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Associação da geoprópolis à quimioterápicos: ação citotóxica e antiproliferativa sobre células HEp-2 e mecanismos envolvidos

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Mestre(a) em Patologia.

Orientador: Prof. Adj. José Maurício Sforcin

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Palavras-chave: Apoptose; Células HEp-2; Geoprópolis; Glicoproteína-P; Quimioterápicos.

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“A ignorância mais frequentemente gera confiança do que o conhecimento: são os que sabem pouco, e não aqueles que sabem muito, que afirmam categoricamente que este ou aquele problema nunca será resolvido pela ciência”

(Charles Darwin)

Resumo

O tratamento concomitante entre dois ou mais fármacos é uma prática comum na terapia antineoplásica. A carboplatina (CARB), metotrexato (MXT) e doxorrubicina (DOX) são os agentes convencionais mais utilizados na terapia antitumoral. Geoprópolis (Geo) é produzida por algumas abelhas sem ferrão a partir de uma mistura que contém resinas, fibras vegetais, secreção glandular das abelhas e terra ou barro. Para determinar a ação antiproliferativa e citotóxica da Geo isolada ou associada à CARB, MXT e DOX, células de carcinoma epidermóide de laringe humana (HEp-2) foram tratadas por 24, 48 e 72 horas com Geo na presença ou ausência desses fármacos. A viabilidade celular, citotoxicidade e apoptose foram obtidos pelo ensaio de 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), quantificação da liberação da lactato desidrogenase (LDH), citometria de fluxo e microscopia eletrônica de transmissão, respectivamente. A influência de Geo sobre a resistência à quimioterápicos foi obtida através da análise da ação da glicoproteína-P (P-gp). A combinação entre Geo e DOX demonstrou efeito inibitório significativo sobre as células HEp-2 mediado por indução de apoptose. Geo não afetou a ação da P-gp, sugerindo que esse produto natural não interfere no efluxo da DOX mediado pela P-gp. Os resultados obtidos indicam o potencial inibitório da associação entre Geo + DOX, e os mecanismos pelos quais esses fenômenos ocorrem merecem futuras investigações, visando novas perspectivas terapêuticas na terapia antineoplásica.

Palavras chaves: geoprópolis, agentes quimioterápicos, apoptose, glicoproteína-P

Abstract

Drug combination therapies are a common practice in cancer treatment. Carboplatin (CARB), methotrexate (MXT) and doxorubicin (DOX) are the most used chemotherapeutic agents for cancer treatment. Geopropolis (Geo) is produced by some stingless bees from a mixture of vegetable resins, gland secretions of the bees and soil. To determine whether Geo enhances the anticancer effect of CARB, MXT and DOX, human laryngeal epidermoid carcinoma (HEp-2) cells were treated for 24, 48 and 72 h with Geo alone or in combination with each drug. Cell growth, cytotoxicity and apoptosis was evaluated using 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) release, flow cytometry and transmission electron microscopy. The influence of Geo on drug resistance was also investigated assessing P-glycoprotein (P-gp) action. Data showed that the combination Geo + DOX led to a higher cytotoxic activity inhibiting the growth of HEp-2 cells and inducing apoptosis. Geo did not affect the action of P-gp, suggesting that this natural product does not interfere with the P-gp-mediated efflux of DOX. Our findings indicate that Geo combined with DOX could be a potential clinical chemotherapeutic approach for laryngeal cancer treatment.

Keywords

Geopropolis, chemotherapeutic agents, apoptosis, P-glycoprotein

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Revisão de Literatura

1. Câncer: características gerais

Câncer é um termo genérico relacionado a um grupo de doenças que podem afetar diversas partes do corpo. Outros termos designados para esse grupo de doenças são tumores e neoplasias. A característica principal do desenvolvimento de um câncer é o crescimento desordenado e anormal de células com capacidade de invadir tecidos adjacentes, se espalhando para outros órgãos, processo este denominado de metástase. As condições para o desenvolvimento tumoral são multifatoriais e podem agir em conjunto ou sequencialmente para promover sua iniciação (1-3).

O câncer é uma das principais causas de morte tanto em países mais desenvolvidos economicamente ou em desenvolvimento. Além disso, a incidência cresce conforme a longevidade e a adoção de hábitos conhecidos como fatores de risco (4, 5). Apesar de tantos anos dedicados a estudos para a sua compreensão, o câncer ainda continua sendo uma doença que carece de maiores investigações e terapias eficazes (6).

1.1. Câncer de laringe

O câncer de laringe é o tipo de neoplasia mais comum dentre os cânceres de cabeça e pescoço (7). A grande maioria destas neoplasias apresenta, como tipo histológico, o carcinoma de células escamosas, sendo raramente do tipo adenocarcinoma (8).

O câncer de laringe é causado principalmente pelo tabagismo e consumo de álcool. Esta malignidade pode atingir diferentes partes anatômicas da laringe dependendo da sua etiologia. O tabaco influencia o desenvolvimento do tumor na região das cordas vocais, enquanto o álcool é o fator de risco mais agravante no desenvolvimento do câncer de supraglote (9, 10). Porém, dados atuais revelam que tumores de cabeça e pescoço, incluindo câncer de laringe, estão acometendo jovens de 30 a 45

anos que não possuem hábitos tabagistas e não fazem consumo de álcool. Médicos e pesquisadores concluíram que o papiloma vírus humano (HPV), microrganismo que pode acometer pessoas de qualquer idade, pode causar infecções que levam subsequentemente ao desenvolvimento desses tumores (11, 12).

O câncer de laringe está entre os mais incidentes mundialmente. Na Europa, 52.000 novos casos são identificados anualmente; nos Estados Unidos da América, a incidência foi de 12 mil novos casos no ano de 2014 (13, 14). No Brasil, o quadro de incidência do câncer de laringe também é alarmante, e estima-se que 6.870 novos casos são observados em homens e 770 em mulheres, estando entre os dez cânceres que mais acometem os homens (15). A taxa de incidência crescente não é exclusividade do sexo masculino, e sua incidência vem crescendo também entre as mulheres por causa dos hábitos tabagistas e aumento no consumo de álcool entre elas (16, 17).

Os sintomas que surgem a partir das lesões levam as pessoas recorrerem aos serviços de saúde. A rouquidão indica o início de lesão na parte da glote que pode tardiamente atingir a parte supraglótica causando disfagia, sensação de corpo estranho e tosse. O diagnóstico pode ser feito através de laringoscopia, imagenologia e exames histológicos (18, 19).

O tratamento para esse tipo de neoplasia é complexo e, em muitos casos, requer conhecimentos multidisciplinares. O tipo de terapêutica vai depender do grau de malignidade do tumor e do estágio em que se encontra. Muitas vezes, a melhor solução é ressecção cirúrgica, embora este método possa ser devastador, pois implica em transformações em uma área muito visível da face. Além disso, a fala e a deglutição podem ser comprometidas (19-21). Além da ressecção, a quimioterapia e a radioterapia são preconizadas para tratamento desse tipo de tumor, porém problemáticas como resistência e alta toxicidade estão implicadas no sucesso dessas duas terapêuticas (22, 23).

Linhagens celulares imortalizadas têm sido uma ferramenta valiosa na investigação molecular, bioquímica, genética e imunológica de células derivadas de câncer de cabeça e pescoço. Diversas células têm sido utilizadas nestes estudos, e as primeiras a serem estudadas foram as células HEp-2, HEp-3 e KB. Muitas outras células foram investigadas posteriormente, tais como as células FaDu, HN-1, UM-SCC-22B, UM-SCC-30, CAL27,MDA-1483, MDA-886LN, MDA-686LN, T1/CUHK, T2/CUHK, entre outras (24-28). Em nosso estudo, utilizamos as células da linhagem HEp-2, as quais foram descritas inicialmente como sendo derivadas de carcinoma epidermóide da laringe, mas subsequentemente foram encontrados marcadores cromossômicos de células HeLa, com base em análise de isoenzimas, provavelmente por contaminação com estas células.

2. Mecanismos de ação e efeitos colaterais dos tratamentos quimioterápicos

Os agentes antitumorais podem agir de várias maneiras, inibindo o processo carcinogênico e a proliferação celular (29). Esses agentes podem ser classificados de acordo com o seu mecanismo e estrutura química.

Os agentes alquilantes causam danos diretamente no DNA, pois seu grupo alquil se liga covalentemente ao DNA ou a proteínas, interferindo em sua ação e impedindo a replicação celular. Esses fármacos agem em todas as fases do ciclo celular e são utilizados para o tratamento de muitos cânceres diferentes, incluindo leucemia, linfoma, doença de Hodgkin, entre outros. Dentre os agentes alquilantes, temos a cisplatina, caboplatina (CARB) e lomustine (30). CARB tem um amplo espectro de ação, podendo atuar contra vários tipos de cânceres, incluindo tumores de cabeça e pescoço (31). Este fármaco é um análogo da cisplatina de segunda geração e apresenta baixa toxicidade. Por este motivo, tem sido escolhido para tratamento de pacientes que sofrem com a toxicidade da cisplatina (23, 31). A CARB se intercala no ácido desoxirribonucléico (DNA), formando ligações

cruzadas e interferindo em sua função (32).

Os fármacos antimetabólitos são definidos como fármacos que interferem em processos metabólicos normais no interior da célula. Um dos antimetabólitos mais conhecidos é o metotrexato (MXT). O conhecimento dos mecanismos intracelulares tem aumentado nos últimos anos, permitindo a identificação de novos alvos potenciais. Tais antimetabólitos interferem em vias importantes que levam à síntese de purinas e pirimidinas: os co-fatores derivados de folato e os que inibem a dihidrofolato redutase, timidilato sintase e glicinamida ribonucleotídeo formiltransferase (33). O metotrexato é um inibidor da dihidrofolato redutase (34, 35), inibindo o metabolismo do ácido fólico (35). É um composto antiproliferativo e imunossupressor, utilizado contra vários tipos de doenças, incluindo tratamento do câncer de laringe (36). O MXT acarreta efeitos colaterais que compreendem desde náuseas e vômitos até cirrose, hepatite e anemia (37).

Antibióticos antitumorais também são amplamente utilizados no tratamento antineoplásico. Como exemplo, as antraciclinas interferem na ação de enzimas envolvidas na replicação do DNA. Esses fármacos agem em todas as fases do ciclo celular. A doxorrubicina (DOX) e a daunorrubicina (DNR) foram isoladas a partir do pigmento produzido por *Streptomyces peucetius* (38). DOX é um dos antineoplásicos prescritos mais potentes, exibindo um amplo espectro de ação tanto contra tumores hematopoiéticos quanto em relação aos tumores sólidos. Estudos têm atribuído a ação antitumoral da DOX à sua capacidade de se intercalar no DNA ou se ligar covalentemente às proteínas envolvidas na replicação e transcrição (39). Assim, essa interação resulta em inibição do DNA, RNA e síntese de proteínas, causando morte celular (40). Apesar de a DOX ter ação contra vários tipos de neoplasias, sua toxicidade limita o sucesso do tratamento (41, 42).

Os inibidores das topoisomerases interferem na ação dessas enzimas, as quais desempenham um importante papel na replicação do DNA (43). A

camptotecina é um antineoplásico que foi isolado pela primeira vez da casca de uma árvore chinesa, a *Camptotheca acuminata* (22). As camptotecinas possuem características farmacológicas distintas, inibindo principalmente a ação da topoisomerase I, tornando-a citotóxica. Os inibidores da topoisomerase I atuam danificando a replicação do DNA na fase S do ciclo celular (44).

Os inibidores de mitose muitas vezes são alcalóides derivados de plantas e outros compostos oriundos de produtos naturais. Agem principalmente induzindo parada da mitose na fase M do ciclo celular, mas também podem agir em outras fases inibindo a proliferação celular. Nessa classe, temos os inibidores de microtúbulos e de cinases (45). Os taxanos e os alcalóides da vinca são os dois grupos mais antigos de inibidores de segmentação dos microtúbulos. Ambos compostos impedem a conclusão do ciclo celular pela sua junção às subunidades beta das tubulinas, resultando na formação de microtúbulos estáveis e não funcionais, aprisionando as células na fase G2 / M (45, 46).

Além dos fármacos supra-citados, outros também podem ser associados à quimioterapia antineoplásica, tais como corticoesteróides, L-asparaginase e o inibidor de proteassoma bortezomib (Velcade ®) (47).

Historicamente, os tratamentos antineoplásicos são conhecidos por sua ampla lista de efeitos colaterais causados pela toxicidade e indução de resistência em células tumorais (48-50). Ademais, um dos grandes fatores implicados na toxicidade dos agentes antitumorais é a falta de seletividade entre as células normais e tumorais. Além dos efeitos colaterais clássicos como náuseas, vómitos e alopecia, estes agentes estão associados a outros efeitos mais graves, tais como cardiotoxicidade; esteatose hepática; pseudocirrose; pancreatite; esclerose biliar, dentre outros (42, 51, 52).

Em nosso trabalho, optamos por utilizar a carboplatina, o metotrexato e a doxorrubicina, por apresentarem diferentes mecanismos de ação, além de serem utilizados também no tratamento de câncer de laringe.

3. Resistência à antineoplásicos

O termo “resistência a drogas” em relação aos tratamentos de tumores é utilizado sinonimamente com “progressão da doença”. Uma vez que o paciente desenvolve resistência adquirida a um determinado fármaco, outra estratégia terapêutica deve ser adotada (49). A resistência de células tumorais é um fenômeno complexo que envolve múltiplas proteínas, porém as mais estudadas estão relacionadas a um único fenótipo, o *Multidrug Resistance* (MDR) (53). Este fenótipo MDR foi descoberto na década de 70, estando relacionado à expressão elevada de uma proteína denominada glicoproteína-P (P-gp) (54). Além da P-gp, também estão envolvidas com o MDR a proteína associada ao MDR 1 (também conhecida como MRP1 ou ABCC1) e BCRP (*Breast Cancer Resistance Protein*). Tais proteínas têm como principal função promover a eliminação de vários compostos hidrofóbicos, incluindo a maioria dos quimioterápicos, assim como taxanos, inibidores da topoisomerase e antimetabólitos (55).

A P-gp foi o primeiro transportador a ser identificado. Esta glicoproteína é expressa na membrana celular de quase todos os tecidos em níveis basais, mas em tecidos com papel excretor o seu nível de expressão é maior (56, 57). Em tumores, geralmente a expressão da P-gp pode aumentar através da sensibilização induzida por quimioterápicos, causando assim, resistência intrínseca a essas drogas e resultando no insucesso da terapia e progressão da doença (58-60).

A administração concomitante de dois ou mais agentes antineoplásicos tem sido uma estratégia adotada para se obter uma melhor resposta no tratamento de tumores em estágio mais avançado (61). Apesar de a adoção de tratamentos concomitantes muitas vezes prolongar a sobrevida do paciente, não há garantias de redução dos efeitos colaterais e de não resistência por parte das células tumorais (49, 62, 63).

Estudos têm relatado a eficácia da quimioterapia combinada com inibidores da P-gp na reversão do quadro de resistência (64, 65). Verapamil

(VRP) é um bloqueador de canais de cálcio comumente utilizado no tratamento de hipertensão, angina e infarto do miocárdio (66) e tem sido mencionado como agente capaz de reverter a resistência causada pela P-gp (67), sendo, portanto, combinado com drogas anticancerígenas (68, 69).

Além dos inibidores de P-gp, dados demonstram que produtos naturais também podem agir como adjuvante de forma sinérgica com agentes antitumorais causando aumento na sensibilidade de células tumorais (70).

4. Indução de apoptose

Apoptose é um tipo de morte celular programada e ocorre com frequência em organismos multicelulares, tanto em situações fisiológicas quanto patológicas. Em condições fisiológicas, a apoptose tem um papel fundamental no controle do crescimento, proliferação celular e na resposta imune, mantendo os tecidos em homeostase (71, 72).

A apoptose pode ser ativada tanto pela via intrínseca quanto pela via extrínseca. A via extrínseca é ativada por ligantes específicos, como o FasL, a um grupo de receptores de membrana da superfamília de receptores de fator de necrose tumoral (rTNF) (73). Esta ligação é capaz de desencadear a cascata das caspases (Figura 1). Todos os membros desta superfamília possuem um subdomínio extracelular rico em cisteína, que permite o reconhecimento de seus ligantes resultando em trimerização e consequente ativação de receptores específicos de morte. Após esse evento, ocorre uma sinalização citoplasmática envolvendo a porção inferior desses receptores conhecida como domínio de morte que interagem com moléculas conhecidas como FADD/MORT-1. Essas moléculas recrutam a caspase-8 que ativará a caspase-3, iniciando a morte por apoptose (74, 75).

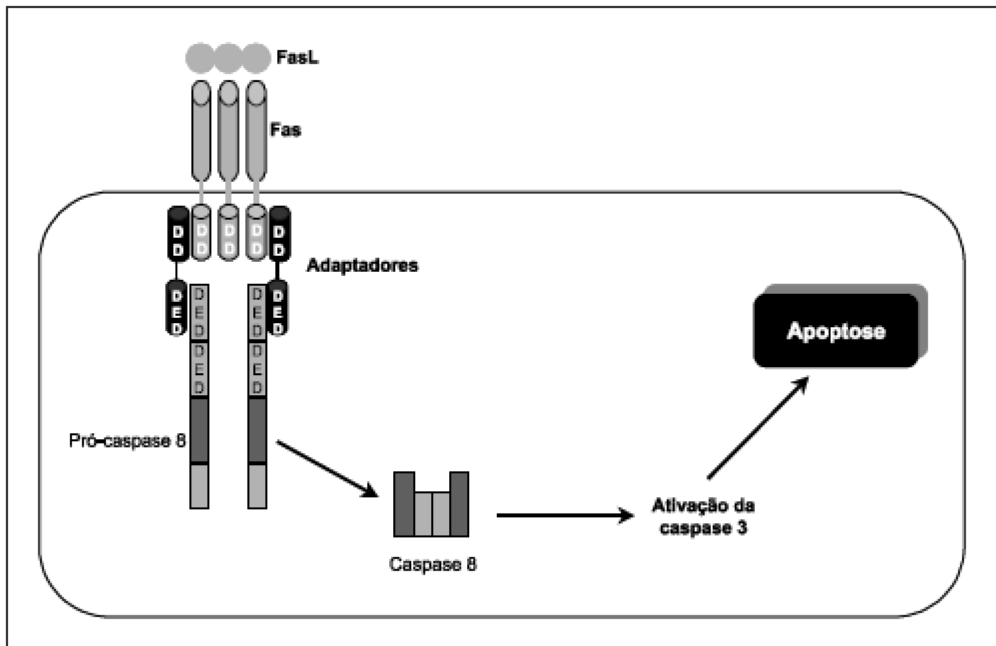

Figura 1. Via extrínseca de ativação de apoptose. DD = domínio de morte; DED = domínio efetor de morte (Fonte: Grivicich *et al.*, 2007).

A via intrínseca pode ser ativada por estresse intracelular ou extracelular. Os sinais transduzidos em resposta à esses estímulos convergem principalmente para a mitocôndria, como sendo o principal mediador desse tipo de morte (77). Essa organela capta os estímulos de morte celular e induz a permeabilidade mitocondrial com consequente liberação de moléculas pró-apoptóticas (78). Quando os sinais de morte alcançam a mitocôndria, o colapso do potencial de membrana mitocondrial interna é desencadeado ($\Delta\psi$) junto com a transição da permeabilidade mitocondrial (TPM) (79, 80). Além da liberação de moléculas pela mitocôndria, a indução do $\Delta\psi$ e TPM acarretam a perda da homeostasia celular, interferindo na síntese de ATP e levando a produção de espécies reativas de oxigênio (EROS) (81). Níveis elevados de EROS desencadeiam a oxidação de lipídeos, proteínas e ácidos nucléicos, elevando o colapso $\Delta\psi$ (82). Além disso, sabe-se que as EROS ativam as caspases -9 e -3 (83, 84). Outro mecanismo relacionado é a formação de um megaporo

constituído de diversas proteínas e que abrange as membranas internas e externas da mitocôndria (85). Por este poro, ocorre a liberação do citocromo *c* para o citoplasma. Diferentes sinais indutores de apoptose são detectados pela mitocôndria, acarretando o desacoplamento da cadeia respiratória e consequente liberação de citocromo *c* e de proteínas ativadoras de apoptose para o citosol (80). Quando no citosol o citocromo *c* forma um complexo com a APAF-1 e a caspase-9, o apoptossomo promove clivagem da pró-caspase-9, tornando a caspase-9 ativa (73). Uma vez ativada, a caspase-9 induz ativação da caspase-3, ocasionando a apoptose (Figura 2) (86, 87).

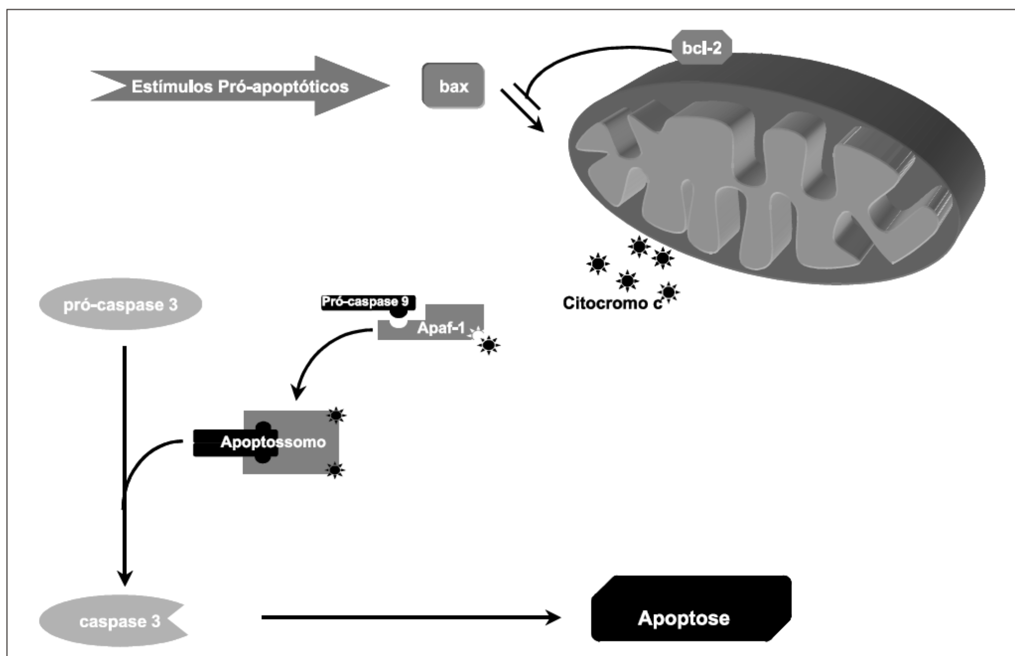

Figura 2. Via intrínseca de ativação da apoptose. APAF-1 = Fator de ativação de protease associada à apoptose 1. (Fonte: Grivicich *et al.*, 2007).

Morfologicamente, a apoptose é caracterizada por condensação da cromatina, condensação do núcleo e do citoplasma, seguida de fragmentação do DNA e formação de corpos apoptóticos, que serão posteriormente engolfados por células fagocíticas e digeridos pela ação de enzimas lisossomais (72). Esse processo é regulado por genes pró-apoptóticos (p53, Bax e c-Myc) ou anti-apoptóticos (Bcl-2, Bcl-xL, sentrin)

(88).

A apoptose tem a importante função de proteger os organismos contra a tumorigênese, sendo, portanto, a desregulação da apoptose uma característica fundamental no desenvolvimento tumoral (89). A regulação da indução da apoptose tem sido descrita como uma das melhores estratégias na terapia antineoplásica (90), e comprometimento no mecanismo de sinalização apoptótica pode possibilitar a proliferação de células tumorais (91).

Há vários quimioterápicos antineoplásicos convencionais que atuam na indução da apoptose (92-94). Além dos quimioterápicos, há também relatos que indicam produtos naturais como indutores de apoptose. Um estudo demonstrou que um polissacarídeo isolado do cogumelo medicinal *Fomes fomentarius* possui propriedades apoptóticas contra células de carcinoma de pulmão (95). Vatansever *et al*., (2010) demonstrou o potencial da própolis oriunda da Turquia ao induzir apoptose por ativação da via das caspases em células de carcinoma mamário.

5. Associação de produtos naturais à fármacos antineoplásicos

Produtos naturais têm sido um excelente recurso em nossa sociedade. O uso de plantas medicinais e metabólitos microbianos tem ampliado a expectativa de vida, seja empiricamente ou a partir da síntese de medicamentos de origem natural (96). A ação antineoplásica de produtos naturais tem sido intensamente investigada e ¾ dos compostos antitumorais são sintetizados a partir de produtos naturais ou relacionado à eles. Dos 140 agentes antitumorais e disponíveis desde a década de 40, mais de 60% são oriundos de compostos naturais (97). Esses agentes agem através de mecanismos que incluem indução de apoptose, clivagem do DNA mediada pela inibição pela topoisomerase I ou II, permeabilização mitocondrial, inibição de enzimas envolvidas na transdução de sinal ou no metabolismo celular, e por inibição da angiogênese (96).

Com o intuito de atenuar os efeitos colaterais causados pelos agentes antitumorais, o estudo da administração de produtos naturais concomitantemente com fármacos tem sido investigado, a fim de obter uma interação entre ambos por efeito aditivo, sinérgico, antagônico ou potencializador (98, 99). Janssen *et al.* (2014) demonstraram que o resveratrol combinado com curcumina potencializou o efeito inibitório da DOX sobre células de câncer de ovário, atenuando seus efeitos cardiotóxicos sobre cardiomiócitos. A curcumina acarretou melhora no efeito supressor da cisplatina sobre células escamosas de carcinoma de cabeça e pescoço, por inibir a via do NF-kB e da proteína IKKβ (100).

Além de plantas medicinais e metabólitos microbianos, há também os opoterápicos obtidos a partir de glândulas, órgãos, tecidos ou secreções animais (101). Um exemplo de opoterápico é a própolis produzida por abelhas africanizadas. A própolis e seus componentes vêm sendo apontados como agente citotóxico, antitumoral e quimiopreventivo contra vários tipos de tumores (102-104). Estudos indicam seu potencial em melhorar sinergisticamente a ação de drogas antitumorais (105, 106).

6. Geoprópolis: características gerais

Diferentemente da própolis produzida por abelhas africanizadas, a geoprópolis é produzida por abelhas sem ferrão (meliponíneos) a partir da mistura de resina, fibras vegetais, cera e, tipicamente, incluindo terra ou barro. Nestas colônias, a geoprópolis é utilizada amplamente, servindo como base para construção de estruturas externas (tubos e entrada) e internas (favos de cria, lamelas de invólucro e potes de alimento) (107).

De modo geral, as amostras de geoprópolis apresentam-se como fragmentos rígidos e com diferentes tamanhos, com grânulos inodoros e de consistência heterogênea, coloração marrom escura e sabor amargo (Figura 3). Assim como para a própolis produzida por abelhas africanizadas, o método de preparo dos extratos de geoprópolis pode influenciar em sua

atividade, uma vez que os diferentes solventes solubilizam e extraem compostos diferentes (108).

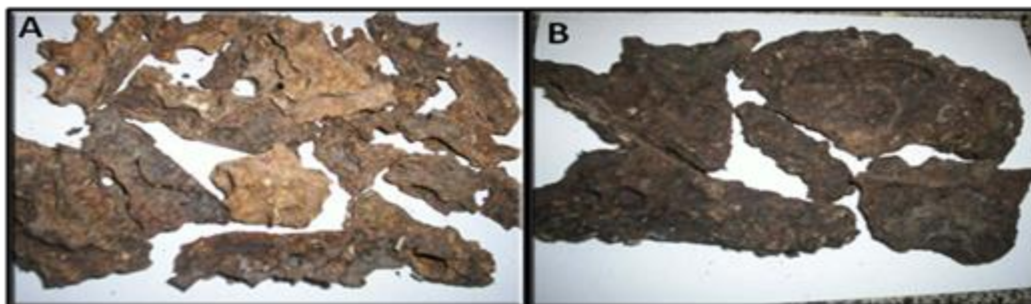

Figura 3. Própolis produzida por *Scaptotrigona* aff. *postica* (A) e geoprópolis produzida por *Melipona fasciculata* Smith (B). Foto: Abigail Araújo.

As ações farmacológicas da geoprópolis têm sido investigadas mais recentemente, tais como antimicrobiana, imunomoduladora e citotóxica, porém essas propriedades não estão bem esclarecidas por falta referências e estudos sobre esse produto (109). Geoprópolis tem sido utilizada na medicina popular para tratamento de doenças respiratórias e dermatoses (110). Também foi verificada sua ação como agente citotóxico contra células de osteossarcoma canino (111). No entanto, estudos demonstraram também seu efeito antiproliferativo sobre outras linhagens tumoral humana (112).

A amostra utilizada em nosso estudo foi analisada quimicamente, revelando que seus principais compostos são lupeol, amirinas, ácido anacárdico dentre outros (109, 113-117). Nossa amostra de geoprópolis foi trazida do Estado do Maranhão por uma doutoranda de nosso grupo, a qual observou sua ação antimicrobiana, imunomoduladora e citotóxica contra células HEp-2. Os dados incitaram-nos a dar continuidade a estas investigações e, neste projeto, avaliamos a ação da geoprópolis associada a diferentes quimioterápicos contra estas células, avaliando o efeito antiproliferativo e citotóxico, indução de apoptose e morfologia celular, e inibição sobre a P-gp.

Os dados obtidos junto a esta dissertação encontram-se apresentados

a seguir junto ao manuscrito intitulado **“Combinatory effects of geopropolis with conventional anticancer drugs towards human laryngeal epidermoid carcinoma (HEp-2) cells”**, que será submetido a publicação após a defesa de mestrado.

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Capítulo II – Manuscrito

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Combinatory effects of geopropolis with conventional anticancer drugs towards human laryngeal epidermoid carcinoma (HEp-2) cells

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Abstract

Objectives Drug combination therapies are a common practice in cancer treatment. Carboplatin (CARB), methotrexate (MXT) and doxorubicin (DOX) are the most used chemotherapeutic agents for cancer treatment. Geopropolis (Geo) is produced by some stingless bees from a mixture of vegetable resins, gland secretions of the bees and soil. To determine whether Geo enhances the anticancer effect of CARB, MXT and DOX, human laryngeal epidermoid carcinoma (HEp-2) cells were treated for 24, 48 and 72 h with Geo alone or in combination with each drug.

Methods Cell growth, cytotoxicity and apoptosis was evaluated using 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) release, flow cytometry and transmission electron microscopy. The influence of Geo on drug resistance was also investigated assessing P-glycoprotein (P-gp) action.

Key findings Data showed that the combination Geo + DOX led to a higher cytotoxic activity inhibiting the growth of HEp-2 cells and inducing apoptosis. Geo did not affect the action of P-gp, suggesting that this natural product does not interfere with the P-gp-mediated efflux of DOX.

Conclusions Our findings indicate that Geo combined with DOX could be a potential clinical chemotherapeutic approach for laryngeal cancer treatment.

Keywords
geopropolis; chemotherapeutic agents; apoptosis; P-glycoprotein

Introduction

Laryngeal cancer is the most common malignant neoplasm among head and neck tumors. Moreover, it represents approximately 1% of all new cancer diagnoses in recent years and chemotherapy has been used for its treatment.[1,2]

Immortalized cell lines have been an valuable tool for investigating detailed molecular, biochemical, genetic, and immunological properties of head and neck cancer, and several cells have been used such as HEp-2, HEp-3, KB, FaDu, HN-1, UM-SCC-22B, UM-SCC-30, CAL27,MDA-1483, MDA-886LN, MDA-686LN, T1/CUHK, T2/CUHK, among others, as reviewed by Lin et al.[3] HEp-2 cells contain HeLa marker chromosomes, and were derived via HeLa contamination. This line was originally thought to be derived from an epidermoid carcinoma of the larynx, but HeLa marker chromosomes and DNA fingerprinting were subsequently found.

Different chemotherapeutic agents such as carboplatin (CARB), methotrexate (MXT) and doxorubicin (DOX) have been widely used against neoplastic cells.[4-6] However, these therapeutic strategies are unsatisfactory due to side effects and drug resistance.[7-9] Multidrug resistance (MDR) may be defined as resistance to various types of chemotherapeutic agents with different mechanisms of action and/or molecular structure, and it is a serious problem in cancer chemotherapy.[10] Among the mechanism of MDR, the overexpression of efflux transporters in tumor cells, especially P-glycoprotein (P-gp), has attracted attention. P-gp acts as an efflux pump to expel chemotherapeutic agents from the cells, decreasing the intracellular concentration of the agents and cell resistance to them.

The identification of natural products exerting a combined effect with therapeutic agents could be an alternative to treat laryngeal cancer, reducing the concentration of the drugs and side effects.[11-13] Geopropolis (Geo) is produced by some stingless bees from a mixture of vegetable resins, gland secretions of the bees and soil. Geo produced in the region of Barra do Corda, Maranhão State, Brazil, has been used popularly because their pharmacological properties, including treatment of respiratory disease and dermatosis.[14] The chemical composition of the Geo of some countries, including Brazil, was analyzed recently.[15,16] The chemical composition of the sample used in this work was previously investigated by gas chromatography coupled with mass spectrometry (GC-MS) revealing that its major compounds were carbohydrates and their derivatives, triterpenes, anacardic acid, alkylresorcinols and sugar alcohols.[15]

In recent years, studies have focused on the potential of Geo against cancer cells.[17-20] However, the effects of Geo combined with chemotherapeutic agents have not been investigated yet, using lower concentrations of drugs and leading probably to fewer side effects. Thus, the goal of this study was to determine whether Geo alone or combined with conventional drugs (CARB, MXT and DOX) could inhibit the growth of HEp-2 cells, induce apoptosis and morphological alterations, and affect the action of P-glycoprotein (P-gp), providing new insights for therapies including this natural product.

Materials and methods

Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), doxorubicin Rubidox® (Bergamo, Brazil), lyophilized methotrexate (Cruz Vermelha, Botucatu, SP Brazil), carboplatin Darrow-Vancel® Laboratories A/S (London, UK), dimethyl sufoxide (DMSO – VETEC Sigma Aldrich, USA), trypsin (TrypLE™ Express Gibco,USA), annexin V-FITC/PI apoptosis kit

(Becton Dickinson, USA), lactate dehydrogenase (LDH) test kit (Sigma Aldrich, USA), resin (Araldite®, Brazil) and verapamil hydrochloride (VRP - Sigma Aldrich, USA) were used.

Cell cultures

HEp-2 cells and African green monkey kidney (Vero) cells were obtained from the Virology Laboratory of Biotechnology Institute (IBTec, UNESP) and were mycoplasma free. Such cells were used in order to investigate the selectivity of Geo alone or associated to drugs against tumor or non-tumoral cells.

HEp-2 cells were grown in Minimal Essential Medium (MEM – Cultilab, Brazil) containing penicillin/streptomycin (1%) and 10% fetal bovine serum (FBS). Vero cells were maintained in Dulbecco's modified Eagle's Medium (DMEM – Cultilab, Brazil) supplemented with 10% FBS and penicillin/streptomycin (1%). Cells were cultured in a humidified atmosphere at 37°C and 5% CO_2.

Sub-confluent cells were detached using trypsin-EDTA, plated at 2 x 10^5 cells/ml in a 96-well plate, and incubated for 24 h at 37°C for adherence.

Geopropolis Sample

Geo was produced in Palmeirândia, Maranhão State, northeast Brazil and extracts were obtained as previously described (19). Briefly, Geo samples were kept at 4°C before extraction. A 40 g geopropolis sample was ground and macerated in 70% ethanol at room temperature under moderate shaking. After 24 h, the extract was filtered and the dry weight of geopropolis hydroalcoholic extract was calculated (9.6 mg/ml). Cells were treated with different Geo concentrations (25, 50 and 100 µg/ml).

Chemotherapeutic agents combined with Geo

HEp-2 cells were incubated with DOX (0.5 and 1 µM), MXT (50 and 100 µM)

and CARB (100 and 200 μM) with or without Geo (25 μg/ml) for 24, 48 and 72 h. Drugs concentrations were established according to literature and on previous assays standardized in our laboratory, in order to obtain the best concentrations to study the combination with Geo.[21-24] Before the assays, Geo and the drugs were filtered using a PES membrane (pore size 0.22 μm - TPP, Switzerland). Control cells were incubated with medium alone. All experiments were performed in triplicate with 3 repetitions of the assays.

Viability assay

MTT assay was performed to assess cell viability (25). HEp-2 and Vero cells were exposed to various concentrations of the variables for 24, 48 and 72 h. After, 100 μl of MTT solution (1 mg/ml) were added to each well and cells were incubated for 3 h. The formazan product were dissolved in DMSO (100 μl) and absorbances were measured using an automated plate reader (BioTek Instruments, USA) at 540 nm. Absorbance from untreated cells was considered as 100% cell viability, and percentage (%) of cell viability was calculated according to the formula: % = [mean experimental absorbance/mean control absorbance] x 100%.

After assessing cell viability, the 50% growth inhibitory concentration (IC_{50}) of Geo extract or chemotherapeutic agents was determined.

Cytotoxicity assay

To analyze the cytotoxic effects of Geo, cell membrane damage was assessed by measuring the release of lactate dehydrogenase (LDH) into the incubation medium using the LDH test kit (Sigma Aldrich, USA) and MRX revelation Dynex technologies analyzer (Germany).

***In vitro* migration assay**

The wound-healing assay was used to assess the in vitro migration ability of HEp-2 cells, culturing the cells in 24-well plates until formation of a single-

layer confluence. After starving overnight in serum-free medium, 200-µl pipette tips were used to make scorings in the cell layer; followed by incubation for 24 and 48 h with Geo (50 and 100 µg/ml). Cell migration was observed in an optical microscope and measures were achieved using the software Image J.

Apoptosis analysis by flow cytometry

The induction of apoptosis by the variables was assessed by annexin V-FITC/PI apoptosis kit (Becton Dickinson, USA). Cells were seeded (1.5 x 10^5 cells/ml) in 24-well plates overnight and treated with the combination that led to the highest cytotoxic activity: DOX (1 µM) and Geo (25 µg/ml) for 72 h. Untreated cells were used as a control. Cells were centrifuged (200 g/10 minutes) and washed twice with PBS. Staining was performed according to the manufacturer's instructions and samples were acquired in a FACSCanto™ II (BD Biosciences, USA) Flow Cytometer with emission filters of 515-545 nm for FITC (green) and 600 nm for PI (red) using FACSDiva (BD Biosciences) software and analyzed using FlowJo software vX 10.6 (Tree Stars Inc.). The percentages of three cell death categories were determined: early apoptotic (AV+, PI-), late apoptosis or necrotic (AV+, PI+) and live cells (AV-, PI-).

Morphological analysis

The morphological characteristics of cells treated with DOX (1 µM), Geo (25 µg/ml) or their combination for 72 h was determined by transmission electron microscopy (FEI Tecnai™, USA). Cells were trypsinized and centrifuged at 200 g for 10 minutes. Afterwards, 2 ml of cell suspension containing $2x10^5$ cells/well were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde buffered with 0.1 M NaH_2PO_4 + $NaHPO_4$ (pH 7.3) and post-fixed in 0.5% osmium tetroxide (OsO_4). After dehydration in ethanol, cells were embedded in resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

Characteristic signs of apoptosis or necrosis (apoptotic bodies formation, condensation of chromatin and loss membrane integrity) were investigated.

Effect of a P-glycoprotein inhibitor (verapamil) on HEp-2 cells

The sensitivity of HEp-2 cells to DOX and the effects of a P-gp inhibitor (verapamil hydrochloride – VRP) were determined using the MTT test (described above). Cells were treated with DOX (1 μM), Geo (25 μg/ml) or their combination in the presence of VRP.HCL (5 μM) for 72 h. VRP was dissolved in ethanol 70% prior to the incubation with the cells. Untreated HEp-2 cells were used as control. Assays were carried out in triplicate.

Statistical analysis

Data were plotted in GraphPad Prism 4.01 using the means and standard-deviation. One-way ANOVA was used for multiple comparisons followed by Tukey test. Significant differences were considered at $P < 0.05$.

Results

Effects of geopropolis extract on the growth of HEp-2 cells

Figure 1 shows the effects of Geo on the growth of HEp-2 cells after 24, 48 and 72 h of treatment at concentrations ranging from 25 to 100 μg/ml. The best inhibitory effect was seen after 72h, and the effects of Geo solvent and LDH release were investigated in this period of time. Ethanol 70% (Geo solvent) show no inhibitory effect on HEp-2 cells (Figure 2), and the effects of Geo on the growth of Vero cells are seen in Figure 3.

Geo inhibited the growth of HEp-2 cells in a dose- and time-dependent manner, with IC_{50} values of 66.86 ± 16.08 at 24 h, 54.42 ± 19.63 at 48 h, and 44.10 ± 23.88 at 72 h. The IC_{50} obtained from three independent experiments for Vero cells was 91.01 ± 10.33, showing that Geo exhibited a selective action for tumors cells over non-tumoral ones.

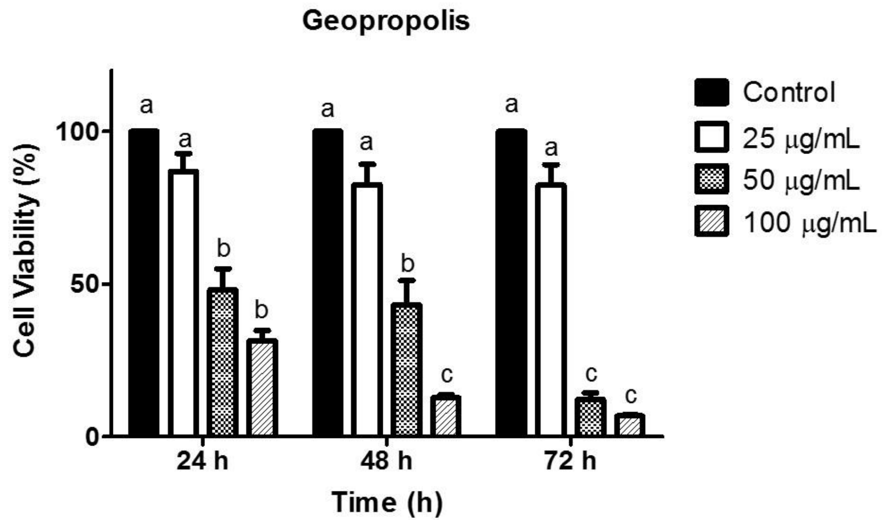


Figure 1. Viability (%) of HEp-2 cells after incubation with Geo (25, 50 and 100 µg/mL) for 24, 48 and 72 h determined by MTT assay. Data represent means of three experiments ± standard-deviation. Different letters indicate significant differences between the treatments ($p < 0.05$).

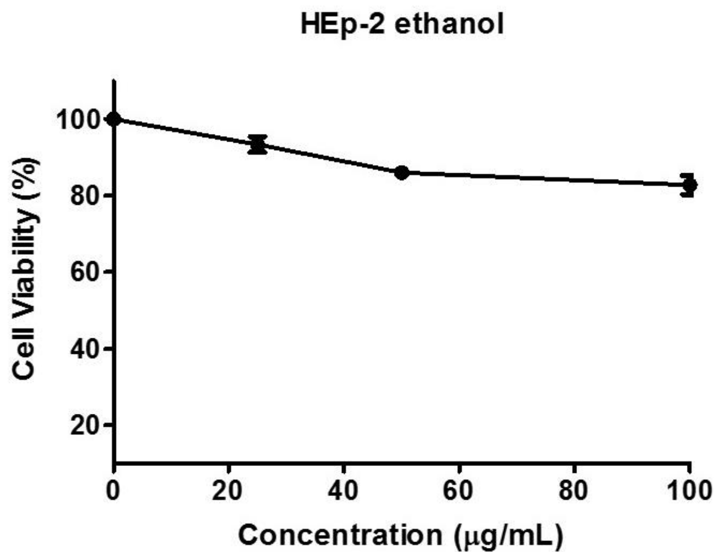


Figure 2. Viability (%) of HEp-2 cells after 72 h incubation with 70% ethanol (Geo solvent) by MTT assay. Ethanol concentrations were equivalent to those found in 25, 50 and 100 µg/mL of Geo (0.15, 0.29 and 0.59%). Data represent means of three experiments ± standard-deviation.

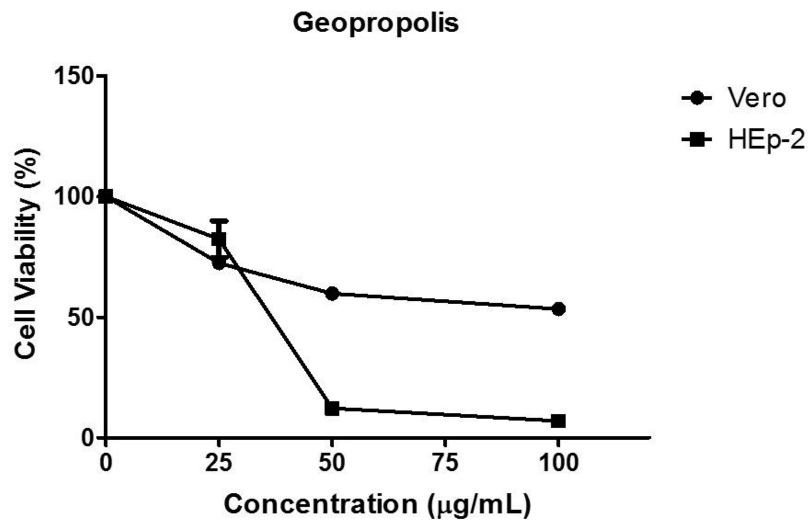


Figure 3. Viability (%) of HEp-2 and Vero cells after 72 h incubation with Geo (25, 50 and 100 µg/mL) by MTT assay. Data represent means of three experiments ± standard-deviation.

LDH release assay

Figure 4 shows LDH release from HEp-2 cell incubated with various concentrations of Geo for 72 h. Geo exhibited a cytotoxic effect using 25 µg/ml (7.0%), 50 µg/ml (29.3%) and 100 µg/ml (60.2%). Thus, the concentration 25 µg/ml was used in the next assays due to its lower cytotoxic effects.

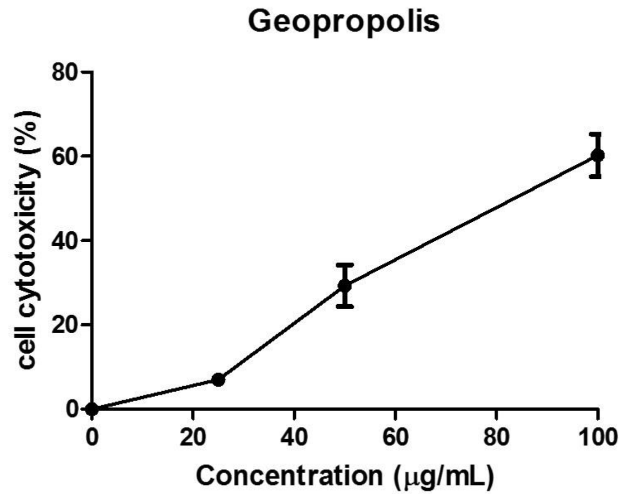
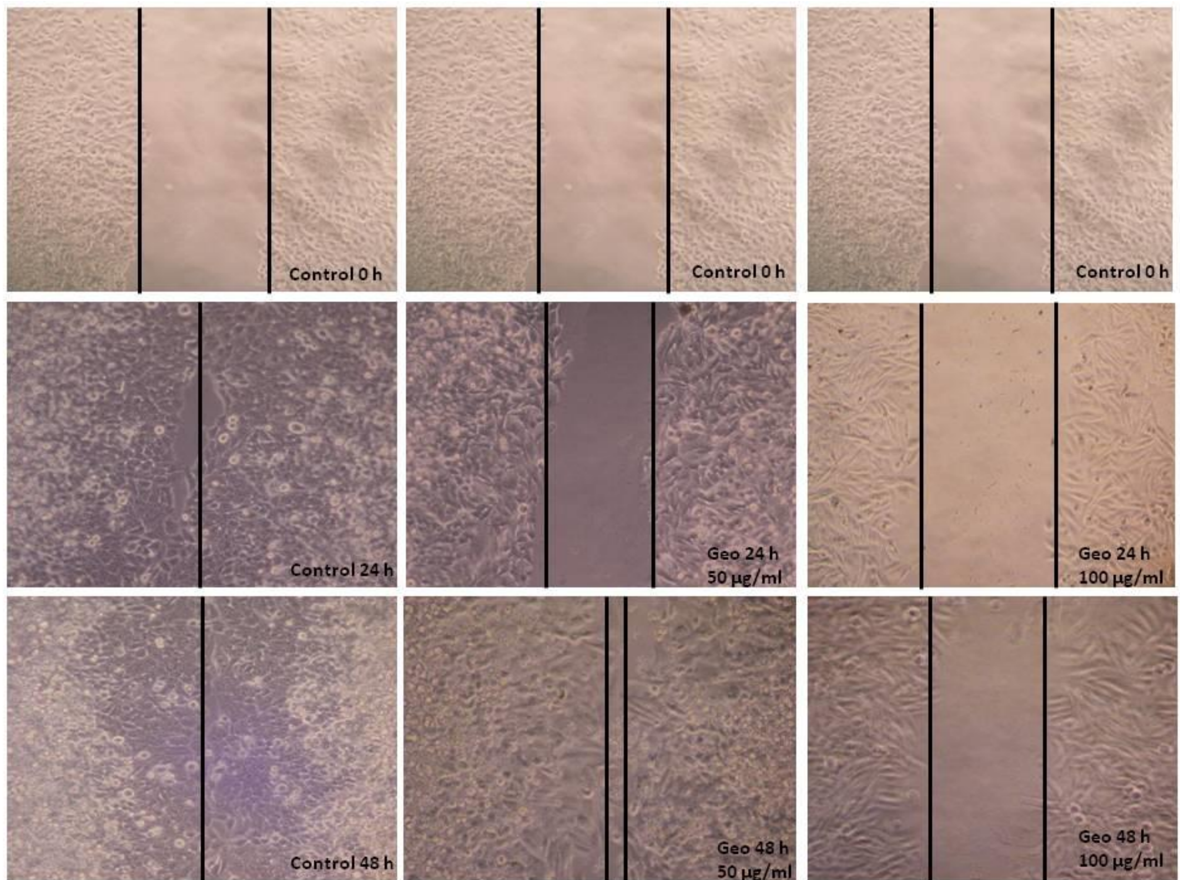


Figure 4. LDH release by HEp-2 cells after incubation with Geo (25, 50 and 100 μg/mL) for 72 h. Data represent means of three experiments ± standard-deviation.

In vitro migration assay

The wound-healing assay showed that Geo treatment (50 and 100 μg/ml) significantly reduced cell migration after 24 h, while after 48 h only the concentration of 100 μg/ml was efficient (Figure 5). The concentration 25 μg/ml was not used because it exerted only a mild effect on cell viability.



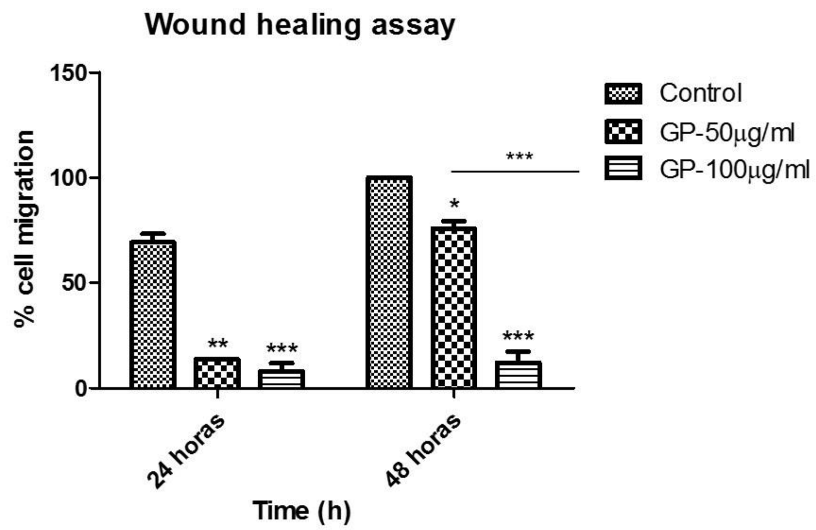


Figure 5. Hep-2 cells migration in vitro after treatment with Geo (50 and 100 µg/mL) by

wound healing assay.

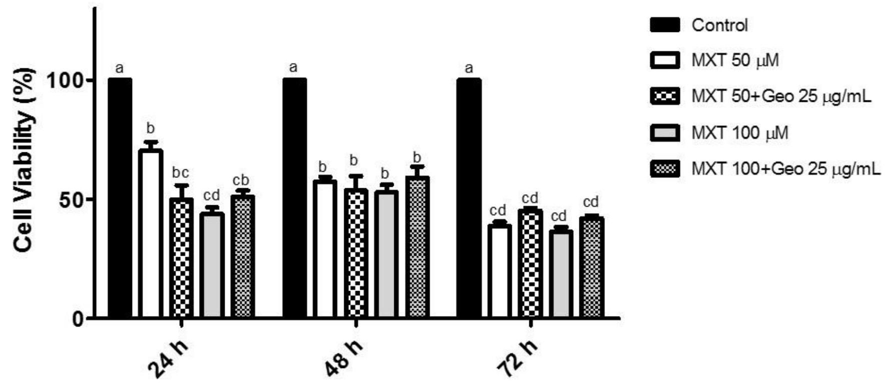
Combined effect of Geo with DOX, MXT and CARB on HEp-2 cells viability

The sensitivity of HEp-2 cells to DOX (0.5 and 1 µM), MXT (50 and 100 µM) and CARB (100 and 200 µM) with or without Geo (25 µg/ml) was examined after 24, 48 and 72 h by MTT assay. MXT combined with Geo affected HEp-2 viability only after 24 h compared to monotherapy (Figure 6a). The co-treatment of Geo with CARB showed no inhibitory effect (Figure 6b). The combination DOX + Geo increased significantly the sensitivity of HEp-2 cells after 72 h, decreasing cell viability (Figure 6c).

Thus, the combination DOX (1 µM) and Geo (25 µg/ml) was chosen to evaluate apoptosis, morphological changes in HEp-2 cells and its effect on P-gp. DOX concentrations did not affect Vero cells viability ($P > 0.05$), indicating that this drug did not exert cytotoxic effects towards non-tumoral cells (Figure 7).

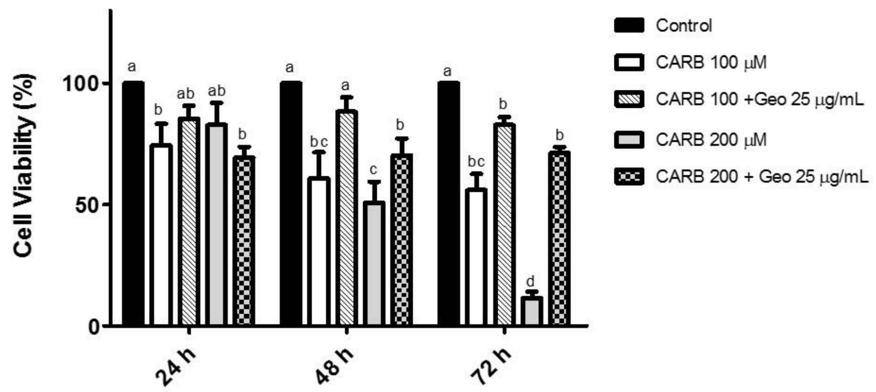
(a)

Associations



(b)

Associations



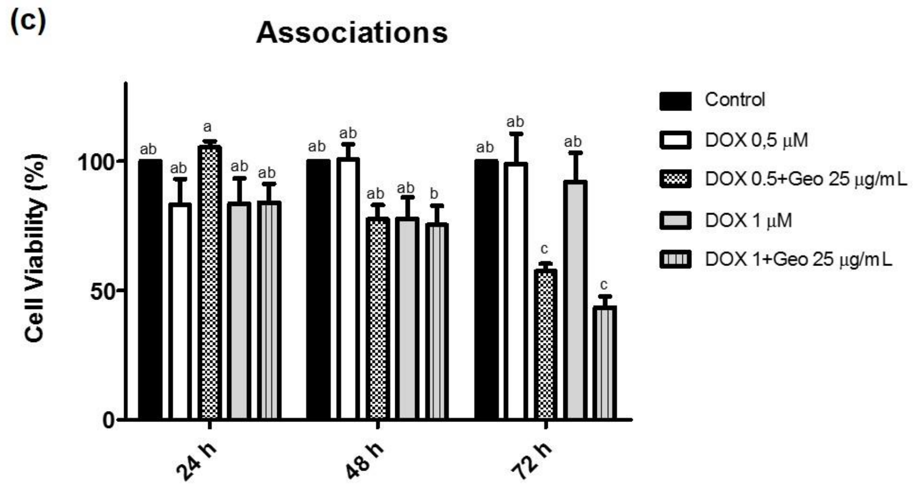


Figure 6. Viability (%) of HEp-2 cells treated with Geo (25 µg/mL) and MXT (50 and 100 µM) **(a)**; CARB (100 and 200 µM) and Geo (25 µg/mL) **(b);** DOX (0.5 and 1 µM) and Geo (25 µg/mL) **(c)** after 24, 48 and 72 h incubation. Different letters indicate significant differences between the treatments ($p < 0.05$).

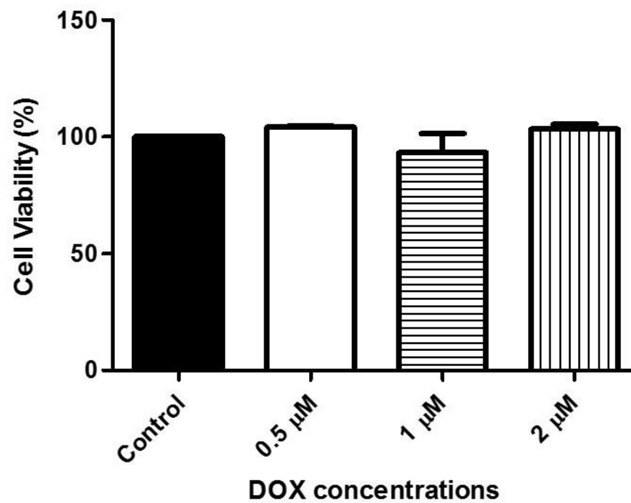


Figure 7. Viability (%) of Vero cells treated with different concentrations of DOX (0.5, 1 and 2 µM) by the MTT assay. Data represent means of three experiments ± standard-deviation.

Apoptotic effect of Geo and DOX on HEp-2 cells

To examine whether Geo (25 µg/ml), DOX (1 µM) and their combination could induce apoptosis after 72 h, cells were stained with Annexin V-FITC and analyzed by flow cytometry. As shown in Figure 8, DOX or Geo alone induced cell apoptosis/necrosis (Q1 + Q2: 16.28 and 11.53%, respectively). The percentage of apoptotic cells increased in comparison to control group (9.31%). The percentage of apoptotic cells was significantly higher after incubation with Geo + DOX (30.11%) compared to DOX and Geo alone. A higher percentage of necrotic cells were seen after incubation of HEp-2 cells with the combination Geo + Dox (Q2 + Q3: 28.57%).

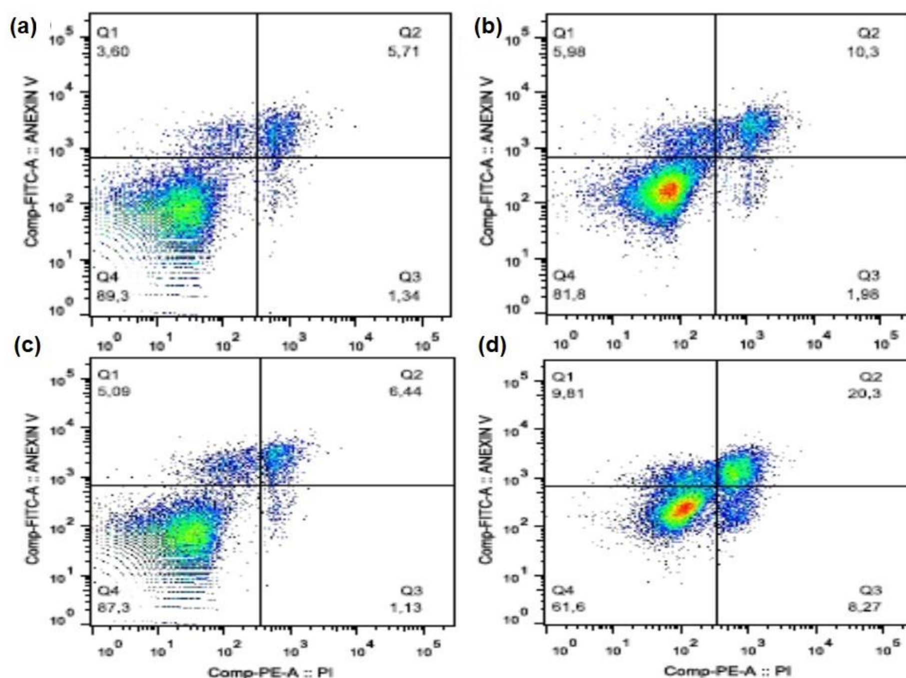


Figure 8. Apoptosis induction in HEp-2 cells using flow cytometry by staining with annexin V/PI. The pseudocolor graphs show **(a)** control; **(b)** Geo (25 µg/mL) **(c)** DOX (1 µM); and **(d)** DOX + Geo (1 µM/25 µg/mL) showing the highest cytotoxicity levels compared to control, as demonstrated by the percentage of early apoptotic cells (AV+), late apoptotic or necrotic cells (AV+/PI+ or PI+). Q1 represent early apoptotic cells; Q2 represents late apoptotic or necrotic cells; Q3 represents necrotic cells; Q4: live cells.

Morphological changes

In order to verify morphological changes, HEp-2 cells were treated with Geo + DOX demonstrated fragmentations into plasma membrane (apoptotic bodies) characteristic of apoptosis and loss membrane integrity characteristic of late apoptotic/necrosis cells. As shown in Figure 9, alterations increased with the combination.

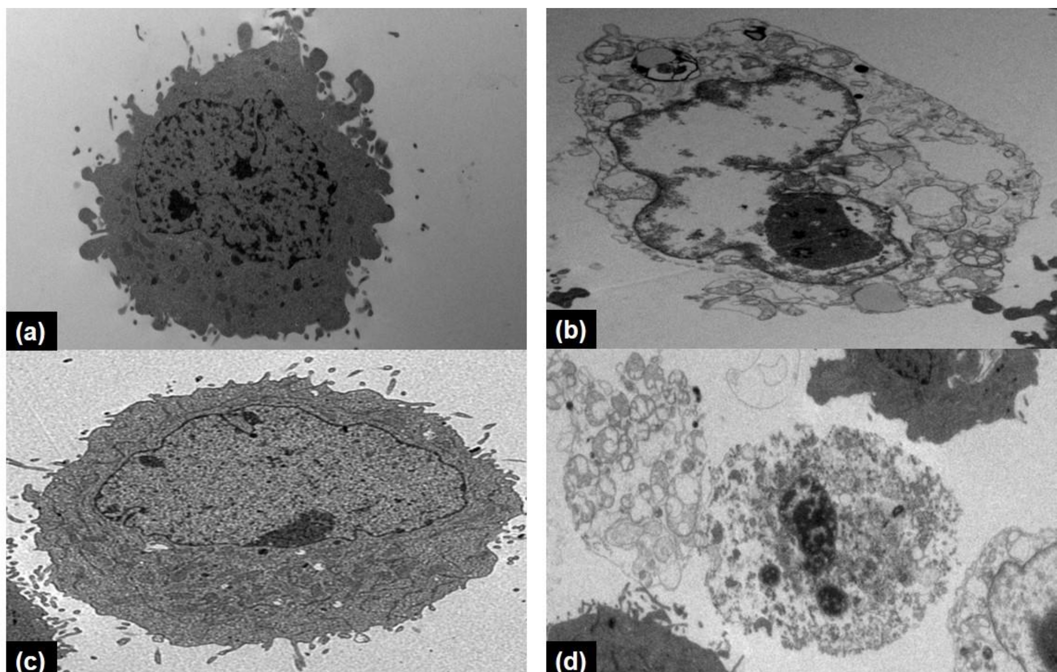


Figure 9. Microscopical analysis of HEp-2 cells. **(a)** control, **(b)** Geo (25 µg/mL), **(c)** DOX 1 µM, and **(d)** combination DOX + Geo (1 µM/25 µg/mL), showing apoptotic/necrotic cells with loss membrane integrity. Scale bars represent 5 µm.

Effects of verapamil on sensitivity of HEp-2 cells to Geo + DOX

The sensitivity of HEp-2 cells to doxorubicin was examined after co-treatment with a P-gp inhibitor: VRP (Figure 10). DOX alone did not influence cell viability, whereas the co-treatment with VRP diminished the

efflux of this drug by inhibiting P-gp, decreasing cell viability.

Geo + DOX decreased cell viability, and the co-incubation with VRP revealed that this combination exerted a similar action to DOX alone.

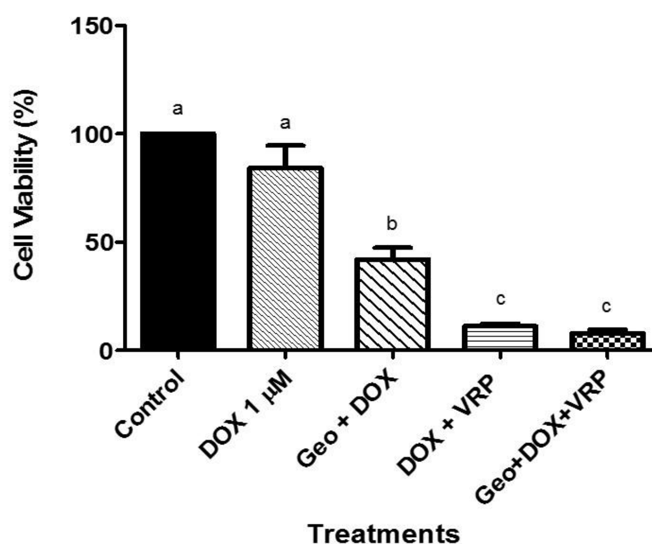


Figure 10. Effects of a P-gp inhibitor (VRP – 5 µM) on the viability (%) of HEp-2 cells incubated with DOX (1 µM), Geo (25 µg/mL), and Geo + DOX. Different letters indicate significant differences between the groups ($p < 0.05$).

Discussion

Drug combination therapies are commonly used in cancer treatment in order to obtain better results and reduce drug resistance.[26] To address these problems, attention has been focused on identifying novel agents that can be combined with conventional antitumor drugs to increase the therapeutic efficacy and decrease side effects. Recently, some studies have reported that some natural agents combined with chemotherapeutic drugs enhanced the anticancer effects against various cell lines.[27-30] In the present study, the effects of Geo, a stingless bee product, combined with DOX, MXT and CARB were investigated on HEp-2 cells. To determine whether Geo

enhanced the anticancer effect of such drugs, HEp-2 cells were treated with Geo alone or in combination with each chemotherapeutic drugs and cell growth, morphological changes, apoptosis and P-gp activity were evaluated using MTT and LDH assay, flow cytometry, transmission electron microscopy and a P-gp inhibitor.

The growth of the HEp-2 cells was reduced by Geo treatment in a time- and dose-dependent manner, showing selectivity against tumor cells compared to non-tumoral Vero cells. LDH leakage assay was also performed as indicator of HEp-2 cytotoxicity. LDH leakage was increased in a dose-dependent manner. Moreover, our data showed that the migration capacity of tumor cells decreased significantly after Geo treatment *in vitro*. The antiproliferative and cytotoxic activity exerted by Geo can be related to the presence of lupeol, amyrins and anacardic acid and derivatives found in our sample.[15] These compounds have already been described by their cytotoxic action against different tumor cell lines.[31-34] Thus, data suggest that the biological effects of Geo may be determined by its chemical composition.

Moreover, the combination Geo + DOX exhibited a higher efficacy by inhibiting HEp-2 cells growth than with CARB and MXT, suggesting that Geo may act differently in combination with different drugs. In this work, Geo enhanced the inhibitory action of DOX against HEp-2 cells; in contrast, it diminished CARB activity.

The antiproliferative potential of different chemotherapeutic agents in association with natural compounds may vary according to the sensitivity and cancer cell lines.[35] DOX is one of the widely used anticancer drugs in the treatment of various malignancies, but its clinical use is limited due to severe side effects to non-tumoral cells.[36,37] The mechanism of action of DOX comprises the inhibition of cell proliferation and induction of apoptosis.[38] However, DOX-mediated cytotoxicity is different towards cancer and normal tissues depending of concentration in vivo and in

vitro.[39,40] The present study showed no cytotoxic effects of DOX (0.5, 1 and 2 μM) on VERO cells and demonstrated the effectiveness of Geo in combination with DOX against HEp-2 cells. It has been reported that differences in DOX-mediated toxicity may be used as an alternative to improve the antitumor therapy with DOX.[29,41]

To study the possible mechanisms involved in the anticancer activity of the combination Geo + DOX, we evaluated induction of apoptosis of HEp-2 cells by flow cytometry. Apoptosis plays a fundamental role in protecting organisms against tumorigenesis.[42] Apoptosis dysregulation is commonly found in cancer cells and its induction has been described as a strategy in cancer therapy.[43-45] Our findings showed that the treatment of HEp-2 cells with Geo in combination with DOX induced apoptosis compared to Geo or DOX alone. These data are in agreement with the images obtained by electron microscopy, showing the presence of apoptotic cells after treatment with Geo + DOX. Additionally, several studies have demonstrated that combination between DOX and different natural products can induce apoptosis.[46-48] Our data indicated for the first time that the combination of Geo extract and DOX exhibited a significant apoptotic potential.

To address whether the combination Geo + DOX could affect P-gp activity, HEp-2 cells were co-treated with VRP. DOX is a P-gp substrate and thus may modulate its expression, inducing cell resistance by increasing the drug efflux.[49] P-gp is expressed by several cells and it is highly specific to eliminate hydrophobic compounds, such as chemotherapeutic agents, taxanes, topoisomerase inhibitors and antimetabolites.[50-51] VRP is a calcium channel blocker and P-gp inhibitor; recently it has been associated with reversion resistance caused by P-gp *in vitro* at concentration of 5-10 μM.[23,52] The treatment concomitantly with VRP increased significantly the sensitivity of HEp-2 cells to DOX, decreasing its efflux. In the presence of VRP, the effects of the combination Geo + DOX were similar to DOX, suggesting that Geo did not affect P-gp-mediated efflux of DOX and

indicating that Geo may affect cell viability by other mechanisms. Similarly, Harbottle *et al*. observed a decreased HEp-2 cell viability after incubation with VRP and DOX, and also with another natural product – curcumin, which had no effects on P-gp.[23] On the contrary, Takara *et al*. suggested that propolis extract inhibited the function of MDR1 and increased sensitivity to substrates of MDR1 in HeLa/TXL cells, and the effects may have been caused by components of the extract other than caffeic acid phenethyl ester (CAPE).[53] Based on our findings *in vitro*, the effect of natural products associated to chemotherapeutic agents may be dependent on the type of agent, the characteristics of the tumor cells and/or the chemical composition of the natural product. The antitumor potential of the combination Geo + DOX should be further investigated to understand its mechanisms of action for clinical chemotherapeutic approaches for cancer treatment.

Conclusions

Our findings have shown that Geo enhanced the anticancer effect of DOX on human laryngeal carcinoma cells *in vitro* by inducing apoptosis, morphological changes including apoptotic bodies, apoptosis/secondary necrosis and membrane dysruption, without interfering on P-gp action.

Declaration

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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