present in microparticle.

A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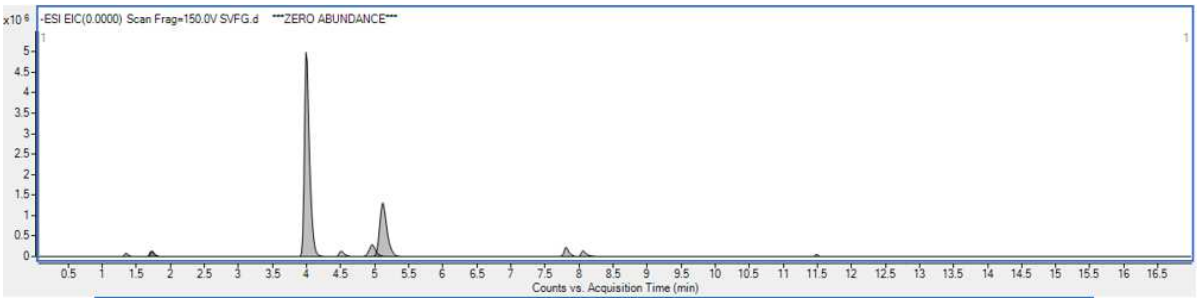
Counts vs. Acquisition Time (min)

B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Counts vs. Acquisition Time (min)

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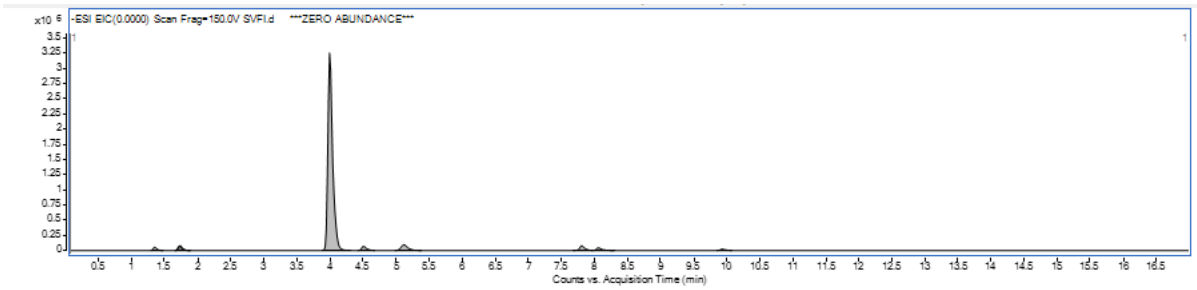
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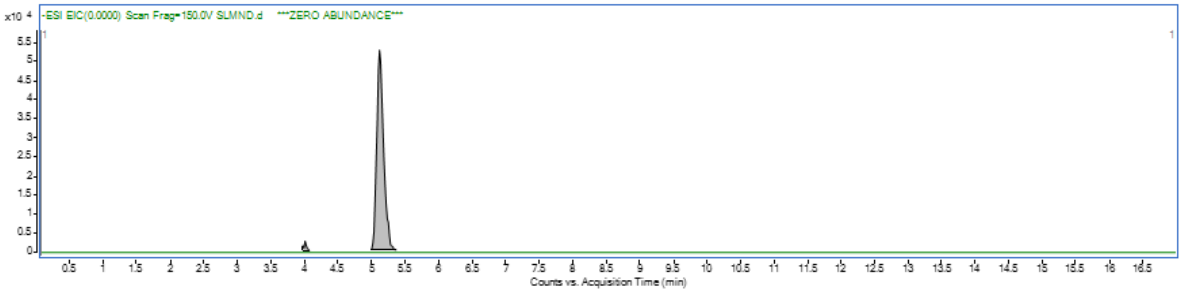
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Counts vs. Acquisition Time (min)

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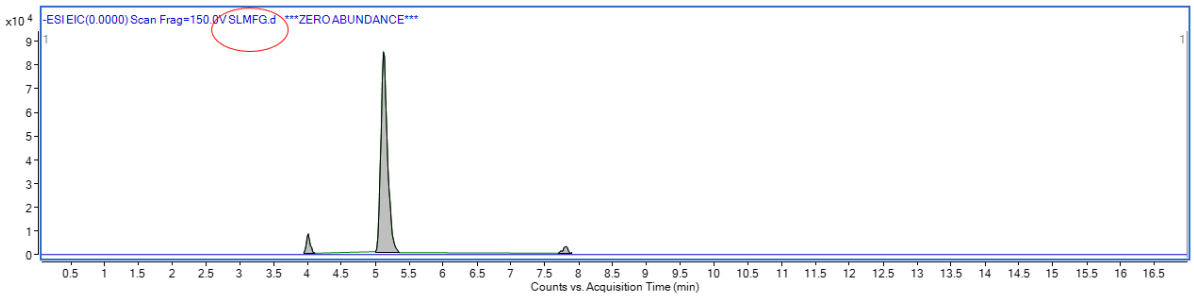

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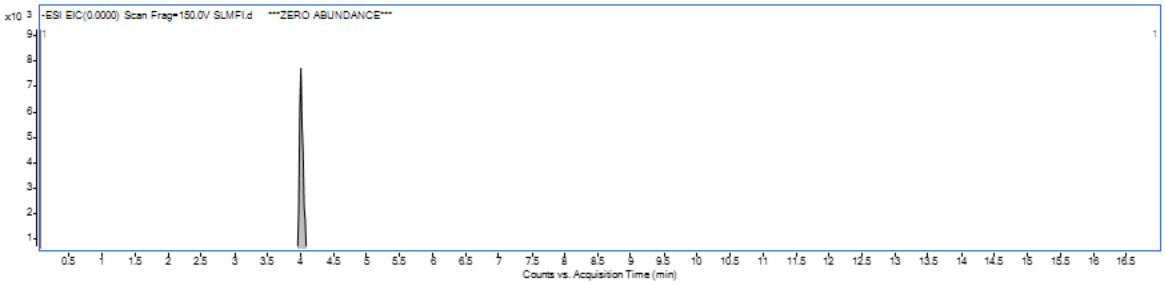
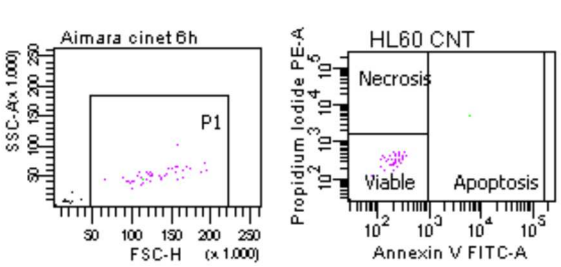


S2. Tentative identification and the amount of the compounds (in terms of absolute or relative area) present in non-digested (ND) and the digested fractions (GP and IP, respectively) of IBBP extract (A-C) and IBBP-SLM (D-F). These fractions were analysed in negative ion mode using a C18 120 SB-Aq 2.7 µm reversed phase column (2.1x100 mm, Agilent) and a Q-ToF iFunnel 6550 mass spectrometer fitted with an electrospray ionization (ESI) source. Mobile phase A consisted of a 0.1% (v/v) solution of formic acid in water, and mobile phase B was a 0.1% (v/v) of formic acid solution in acetonitrile. The mass spectrometer parameters used were: VCap 3000 V, fragmentor voltage at 150 V, OCT 1RF Vpp at 750 V, Gas Temperature at 290oC, Sheath Gas Temperature at 350 °C, Drying Gas at 12 L/min-1. Mass spectra were acquired in profile mode and the acquisition range was 100 - 1500 m/z.

FACSDiva Version 6.1.3



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Population	#Events	%Parent
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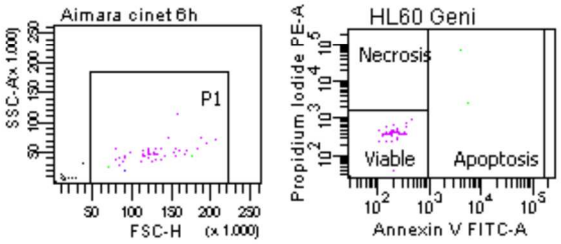
Apoptosis	1	2.3
Necrosis	0	0.0
Viable	42	97.7



Tube Name: HL60 Geni
Record Date: Sep 8, 2017 5:10:43 PM
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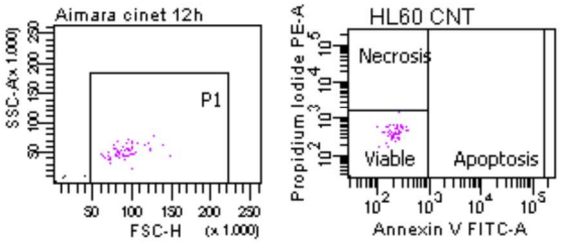
Apoptosis	2	4.4
Necrosis	1	2.2
Viable	42	93.3



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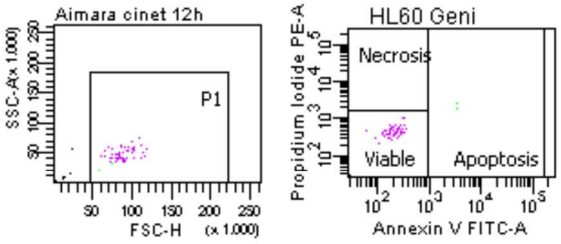
Viable	55	100.0



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Population	#Events	%Parent
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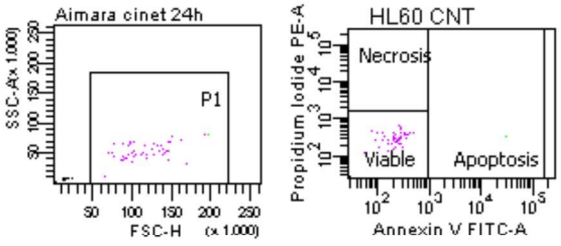
Apoptosis	2	3.9
Necrosis	0	0.0
Viable	49	96.1



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Population	#Events	%Parent
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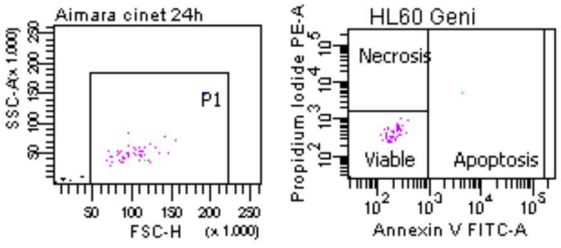
Apoptosis	1	2.1
Necrosis	0	0.0
Viable	46	97.9



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Population	#Events	%Parent
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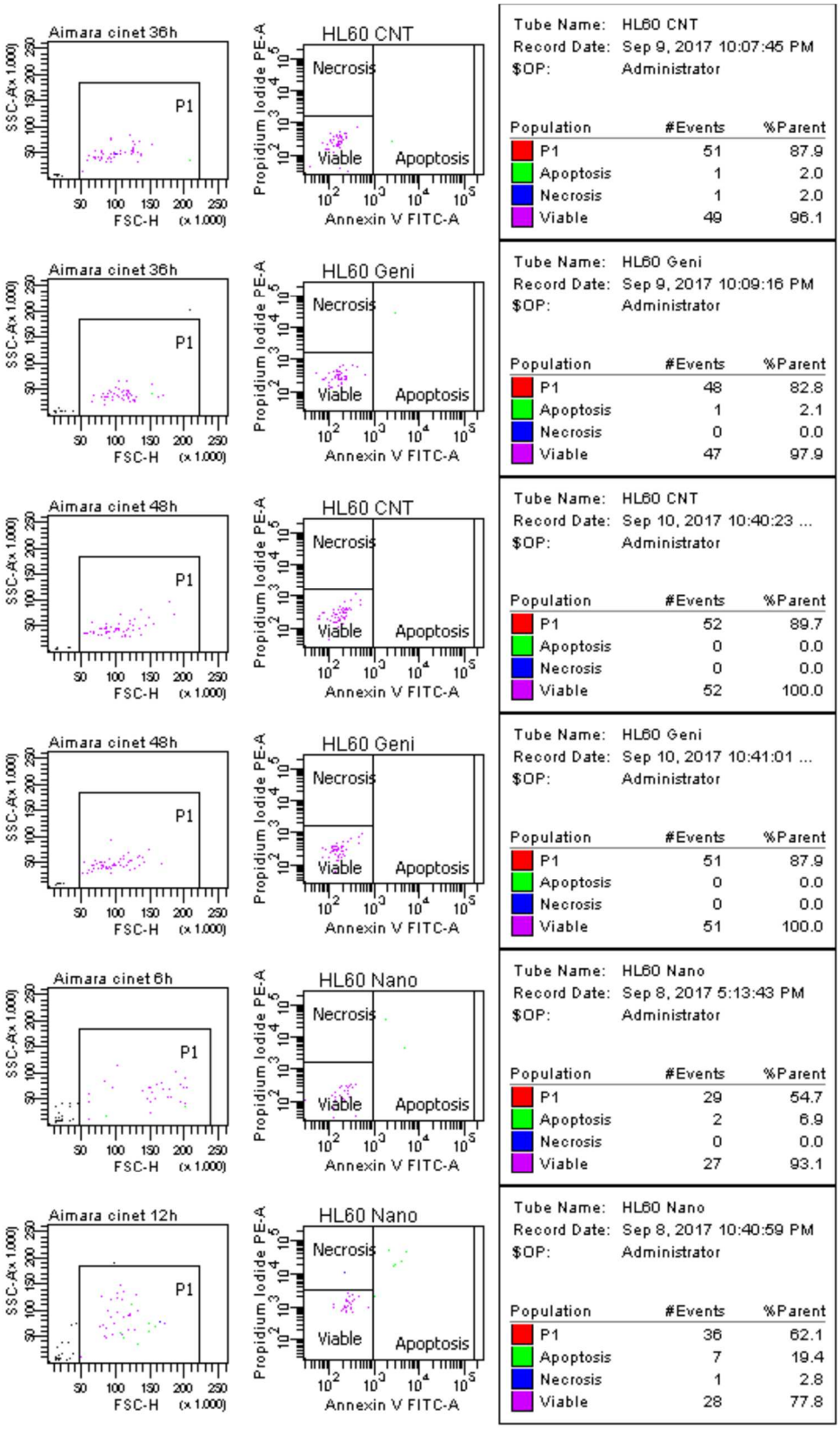
Apoptosis	1	2.1
Necrosis	0	0.0
Viable	46	97.9

FACSDiva Version 6.1.3



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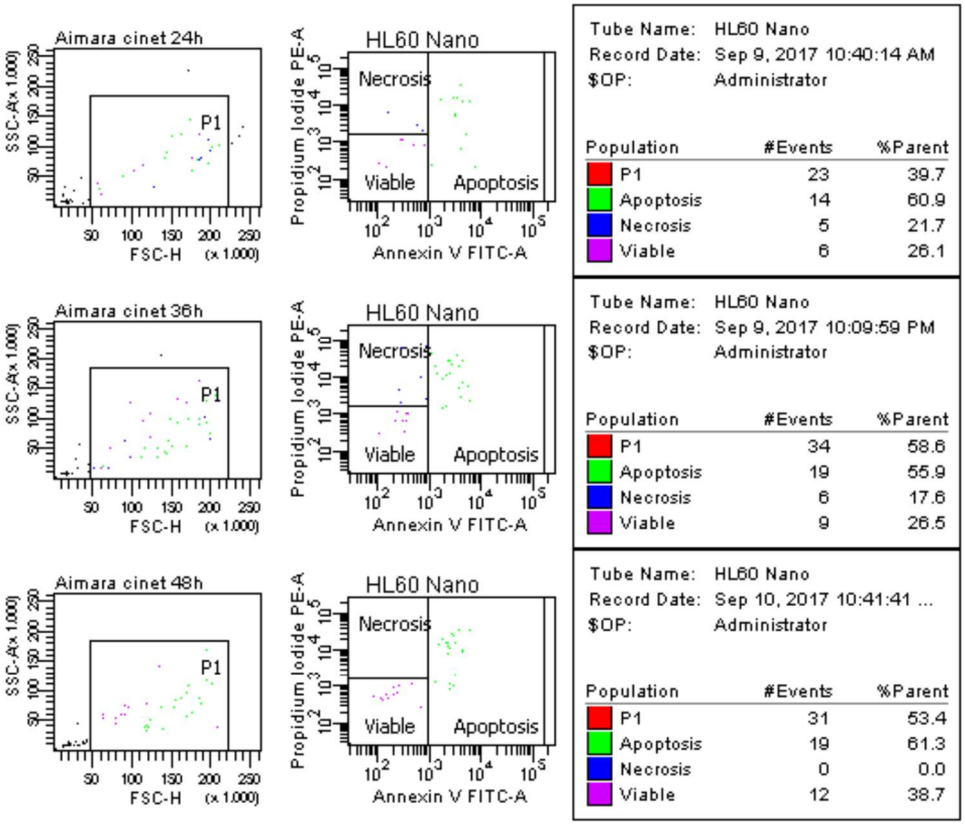

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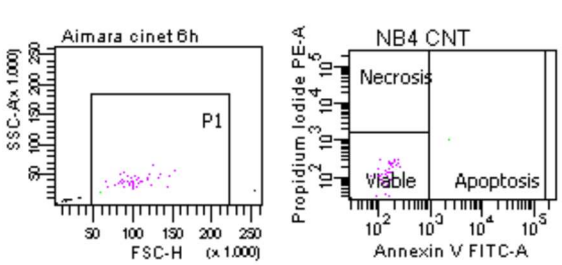

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Population	#Events	%Parent
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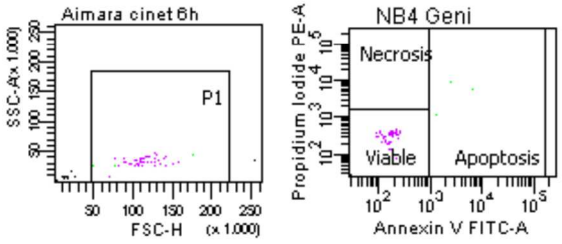
Apoptosis	1	2.4
Necrosis	0	0.0
Viable	40	97.6



Tube Name: NB4 Geni
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Population	#Events	%Parent
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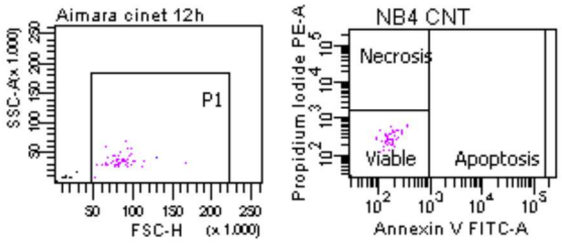
Apoptosis	3	6.5
Necrosis	0	0.0
Viable	43	93.5



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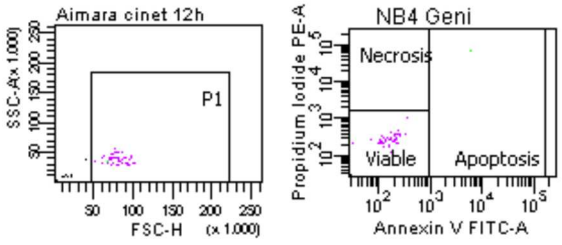
Apoptosis	0	0.0
Necrosis	1	2.0
Viable	50	98.0



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Population	#Events	%Parent
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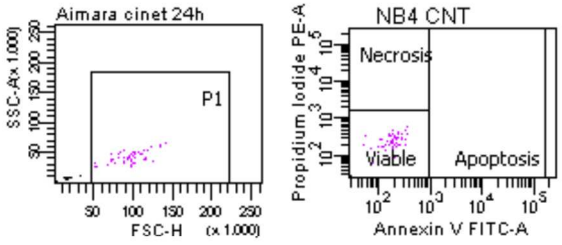
Apoptosis	1	2.0
Necrosis	0	0.0
Viable	49	98.0



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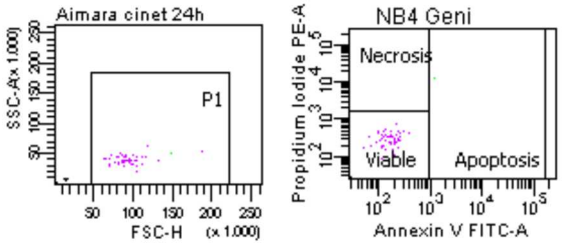
Necrosis	0	0.0
Viable	49	100.0



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Population	#Events	%Parent
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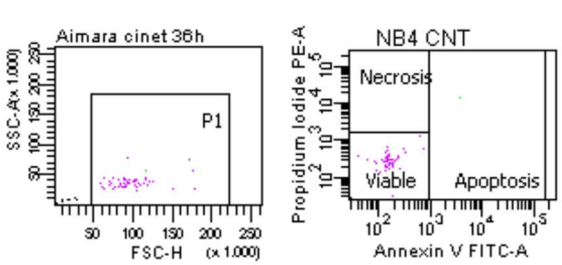
Apoptosis	1	2.0
Necrosis	0	0.0
Viable	50	98.0

FACSDiva Version 6.1.3



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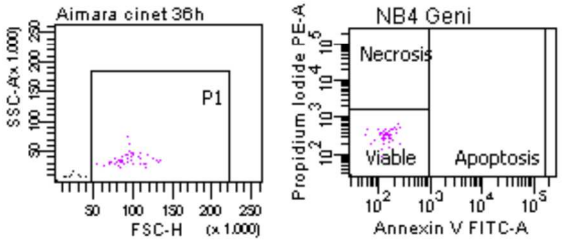
Population	#Events	%Parent
P1	51	87.9
Apoptosis	1	2.0
Necrosis	0	0.0
Viable	50	98.0



Tube Name: NB4 Geni
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Population	#Events	%Parent
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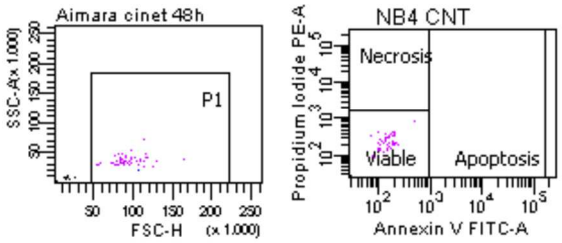
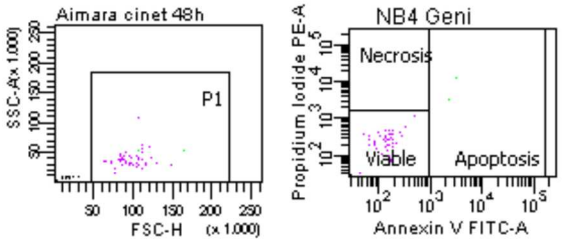
Apoptosis	0	0.0
Necrosis	0	0.0
Viable	47	100.0



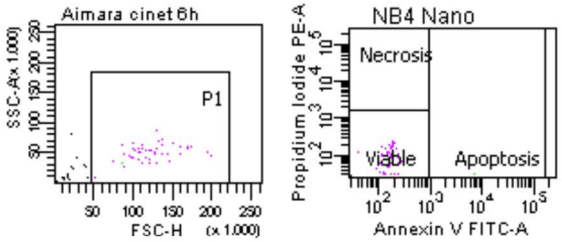
Tube Name: NB4 CNT
Record Date: Sep 10, 2017 10:42:42 ...
$OP: Administrator

Population	#Events	%Parent
P1	51	87.9
Apoptosis	1	2.0
Necrosis	1	2.0
Viable	50	98.0



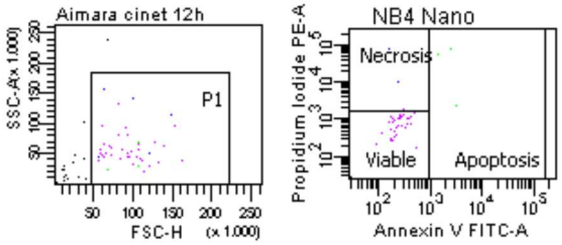
Tube Name: NB4 Geni
Record Date: Sep 10, 2017 10:43:17 ...
$OP: Administrator

Population	#Events	%Parent
P1	46	79.3
Apoptosis	2	4.3
Necrosis	0	0.0
Viable	44	95.7



Tube Name: NB4 Nano
Record Date: Sep 8, 2017 5:16:13 PM
$OP: Administrator

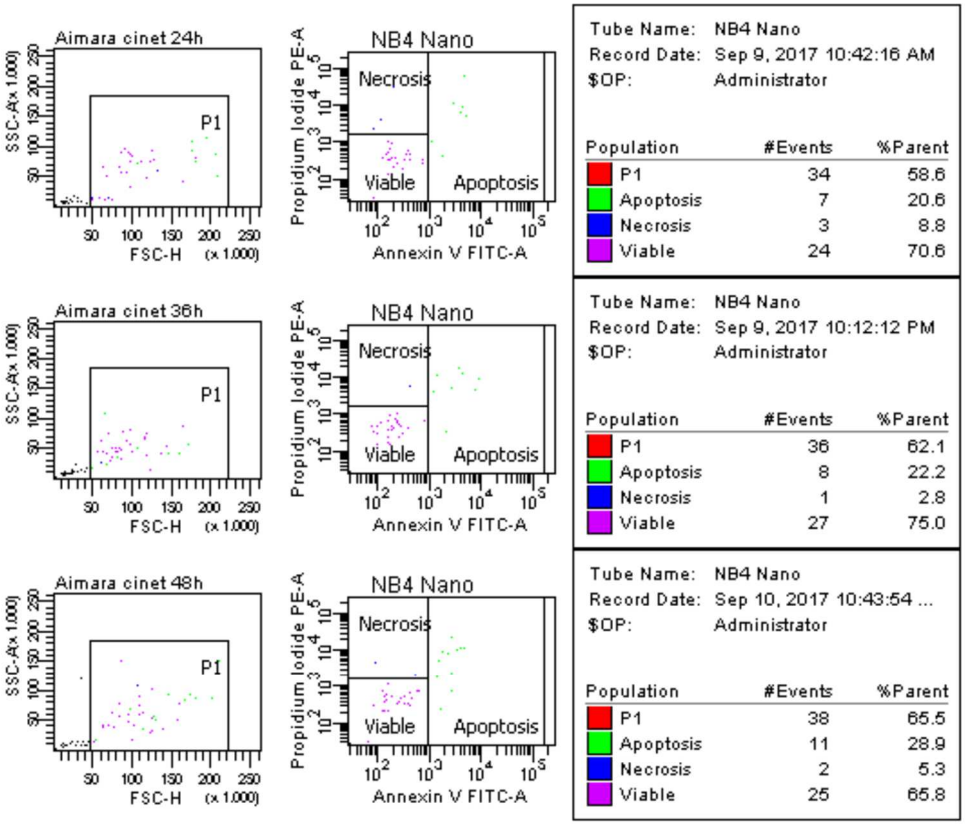
Population	#Events	%Parent
P1	44	75.9
Apoptosis	1	2.3
Necrosis	0	0.0
Viable	43	97.7



Tube Name: NB4 Nano
Record Date: Sep 8, 2017 10:44:07 PM
$OP: Administrator

Population	#Events	%Parent
P1	42	72.4
Apoptosis	3	7.1
Necrosis	5	11.9
Viable	35	83.3

FACSDiva Version 6.1.3



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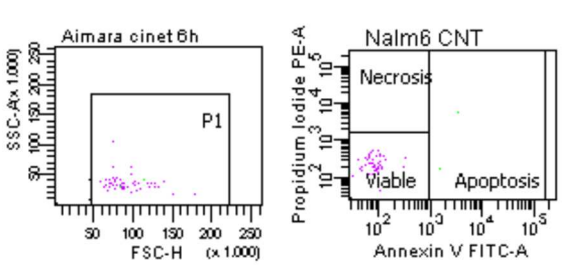
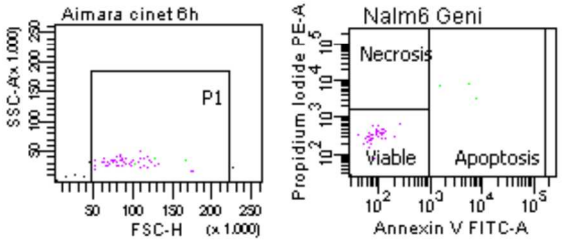


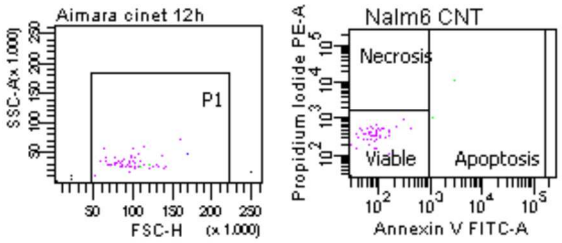
Tube Name: Nalm6 CNT
Record Date: Sep 8, 2017 5:26:45 PM
$OP: Administrator

Population	#Events	%Parent
P1	55	94.8
Apoptosis	2	3.6
Necrosis	0	0.0
Viable	53	96.4



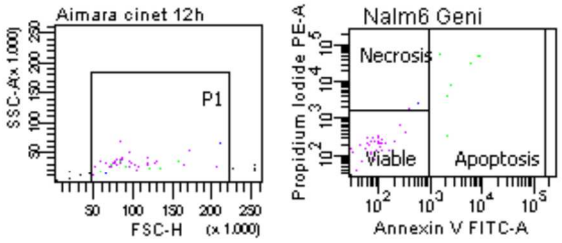
Tube Name: Nalm6 Geni
Record Date: Sep 8, 2017 5:28:20 PM
$OP: Administrator

Population	#Events	%Parent
P1	53	91.4
Apoptosis	3	5.7
Necrosis	0	0.0
Viable	50	94.3



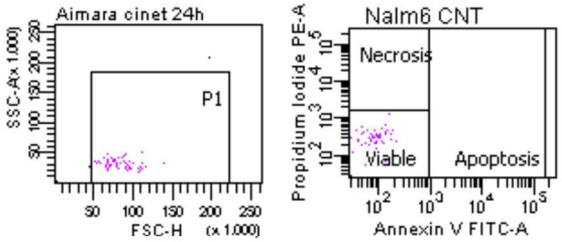
Tube Name: Nalm6 CNT
Record Date: Sep 8, 2017 10:52:14 PM
$OP: Administrator

Population	#Events	%Parent
P1	55	94.8
Apoptosis	2	3.6
Necrosis	1	1.8
Viable	52	94.5



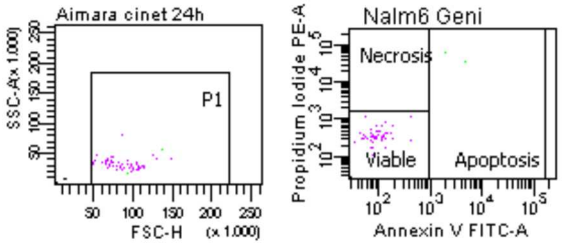
Tube Name: Nalm6 Geni
Record Date: Sep 8, 2017 10:53:01 PM
$OP: Administrator

Population	#Events	%Parent
P1	46	79.3
Apoptosis	7	15.2
Necrosis	2	4.3
Viable	38	82.6



Tube Name: Nalm6 CNT
Record Date: Sep 9, 2017 10:48:00 AM
$OP: Administrator

Population	#Events	%Parent
P1	56	96.6
Apoptosis	0	0.0
Necrosis	0	0.0
Viable	56	100.0



Tube Name: Nalm6 Geni
Record Date: Sep 9, 2017 10:48:34 AM
$OP: Administrator

Population	#Events	%Parent
P1	56	96.6
Apoptosis	2	3.6
Necrosis	0	0.0
Viable	54	96.4

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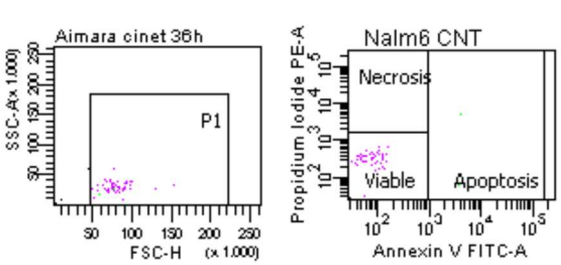

Tube Name: Nalm6 CNT
Record Date: Sep 9, 2017 10:17:59 PM
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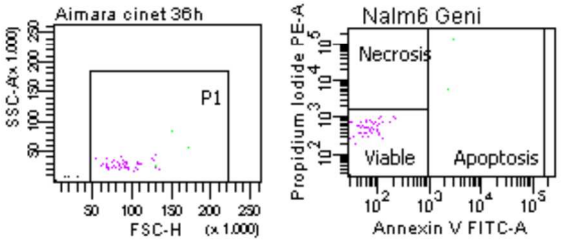
Population	#Events	%Parent
P1	56	96.6
Apoptosis	2	3.6
Necrosis	0	0.0
Viable	54	96.4



Tube Name: Nalm6 Geni
Record Date: Sep 9, 2017 10:19:26 PM
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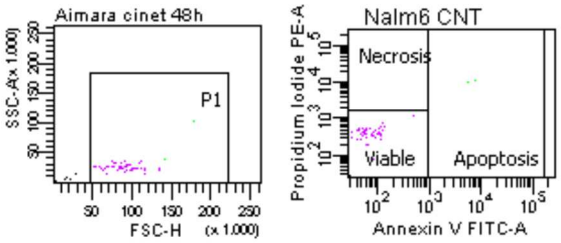
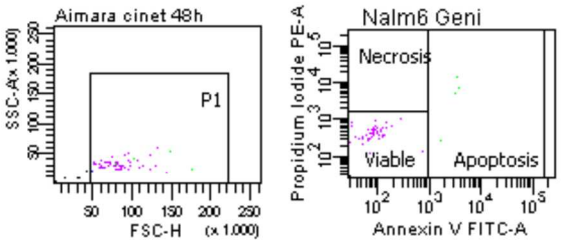
Population	#Events	%Parent
P1	55	94.8
Apoptosis	3	5.5
Necrosis	0	0.0
Viable	52	94.5



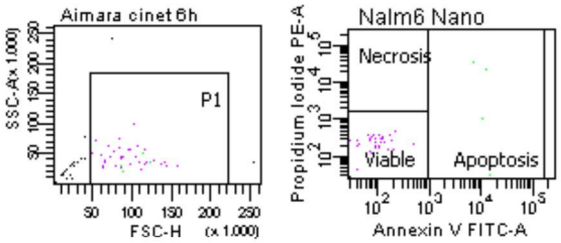
Tube Name: Nalm6 CNT
Record Date: Sep 10, 2017 10:50:15 ...
$OP: Administrator

Population	#Events	%Parent
P1	52	89.7
Apoptosis	2	3.8
Necrosis	0	0.0
Viable	50	96.2



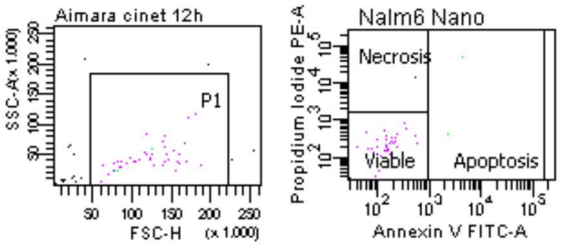
Tube Name: Nalm6 Geni
Record Date: Sep 10, 2017 10:50:58 ...
$OP: Administrator

Population	#Events	%Parent
P1	54	93.1
Apoptosis	4	7.4
Necrosis	1	1.9
Viable	49	90.7



Tube Name: Nalm6 Nano
Record Date: Sep 8, 2017 5:29:26 PM
$OP: Administrator

Population	#Events	%Parent
P1	40	69.0
Apoptosis	4	10.0
Necrosis	0	0.0
Viable	36	90.0



Tube Name: Nalm6 Nano
Record Date: Sep 8, 2017 10:53:46 PM
$OP: Administrator

Population	#Events	%Parent
P1	40	69.0
Apoptosis	2	5.0
Necrosis	1	2.5
Viable	37	92.5

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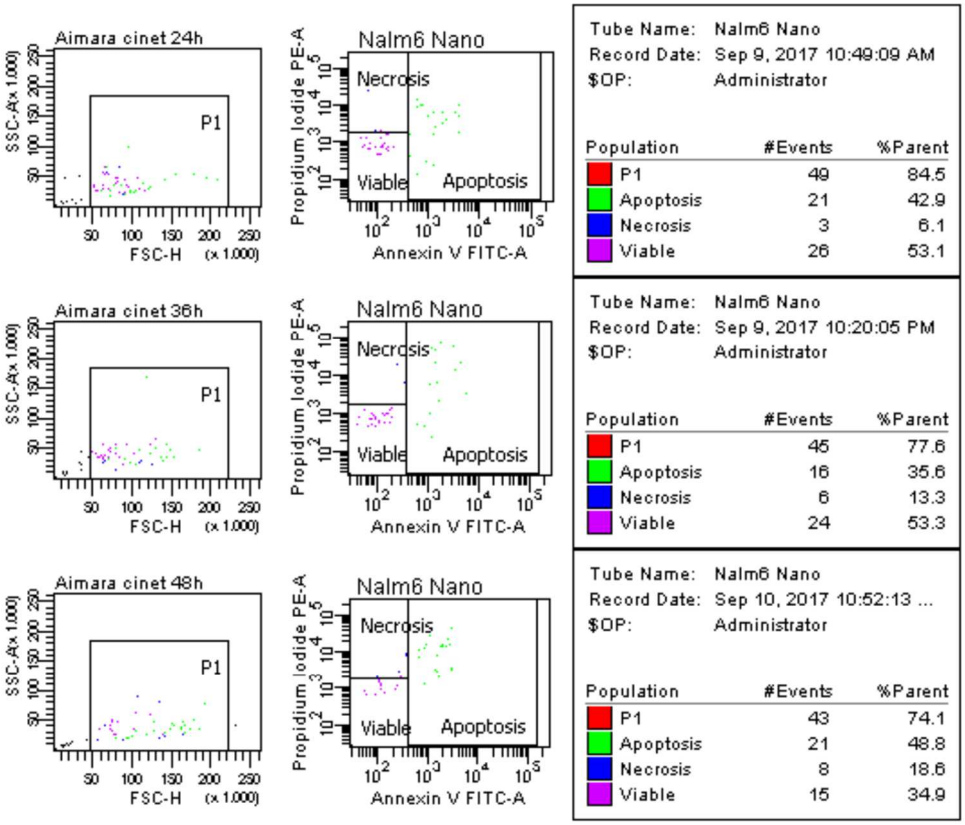
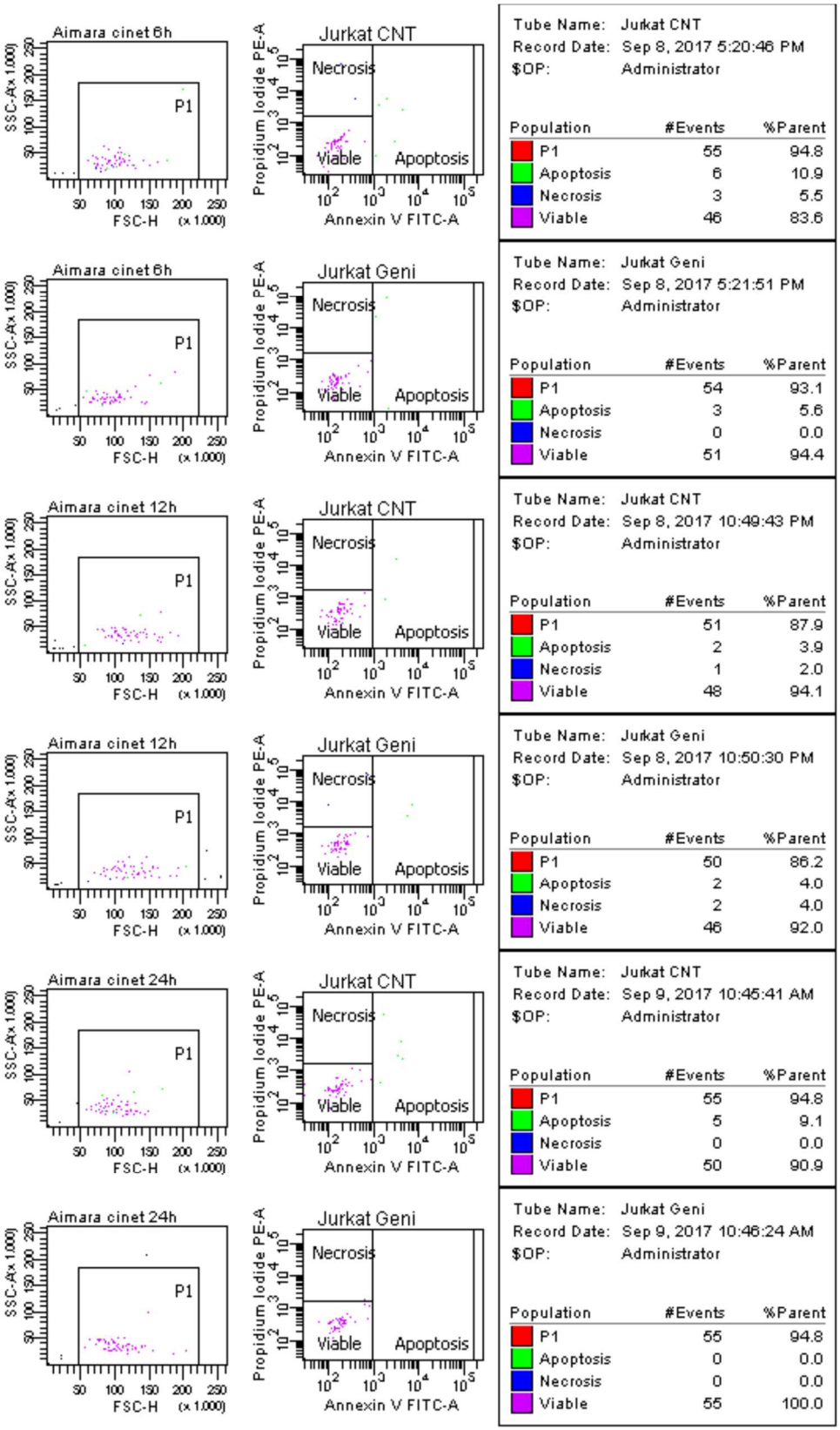
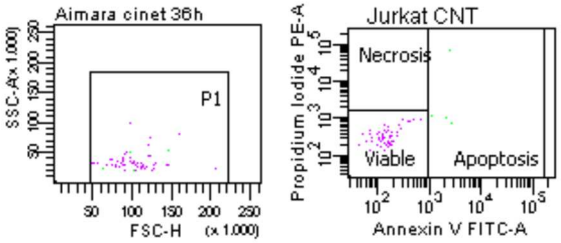


FACSDiva Version 6.1.3

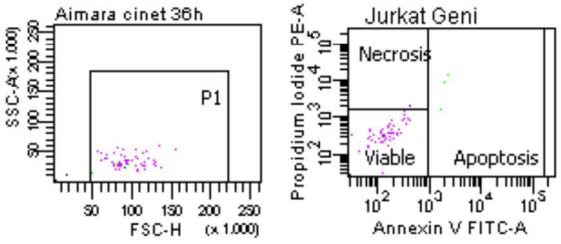


FACSDiva Version 6.1.3



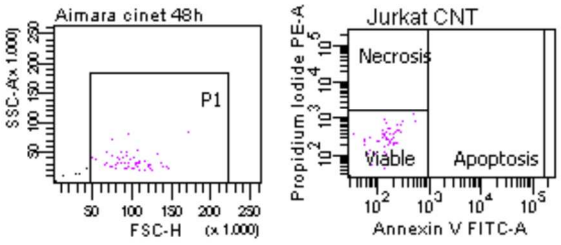
Tube Name: Jurkat CNT
Record Date: Sep 9, 2017 10:15:34 PM
$OP: Administrator

Population	#Events	%Parent
P1	58	100.0
Apoptosis	4	6.9
Necrosis	0	0.0
Viable	54	93.1



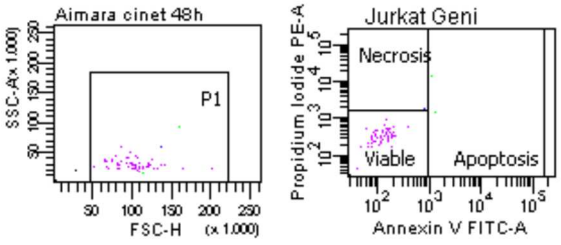
Tube Name: Jurkat Geni
Record Date: Sep 9, 2017 10:16:19 PM
$OP: Administrator

Population	#Events	%Parent
P1	57	98.3
Apoptosis	3	5.3
Necrosis	1	1.8
Viable	53	93.0



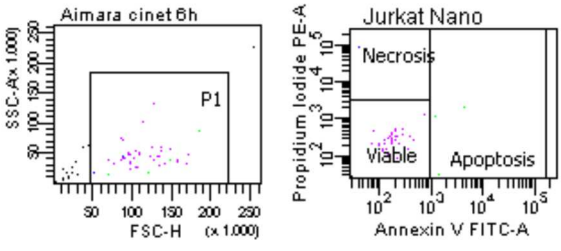
Tube Name: Jurkat CNT
Record Date: Sep 10, 2017 10:47:31 ...
$OP: Administrator

Population	#Events	%Parent
P1	54	93.1
Apoptosis	0	0.0
Necrosis	0	0.0
Viable	54	100.0



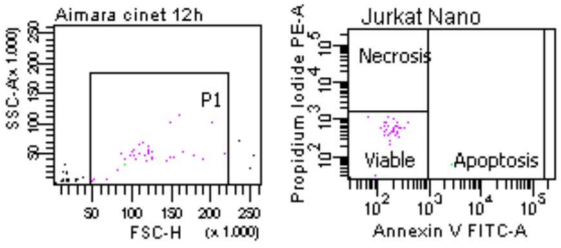
Tube Name: Jurkat Geni
Record Date: Sep 10, 2017 10:48:13 ...
$OP: Administrator

Population	#Events	%Parent
P1	57	98.3
Apoptosis	2	3.5
Necrosis	1	1.8
Viable	54	94.7



Tube Name: Jurkat Nano
Record Date: Sep 8, 2017 5:23:47 PM
$OP: Administrator

Population	#Events	%Parent
P1	42	72.4
Apoptosis	4	9.5
Necrosis	2	4.8
Viable	37	88.1



Tube Name: Jurkat Nano
Record Date: Sep 8, 2017 10:51:12 PM
$OP: Administrator

Population	#Events	%Parent
P1	35	60.3
Apoptosis	1	2.9
Necrosis	0	0.0
Viable	34	97.1

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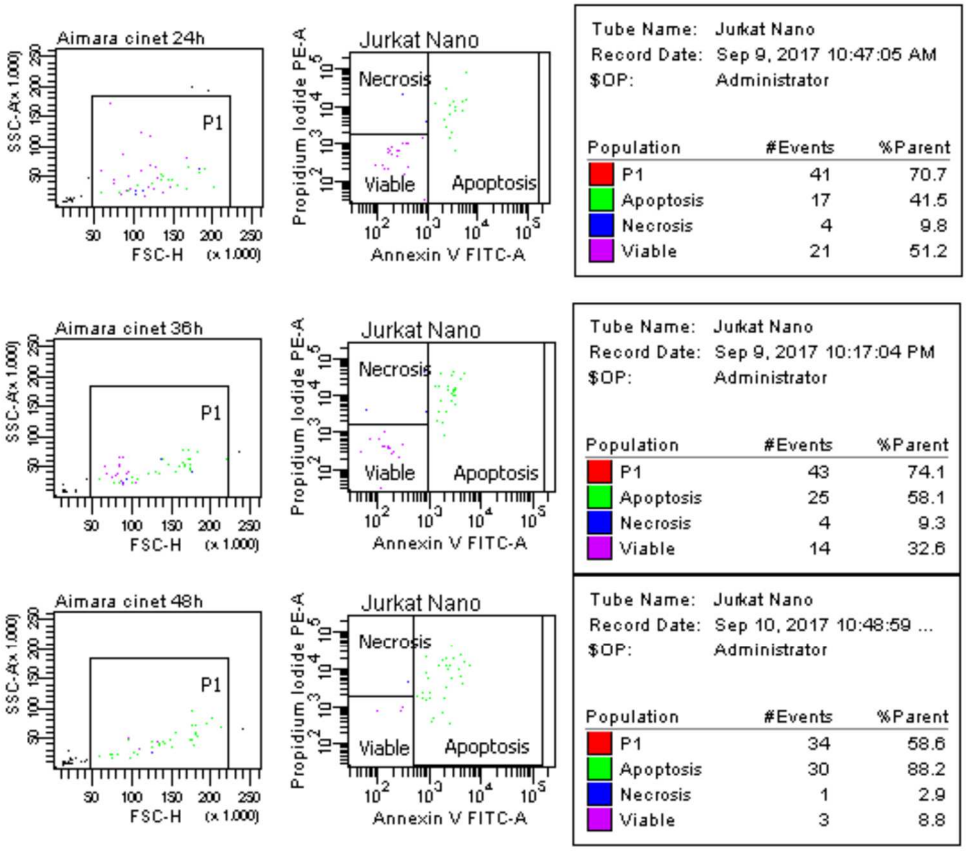

DISCUSSÃO GERAL

Este estudo visou avaliar a propriedades funcionais do iridóides presentes no corante azul extraído do jenipapo (*Genipa americana* L.), assim como na micropartícula lipídica produzida a partir deste extrato.

Inicialmente foi feita uma extensiva revisão de literatura apresentando os recentes avanços e aplicações da genipina (principal iridóide presente gardênia e no jenipapo) para fins terapêuticos, farmacêuticos e industriais. Foi possível constatar a existência de milhares de reivindicações de patente, cujos domínios principais estão alocados nos campos da tecnologia médica, biotecnologia, farmacêutico, química de materiais básicos, química de macromoléculas e polímeros. Mas, apesar de se tratar de uma alternativa natural aos corantes azuis sintéticos, considerada *"eco-friendly"* e detentora de diversas propriedades terapêuticas, a genipina e o corante azul proveniente da sua oxidação apresentam algumas desvantagens com relação a sua estabilidade e toxicidade. Além disso, a maioria dos requerimentos de patente e trabalhos encontrados na literatura, referem-se à genipina obtida dos frutos da gardênia, sendo o jenipapo sub explorado. Assim, há uma demanda por estudos que foquem não somente nos processos de extração e purificação da genipina obtida dos frutos do jenipapeiro, mas também em trabalhos que abordem seus efeitos biológicos, especialmente os de atividade anticâncer.

Na sequência, o trabalho prático visou preliminarmente determinar o estágio de maturação das frutas bem como identificar e caracterizar os constituintes químicos presentes nos frutos do jenipapeiro. Assim, as diferenças entre frutos maduros e verdes foram determinadas com base em parâmetros instrumentais de cor, firmeza, características físico-químicas e análise de açúcares por cromatografia de troca iônica. Como esperado, o peso e o diâmetro do fruto maduro foram maiores do que os frutos verdes, enquanto a textura diminuiu após o amadurecimento, sendo que a coloração da casca e polpa sofreram alteração durante o amadurecimento. De acordo com a coordenada de cromaticidade b*, a casca e a polpa dos frutos maduros tendem mais para a coloração amarela do que os frutos verdes. Observou-se também que os teores de sólidos totais, acidez titulável e açúcares totais aumentaram significativamente após o amadurecimento, enquanto a relação SST/AT mostrou um ligeiro aumento.

Com relação aos compostos de interesse presentes no endocarpo do jenipapo, dez compostos foram separados por UHPLC-ESI-MS/MS, sendo cinco destes encontrados na fruta verde e seis na fruta madura. Dentre estes, a genipina, a genipina 1-β-gentiobiosídeo (presente tanto no endocarpo verde quanto no maduro), o geniposídeo, e o ácido geniposidico foram confirmados com padrões de referência, enquanto o ácido geniposidico o 6''-O-*p*-coumaroil-1-β-gentiobiosídeo, o 6''-O-*p*-coumaroilgenipina-gentiobiosídeo, o ácido geniposidico 6'-O-p-coumaroil e o ácido geniposidico 6'-O-feruloil foram tentativamente identificados considerando suas massas exatas e padrão de fragmentação. Estes resultados estão de acordo com Ono et al (2005), pioneiros no isolamento e elucidação estrutural de monoterpenóides de *G. americana*. A genipina livre foi encontrada apenas em endocarpo da fruta verde.

Além disso, também foi observado que a quantidade de genipina-1-β-D-gentiobiosídeo no extrato do endocarpo da fruta madura foi 3,4 vezes maior do que no extrato do endocarpo da fruta verde. Por outro lado, o geniposídeo e o ácido geniposídico foram encontrados apenas no endocarpo da fruta madura. Contrariamente, Bentes & Mercadante (2014) relataram que o geniposídeo e o ácido geniposídico foram os compostos majoritários encontrados na fruta verde, enquanto a genipina-1-β-D-gentiobiosídeo restringiu-se somente ao endocarpo da fruta madura. No entanto, essa discrepância foi atribuída ao lote de frutos, onde provavelmente o "ponto de viragem" do amadurecimento biológico identificável na fruta no momento da extração pode ter influenciado no resultado observado.

Para entender como os principais parâmetros do processo de estocagem podem afetar a estabilidade de cor ou o conteúdo de genipina presente no extrato rico em iridóides (IBBP), avaliou-se as variáveis pH e temperatura de incubação utilizando um delineamento composto central. Foi constatado que a diferença de coloração do extrato pode aumentar até o 21º dia de vida de prateleira e depois se estabiliza. Verificou-se também que as diferenças de coloração assim como os a perda do conteúdo de genipina podem ser minimizadas utilizando condições de armazenamento com pH e temperatura baixos. Os resultados obtidos foram similares aos encontrados para corantes azuis de gardênia reportado por Fujikawa et al (1996). Além disso, alguns estudos mencionaram que o pH ideal (4,0 ~ 6,0) para a produção de pigmento é semelhante ao pH ideal para manter a estabilidade da cor (Cho et al 2006,

Bentes et al., 2015; Wu & Horn, 2017). Com relação ao conteúdo de genipina, notou-se maior estabilidade em pHs menores que 5 e temperaturas inferiores a 25 ^{o}C, enquanto que em pHs maiores de 6 e temperaturas mais altas, a degradação de genipina é maximizada.

Em relação à caracterização dos compostos por UHPLC-ESI-MS/MS e ao perfil antioxidante do extrato IBBP antes e depois da digestão *in vitro*, foi possível separar e identificar dez iridóides, sendo a genipina e genipina 1-β-gentiobiosídeo as substâncias majoritárias do extrato, cuja confirmação foi realizada utilizando padrões de referência.

Após o emprego da digestão *in vitro*, o conteúdo de genipina foi reduzido até níveis abaixo do limite de quantificação, enquanto a quantidade relativa de genipina 1-β-gentiobiosídeo aumentou 49% na digesta. Provavelmente a redução no teor de genipina se deve a instabilidade deste composto em valores de pH neutros, como os encontrados na fase intestinal da digestão. No entanto, alguns estudos relataram que a genipina é mais estável em pH alcalino do que em pHs neutros ou ácidos, permanecendo estável mesmo após altas temperaturas (Lee et al., 2003; Wu & Horn, 2017). Assim, neste estudo foi demonstrado que a estabilidade da genipina em função do pH não apresenta um comportamento linear. Portanto, dependendo da combinação de pH e temperatura, a genipina pode ser bastante instável.

Além disso, foi observado que algumas formas glicosídicas (gardenosídeo e geniposídeo) e ésteres glicosilados do ácido geniposídico diminuíram durante a fase intestinal da digestão. Estudos anteriores também relataram a associação da hidrólise das ligações éster e glicosídica utilizando enzimas intestinais com a diminuição de alguns compostos fenólicos como flavonóis e conteúdos de antocianinas (Sangiovanni et al., 2015, Cassani et al., 2018). O mesmo acontece com os compostos fenólicos, que são sensíveis às condições alcalinas do intestino delgado (Wang et al., 2017).

Quanto à capacidade antioxidante, pode-se observar um aumento de 17% e 18% após a fase gástrica e intestinal, respectivamente. Este comportamento também tem sido relatado para diversos polifenóis devido à liberação da forma aglicona, que geralmente é um antioxidante mais eficiente (Bohn et al., 2015; Tagliazucchi et al., 2010; Pino-García). No entanto, não foi possível verificar um aumento nas formas agliconas em nosso estudo,

sugerindo que a maior capacidade antioxidante da fase intestinal pode estar relacionada aos produtos desconhecidos formados durante a digestão *in vitro*, podendo apresentar diferentes propriedades químicas e consequentemente, bioacessibilidade, biodisponibilidade e efeitos biológicos diferentes (Wang et al., 2017).

Atualmente, a maioria dos estudos sobre avaliação dos potenciais riscos dos corantes naturais azuis a base de iridóides, referem-se aos extratos obtidos da *Gardenia jasminoides* J. Ellis, outro representante da família Rubiaceae que é amplamente utilizado na medicina tradicional chinesa (Sohn et al., 2017). No entanto, os estudos sobre os fatores preditivos de toxicidade do jenipapo, do ponto de vista citotóxico, genotóxico e mutagênico são praticamente inexistentes. Assim, uma das abordagens de potencial biológico do trabalho foi verificar se o extrato IBBP na concentração testada (25 µg/mL), poderia exercer algum papel antiproliferativo ou ser capaz induzir danos ao DNA na sua forma original ou se precisa ser ativada/metabolizada para se tornar mutagênica.

Dentre as linhagens tumorais testadas, verificou-se que o extrato IBBP foi capaz de inibir (IC_{50}) o crescimento das linhagens de glioma (U251) e câncer de mama (MCF-7), respectivamente. Previamente, Chen et al., 2017 demonstrou que uma formulação a base do extrato de *G. jasminoides* (1,03% de genipina gentiobiosídeo, 5,90% de gardenosídeo, 1,26% de crocina 1 e 0,17% de crocina 2) exerceu forte efeito inibitório em células HepG2 (80,2% de inibição a 400 µg/mL). Da mesma forma, Zuo et al (2017) e Ko et al (2015) demonstraram que a genipina a 100 µM é capaz de inibir o crescimento de células da granulosa humana com perfil esteroidogênico e do carcinoma gástrico, respectivamente. Por fim, Huang et al (2017) observaram que o genipisídeo (50-100 µM) pode aumentar a eficácia de DOX contra xenoenxertos derivados de células de câncer MG63/DOX em camundongos pelados, indicando que este iridoide pode ser empregado em combinação com outros anti-neoplásicos convencionais para prevenir a resistência a múltiplos medicamentos.

Com relação ao potencial genotóxico, nenhum dano ao DNA foi detectado pelo ensaio cometa utilizando células CHO-K1 após 4 h de exposição ao extrato IBBP (25 µg/mL), quando comparado com as células não tratadas, ambos na ausência e presença de ativação metabólica ($S9^+$/$S9^-$). Portanto, sendo considerado não genotóxico na concentração de 25 µg/mL além de exercer efeito protetor contra danos no DNA em células CHO-K1.

Considerando que o extrato IBBP pode ser quimicamente instável, degradando-se rapidamente quando exposto ao ambiente externo (O_2, temperatura, luz, pH, etc.), o emprego de uma técnica de encapsulação mostrou-se uma opção adequada para superar tais desvantagens. Assim, foram produzidas micropartículas lipídicas sólidas a partir do extrato IBBP (IBBP-SLM) utilizando ácido esteárico através da técnica de *spray chilling*.

Devido à complexidade do trato gastrointestinal, o trabalho ainda visou desenvolver um estudo comparativo entre o extrato IBBP e a IBBP-SLM utilizando o modelo de digestão *in vitro* a fim de avaliar o comportamento das micropartículas após ingestão, bem como as taxas de liberação e biodisponibilidade dos iridóides-alvo. Neste enfoque, foram observadas as diferenças referentes ao perfil cromatográfico e capacidade antioxidante, bem como a determinação da capacidade de induzir apoptose pela exposição da fosfatidilserina (PS).

Em relação à produção e caracterização das IBBP-SLM, a microscopia eletrônica revelou formas esféricas com superfície lisa e formação de cristais de diferentes diâmetros na superfície das micropartículas. Foram observadas também aglomerações de partículas com partículas de fusão e partículas danificadas ocasionais, sugerindo fraca proteção do material presente no núcleo da micropartícula e também redução da funcionalidade do mesmo. Os resultados estão de acordo com Consoli et al (2016) que também mencionaram algumas imperfeições e rugas na superfície das micropartículas de ácido gálico produzidas por formulações de ácido esteárico, bem como a ocorrência de cristais e aglomerados de partículas como resultado de solidificação incompleta.

Já na análise do comportamento térmico, a curva DSC da IBBP-SLM apresentou dois picos claramente definidos: um pico endotérmico a 66^{o}C (676.69 mJ) e um pico exotérmico a 70^{o}C (677.02 mJ), correspondente aos pontos de fusão e cristalização, respectivamente. Estes resultados sugerem que o ponto de fusão SLM pode ser um gatilho para aplicações alimentares. Pois, dependendo do tipo de aplicação, a IBBP pode ser liberada com temperaturas acima de 66°C, significando um ponto positivo, porque o ativo só terá contato com o alimento se houver um aumento na temperatura. Nossos resultados estão de acordo com Feldman et al. (1989) que mencionaram que os ácidos graxos como ácido cáprico,

láurico, palmítico e esteárico são bons candidatos para o armazenamento de energia térmica de calor latente em aplicações de aquecimento.

Por outro lado, os resultados da eficiência total de encapsulação (TE) mostraram que, em geral, o processo de microencapsulação resultou em valor similar para todos os compostos analisados, com aproximadamente 15% de IBBP (ácido geniposídico, geniposídeo e genipina) incorporado na micropartícula (SLM). Sendo que todo o conteúdo do geniposídeo ficou retido na superfície da micropartícula. Provavelmente devido à composição do material da parede (ácido esteárico) que não tem afinidade com este composto, resultando na sua expulsão através da matriz lipídica. Em contraste, o ácido geniposídico apresentou maior afinidade com a matriz lipídica, haja vista que nenhuma quantidade deste composto foi encontrada na superfície da SLM. Assim, para estudos futuros envolvendo a relação entre IBBP-SLM e seus efeitos biológicos, será necessária reavaliar as condições de processo, considerando a concentração de iridóides e do tipo de material de parede empregado durante a produção de SLM.

Quanto aos efeitos da digestão nas amostras, observou-se que o conteúdo dos iridóides mudou durante o processo de digestão. Particularmente, os iridóides da IBBP-SLM se degradaram durante o processo de digestão, restando apenas a genipina 1-β-gentiobiosídeo. Esta perda do conteúdo dos iridóides pode estar relacionada a degradação dos compostos presentes na micropartícula. Outra explicação seria a instabilidade destes compostos em presença de soluções com valores de pH neutros ou alcalinos, ou devido a hidrólise das ligações éster e glicosídicas com enzimas intestinais na qual têm sido citadas por alguns autores que avaliaram a estabilidade da genipina (e suas formas de glicosídicas) durante o processo digestivo (Lee et al., 2003; Wu & Horn, 2017).

Ademais, a capacidade antioxidante das amostras digeridas e não digeridas (extrato de IBBP e IBBP-SLM) foi avaliada. Os resultados mostraram que o processo de microencapsulação influencia a capacidade antioxidante do IBBP-SLM. Apesar dos valores de ORAC do extrato IBBP e da IBBP-SLM serem significativamente maiores ($p < 0,05$) após a digestão (fase intestinal (IP)= 46% e 100%, respectivamente), notou-se que a liberação de iridóides presente no IBBP extrato (não digerido (ND) e IP com valores ORAC de 843.47 e 817.54 μM TE / g, respectivamente) foi maior do que nas frações IBBP-SLM.

Portanto, os valores obtidos estão relacionados às características das micropartículas (ex.: tamanho, concentração de iridóides encontrados na formulação, tipo de material de parede) e as condições de ensaio (ex.: soluções aquosas e temperatura), onde os iridóides retidos nas micropartículas produzidas com o ácido esteárico não estavam disponíveis para reagir com os agentes oxidantes antes do processo de digestão (ex.: fração ND sem capacidade antioxidante). Por outro lado, o processo de digestão favoreceu a liberação das formas de agliconas dos iridóides (ex.: genipina 1-β-gentibiosídeo) presentes na IBBP-SLM após a digestão (valor ORAC após a IP= 173,05 μM TE / g).

Além disso, o aumento da capacidade antioxidante da IP pode estar relacionado aos produtos desconhecidos formados durante a digestão *in vitro* ou aos produtos de degradação da aglicona. Portanto, esses compostos desconhecidos podem ser bons alvos para estudos futuros envolvendo a relação entre IBBP-SLM e biodisponibilidade, incluindo experimentos que utilizem modelos *in vivo* para elucidar seus mecanismos de ação nos metabolismos oxidativo e de proliferação celular.

Em relação aos estudos de atividade anticâncer, a IBBP-SLM apresentou uma forte atividade antiproliferativa, inibindo o crescimento da maioria das linhagens de células tumorais (IC_{50}= 0,97 – 43,5 5 μg / mL), com exceção das de glioma (U251) e tumor renal (786-O), onde foi inativa. Por outro lado, observou-se que o ácido esteárico em baixas concentrações (material de parede) também inibiu o crescimento de todas as linhagens (IC_{50}= 1,4 – 26,2 μg / mL) estudadas. Dessa forma, estes resultados somados aos da análise morfológica da SLM, TE e digestão *in vitro*, sugerem que o tipo de matriz lipídica (ácido esteárico) interferiu na qualidade do experimento, pois a alta rigidez da superfície das micropartículas não permitiu a completa dissolução da IBBP-SLM e a liberação do conteúdo de iridóides retido no interior da partícula sobre as condições de análise empregadas.

Alguns estudos relatam que a liberação do composto ativo da SLM pode ser influenciada pelo tipo de matriz lipídica, parâmetros de produção, bem como a solubilidade do bioativo no lipídio empregado como carreador ou devido ao coeficiente de partição (Wissing et al., 2004; Hu et al. 2005). Em relação à capacidade do extrato IBBP inibir o crescimento de células tumorais, tanto o geniposídeo quanto a genipina têm se mostrado promissores contra vários tipos de células tumorais (Habtemariam & Lentini, 2018). Em um

estudo anterior, Feng et al (2011) relataram que a genipina pode inibir a proliferação das células leucêmicas K562 com um valor de IC_{50}=250 µmol/L. Da mesma forma, Yang et al (2013) observaram que a genipina foi capaz de inibir a proliferação de uma linhagem de adenocarcinoma de pulmão (H1299) com IC_{50}=351,5µM. No entanto, Habtemariam & Lentini (2018) consideraram os efeitos antiproliferativos do geniposídeo e genipina como fracos, uma vez que a dose efetiva de ambos deveria ser superior a 100- 200µM. Portanto, percebe-se a necessidade de mais estudos para confirmar as propriedades farmacológicas dos iridóides. Finalmente, no ensaio para detecção da indução da apoptose pela exposição da fosfatidil serina, observou-se que a IBBP-SLM a (25 µg/mL) atingiu a exposição máxima de PS em 12 h para HL60 (62,1% de células positivas para Anexina V / PI), 24h para NALM6 e JURKAT (42,9 e 41,5%, respectivamente) e 48h para NB4 (28,9%) quando comparadas com células não tratadas (0%), respectivamente.

Recentemente Feng et al (2015) observaram que a genipina nas concentrações de 200 – 400 µmol/L foi capaz de inibir a proliferação das células leucêmicas K562 bem como induzir a apoptose (17,9 – 27,6% de células positivas para PI). Cao et al (2010) também observaram que células HeLa tratadas com genipina (135 µg/mL) por 24 horas resultam em um aumento significativo na porcentagem de apoptose (3,7 - 35,1%) devido a ativação da quinase NH2 terminal c-Jun e proteína p53. Da mesma forma, Li et al (2018), relataram que a genipina promove a apoptose em células de câncer de bexiga (T24 e 5637), quando expostas a uma concentração de 60 µM de genipina por 48h sendo inibidas via inativação da sinalização PI3k / Akt, significando um aumento na porcentagem de apoptose de 4,5 - 34,6% para células T24 e de 3,9 - 28,5% para células 5637. No entanto, estudos com foco nos efeitos protetores do extrato IBBP em células leucêmicas são ainda incipientes e não há nenhum estudo sobre iridóides encapsulados de *G. americana* L.

CONCLUSÃO GERAL

O presente trabalho de doutorado apresentou o desenvolvimento e validação de um método de análise cromatográfica de ultra eficiência para a separação e quantificação simultânea dos iridóides ácido geniposídico, geniposídeo e genipina presentes no mesocarpo e endocarpo de frutos verdes e maduros de jenipapo. Os resultados obtidos nos parâmetros de validação, como seletividade, limite de detecção, limite de quantificação, linearidade, precisão e exatidão mostram que o método pode ser empregado como uma ferramenta rápida para o *screening* de iridoides do gênero Genipa de interesse para os fabricantes de alimentos, cosméticos ou farmacêuticos.

O extrato IBBP mostrou ser mais estável em sistemas com pH e temperaturas baixas, demonstrando assim ser compatível em aplicações alimentares que utilizem pHs ácidos e que necessitem ser mantidos sob temperatura de refrigeração. Adicionalmente, esse extrato não apresentou efeito genotóxico e tampouco interferiu na proliferação de células não tumorais (CHO-K1 e HaCaT), quando utilizada a dosagem de 25 μg/mL, demonstrando seu papel protetor contra lesões no DNA.

Dessa forma, o extrato IBBP pode ser considerado uma opção segura de corante natural azul (a 25 μg/mL), além de ser de aumentar a funcionalidade dos produtos alimentares.

A técnica de *spray chilling* para a produção de micropartículas solidas lipídicas contendo extrato de IBBP possui algumas desvantagens quando o ácido esteárico é utilizado. As micrografias SEM confirmaram que o SLM tinha perfis relativamente esféricos e o DSC indica boa estabilidade térmica, apesar do fraco desempenho em proteger a IBBP da degradação. No entanto, a IBBP-SLM parece exercer efeitos biológicos, especialmente na viabilidade celular, bem como na indução de apoptose (na dose de 25 μg/mL). Também enfatizamos que o metabolismo dos pigmentos iridóides de base azul do jenipapo não é completamente compreendido e que estudos abrangentes e comparativos são necessários. Assim, os próximos passos para o nosso trabalho serão focados na melhoria da eficiência de encapsulação, variando a proporção do material de parede, bem como o uso de outras matrizes lipídicas. Também é necessária uma avaliação do metabolismo e distribuição de

micropartículas e bem sua segurança, já que o seu uso é pretendido para fins alimentícios e farmacêuticos.

Este foi o primeiro trabalho que visou analisar potencial genotóxico do extrato IBBP na presença e ausência de ativação metabólica bem como produzir e avaliar o potencial biológico de micropartículas lipídicas de extrato IBBP contento ácido esteárico, caracterizando um objetivo inovador deste projeto de doutorado.

SUGESTÕES PARA TRABALHOS FUTUROS

O uso de substâncias consideradas naturais, não tóxicos, *"ecofriendly"* e com efeitos biológicos desejáveis é uma tendência. Dentre deste contexto, os iridóides presentes no jenipapo representam uma alternativa interessante para aplicações industriais e terapêuticas. No entanto, o metabolismo dos pigmentos azuis à base de iridóides do genipapo não é completamente compreendido, exigindo estudos abrangentes e comparativos. Assim, a partir dos resultados obtidos com a realização do presente trabalho foram identificadas as seguintes linhas de ação para futuras pesquisas:

- Estudar novos métodos de extração, concentração e purificação para obtenção da genipina e/ou conversão do geniposideo em genipina,
- Avaliar a estabilidade dos iridóides presentes no extrato ou da genipina/geniposideo purificados empregando condições mais drásticas de atividade de água, pH, temperatura e presença de oxigênio para determinação dos limites críticos de vida de prateleira,
- Estudar a toxicidade do extrato ou da genipina/geniposideo purificados em modelos in vivo utilizando dosagens aguda, sub-crônica e crônica,
- Estudar o emprego destes componentes para diversas possibilidades terapêuticas,
- Estudar o processo de produção de micropartículas contendo IBBP, utilizando diversas matrizes. Por exemplo, lectina, quitosana, alginato, pectina, entre outros. Verificar se a mistura resulta em melhores propriedades de barreira e se mantém a viabilidade do composto,
- Avaliar a oxidação das micropartículas durante a estocagem,
- Estudar a liberação controlada das micropartículas em condições gastrointestinal simuladas *in vitro* e *in vivo*,
- Estudar a aplicação das micropartículas em produtos alimentícios compostos por matrizes diversas, tais como bebidas, yogurtes ou produtos de panificação,
- Estudar a aplicação das micropartículas de IBBP para diversas possibilidades terapêuticas.

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ANEXO 1

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Author: Iramaia Angélica Neri-Numa,Marina Gabriel Pessoa,Bruno Nicolau Paulino,Glaucia Maria Pastore

Publication: Trends in Food Science & Technology

Publisher: Elsevier

Date: September 2017

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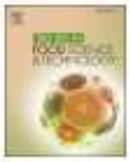

Review

Genipin: A natural blue pigment for food and health purposes

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ARTICLE INFO

Article history:
Received 30 March 2017
Received in revised form
13 June 2017
Accepted 24 June 2017
Available online 27 June 2017

1. Introduction

Natural and synthetic pigments are widely used to colour foodstuffs in order to make the processed food more attractive to consumers (Carocho, Morales, & Ferreira, 2015; Spence, 2015; Stich, 2016). Whether natural or synthetic, all colour additives must meet stringent requirements before their use in spite of a growing trend the towards replacement of synthetic ones for healthier alternatives as well as eco-friendly and biodegradable commodities (Martins, Roriz, Morales, Barros, & Ferreira, 2016; Newsome, Culver, & van Breemen, 2014).

Concerning natural pigments, they can be obtained from plants, microorganisms and insects or animals tissues (Zhang et al., 2014). Structurally, natural pigments can be classified in different groups that comprises several compounds with specific characteristics, such as isoprenoid derivatives (carotenoids and iridoids), benzopyran derivatives (oxygenated heterocyclic compounds like anthocyanins and others flavonoid piments), quinones (benzoquinone, naphthoquinone, and anthraquinone), tetrapyrrole derivatives (chlorophylls and heme colours), *N*-heterocyclic compounds different from tetrapyrroles (purines, pterins, flavins, phenazines, phenoxazines, and betalains) and melanins (Bauernfeind, 1981; Delgado-Vargas, Jimenez, & Paredes-Lopez, 2000; Hari, Patel, & Martin, 1994). Among natural pigments from plant sources, the main are either water- or lipid-soluble represented by carotenoids, chlorophylls, anthocyanins and betalains wich differ both in structure and metabolic pathway (Buchweitz, 2016; Tanaka, Sasaki, & Ohmiya, 2008; Zhang et al., 2014).

From the commercial point of view, anthocyanins, betalains and carotenoids are used in large scale for red, orange and yellow hues while natural green and blue colorants are few, thus making room to search for novel pigment sources such as unexploited fruits and vegetables (Brauch, 2016; Buchweitz, 2016).

Furthermore, these compounds have drawn considerable attention to the food and pharmaceutical segments, not only because of their coloring properties, but due to their biological activities such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective activities (Pangestuti & Kim, 2011; Rodriguez-Amaya, 2016). For this reason, much effort has been focused on improving colorant extraction, purification and stability as well as to satisfy the related hygiene and safety criteria as prerequisite to the approval of food regulatory authorities (Rodriguez-Amaya, 2016; Shahid, Shahid-ul-Islam, & Mohammad, 2013).

Thus, being motivated by the possible applications of genipin isolated not only from *Gardenia* sp but also from genipap (*Genipa americana* L.) as a precursor of natural blue pigment, the proposed review emphasized their potential health benefits for the food and pharmaceutical areas.

1.1. A brief overview of food colour additives

Since ancient times, foods appearance represents an important role in the lives of people, mainly when early humans searched for food and they had to learn how to identify edible or ripe fruits, to avoid toxic and spoiled objects (Adam Burrows, 2009; Stich, 2016). Now in much the same way, the practice of adding colours to food is intended to serve as a visual cue to quality, to look more appealing and meet consumer expectations (Spence, 2015). In addition, archaeological finds have shown that ancient cultures used dyes from plant, animal or mineral sources relates to the use of both for colouration or medical purposes (Adam Burrows, 2009; Mapari, Thrane, & Meyer, 2010).

One of the earliest records of the use of food colorants relates to the use of wine in order to improve a products appearance in 400 B.C. (Stich, 2016). The Egyptians coloured food, drugs and cosmetics adding saffron, turmeric and paprika as far back as 1.500 B.C., while

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http://dx.doi.org/10.1016/j.tifs.2017.06.018
0924-2244/

ANEXO 3

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

Espécie

Genipa americana

Título da Atividade:	**AVALIAÇÃO DAS PROPRIEDADES FUNCIONAIS DOS IRIDÓIDES OBTIDOS DO JENIPAPO (Genipa americana L.)**

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Situação cadastral conforme consulta ao SisGen em **14:43** de **21/03/2018**.

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