Universidade Estadual de Londrina

Priscila de Matos Cândido-Bacani

"Citotoxicidade, genotoxicidade, mutagenicidade e potencial apoptótico da isatina em células de mamíferos"

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Londrina, 22 de fevereiro de 2010

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Dedico este trabalho

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CÂNDIDO-BACANI, PRISCILA DE MATOS**. "Citotoxicidade, genotoxicidade, mutagenicidade e potencial apoptótico da isatina em células de mamíferos"**. 2010. Dissertação (Mestrado em Genética e Biologia Molecular) – Universidade Estadual de Londrina, Londrina-PR.

RESUMO

A isatina (1H-indole-2,3-dione) é uma molécula sintética versátil, encontrada em várias espécies de plantas medicinais. Várias propriedades farmacológicas e biológicas que podem ser benéficas à saúde humana têm sido atribuídas a isatina, incluindo anticolinesterase, anticonvulsiva, anti-inflamatóra, anti-hipertensiva, anti-hipóxia, antimicrobiana, antiviral e anticâncer. O objetivo do presente estudo foi avaliar a isatina quanto aos seus potenciais citotóxico, genotóxico e mutagênico em testes *in vivo* e as possíveis atividades citotóxica, mutagênica, apoptótica e antiproliferativa em testes *in vitro*. Os testes empregados *in vivo* foram o ensaio cometa e o teste do micronúcleo em células de camundongos. Para a avaliação da genotoxicidade e mutagenicidade, três doses foram testadas: 50, 100 e 150 mg/kg p.c., via gavagem. Estas doses foram selecionadas de acordo com a DL_{50} da isatina (1000 mg/kg p.c.), estimada em um teste preliminar. Os testes empregados *in vitro* foram o ensaio de citotoxicidade (MTT), teste do micronúcleo e índice de apoptose em culturas de células de ovário de hamster Chinês (CHO-K1) e células epiteliais de carcinoma do colo uterino humano (HeLa). As cinco concentrações avaliadas nos testes de mutagenicidade e de apoptose (0,5; 1,0; 5,0; 10,0 e 50,0μM) foram escolhidas através do ensaio MTT. Grupos controle positivo (DXR) e negativo (PBS) também foram incluídos na análise. Os resultados obtidos nos testes *in vivo* mostraram que para todas as doses testadas (50, 100 e 150mg/Kg p.c) não foram observadas alterações na migração do DNA (ensaio do cometa) e nenhum efeito mutagênico foi encontrado no teste do micronúcleo em células do sangue periférico após tratamento agudo com isatina. No entanto, após tratamento com doses repetidas por 14 dias consecutivos, em intervalos de 24 horas, os resultados obtidos em células da medula óssea e sangue periférico de camundongos nos testes do micronúcleo e ensaio do cometa mostraram claramente que somente a maior dose da isatina (150 mg/kg p.c.) foi capaz de induzir alterações no DNA, dando origem a micronúcleos em células da medula óssea e sangue periférico. A isatina não mostrou efeito mutagênico em células CHO-K1 e HeLa nos tempos avaliados (3 e 24 horas). As concentrações de 10 e 50 μM foram capazes de inibir a proliferação celular e promover a apoptose nas duas linhagens celulares (CHO-K1 e HeLa). De acordo com as condições experimentais utilizadas no presente estudo, pode-se observar que os efeitos genotóxico e mutagênico da isatina após tratamento com doses repetidas em teste *in vivo* foram concentração e período de exposição dependentes. Os efeitos citotóxico, apoptótico e antiproliferativo da isatina observados nos testes *in vitro* foram dependentes da concentração e independentes da linhagem celular utilizada. As concentrações da isatina utilizadas no presente estudo poderão ser avaliadas em estudos futuros quanto ao seu potencial farmacológico e determinar a dose limite deste alcalóide em células de mamíferos.

Palavras-chave: isatina, citotoxicidade, genotoxicidade, mutagenicidade e potencial apoptótico

CÂNDIDO-BACANI, PRISCILA DE MATOS. **"Cytotoxicity, genotoxicity, mutagenicity and apoptotic potentials of isatin in mammalian cells"**. 2010. Dissertation (Master in Genetics and Molecular Biology) – Universidade Estadual de Londrina, Londrina-PR.

ABSTRACT

Isatin (1H-indole-2,3-dione) is a synthetically versatile molecule, found in various medicinal plants species. Several pharmacological and biological properties that may be beneficial to the health of humans have been ascribed to isatin, including anticholinesterase, anticonvulsant, anti-inflammatory, anti-hypertensive, antihipoxia, antimicrobial, antiviral and anticancer. The aim of the present study was to assess cytotoxic, genotoxic and mutagenic potentials of isatin in *in vivo* tests and the possible cytotoxic, mutagenic, apoptotic and antiproliferative activities in *in vitro* tests. The tests employed *in vivo* were comet assay and micronucleus test in mice cells. For genotoxic and mutagenic evaluation, three different doses were tested: 50, 100 and 150 mg/kg b.w., via gavage. These doses were selected according to the LD_{50} of isatin (1000 mg/kg b.w.), estimated in a preliminary test. The tests employed *in vitro* were MTT assay, micronucleus test and apoptosis index in cultures of Chinese hamster ovary cells (CHO-K1) and human cervical cancer cells (HeLa). The five concentrations evaluated in the mutagenicity and apoptosis tests (0.5, 1.0, 5.0, 10. and 50.0μM) were chosen through MTT assay. Positive (DXR) and negative (PBS) control groups were also included in the analysis. The results obtained in *in vivo* tests showed that for all tested doses (50, 100 and 150mg/kg b.w.) it was not observed alterations in DNA migration (comet assay) and no mutagenic effect was found in the micronucleus test in peripheral blood cells after acute treatment with isatin. Nevertheless, after repeated doses treatment for 14 days consecutives with 24 hour intervals, the results obtained in the in bone marrow and peripheral blood cells of mice in the micronucleus tests and comet assay showed clearly that only the highest dose of the isatin (150 mg/kg b.w.) was able to induced alterations in DNA giving rise to micronuclei in the bone marrow and peripheral blood cells of the treated mice. Isatin did not show mutagenic effect in CHO-K1 and HeLa cells in all times evaluated (3 and 24 hours). Nevertheless, the concentrations 10 and 50 μM were able of inhibit the cell proliferation and promote apoptosis both CHO-K1 and HeLa cells. According to the experimental conditions used in this study, it may be observed that the mutagenic and genotoxic effects of isatin after repeated doses treatment in *in vivo* tests depend on the alkaloid concentration and period of exposition used. The cytotoxic, apoptotic and antiproliferative effects of isatin observed in *in vitro* tests were concentration-dependent and cell line independent. The concentrations of isatin used in the present study should be used in future studies to evaluate its properties in terms of pharmacological potential and determine the safe dose-limit this alkaloid in mammal cells.

Key Words: isatin, cytotoxicity, genotoxicity, mutagenicity and apoptotic potential

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1 INTRODUÇÃO

1.1 Considerações gerais sobre mutagênese

No decorrer da vida o DNA sofre alterações denominadas mutações, que podem ser causadas por erros durante a duplicação do material genético ou durante a divisão celular (RIBEIRO e MARQUES, 2003). Substâncias químicas, físicas e biológicas capazes de causar tais mudanças são chamadas de mutagênicas, e a ciência que estuda o processo de indução de danos no DNA pela ação de tais agentes é conhecida como mutagênese (HODGSON, 2004).

Os agentes mutagênicos que causam danos no DNA podem ser classificados como: a) aqueles que atuam diretamente sobre a molécula; b) agentes indiretos, que precisam ser metabolizados para que seus metabólitos causem danos; c) os que promovem a produção de espécies reativas de oxigênio e d) aqueles que causam inibição no reparo e na síntese do DNA (LEE e STEINERT, 2003).

Agentes que interagem com o DNA e ou com seus componentes celulares (fibras do fuso) e enzimas (topoisomerases) são conhecidos como genotóxicos. A genotoxicidade refere-se à capacidade de tais agentes causarem lesões genômicas que são passíveis de correção por vias de reparo do DNA nas células. No entanto, a mutagenicidade é uma alteração permanente no conteúdo ou na estrutura do material genético de um organismo. O termo refere-se especificamente à indução de mutação ao nível gênico ou cromossômico (DEARFIELD et al., 2002).

Segundo Zaha (1996) as mutações são modificações súbitas e hereditárias no conjunto gênico de um organismo que não são explicáveis pela recombinação da variabilidade genética pré-existente. Estas alterações podem ser decorrentes de processos celulares normais ou de interações aleatórias com o ambiente. Tais alterações são denominadas de mutações espontâneas e a freqüência na qual elas ocorrem é característica para cada organismo em particular. A ocorrência de mutações pode ser aumentada pela exposição do organismo a agentes mutagênicos e as alterações que eles causam são definidas como mutações induzidas (LEWIN, 2001).

A mutação é a principal fonte de toda a variação genética, e é fundamental para o processo evolutivo. Sem as mutações, não há variabilidade genética e assim não há evolução (SNUSTAD e SIMMONS, 2001; RIBEIRO e MARQUES, 2003). Apesar dessa grande relevância evolutiva, a maioria das mutações pode ter efeitos prejudiciais, incluindo malformações, desenvolvimento de câncer, envelhecimento e morte (ERDTMANN, 2003).

Kirsch-Volders et al. (2002) classificam as mutações em três níveis: as mutações gênicas, as aberrações cromossômicas estruturais, como as deleções, duplicações, inversões e translocações e as aberrações cromossômicas numéricas, como as aneuploidias e euploidias. Estas são originadas a partir de perdas cromossômicas e defeitos na citocinese. As aberrações cromossômicas numéricas contribuem para um grande número de abortos, morte pré-natal e anomalias congênitas em recém-nascidos. Análises cromossômicas de embriões provenientes de abortos espontâneos indicam que 50% deles contêm anormalidades genéticas (NATARAJAN, 2002).

As manifestações das mutações dependem da natureza celular que ela ocorre: somática ou germinativa (RABELLO-GAY et al., 1991). Essas mutações podem ser expressas em genes individuais, grupo de genes ou em nível cromossômico (DEARFIELD et al., 2002). A indução de mutações em células germinativas pode resultar no aumento da frequência de doenças genéticas, baixa fertilidade, proles defeituosas e abortos (MALLING, 2004). As mutações em células somáticas, por sua vez, apresentam um papel relevante na patogênese de doenças crônicas degenerativas, como arteriosclerose e doenças cardiovasculares, podem ser à base do processo de envelhecimento, além de estar relacionadas com o processo de carcinogênese (DE FLORA et al., 1996; RIBEIRO e MARQUES, 2003; DE FLORA e FERGUNSON, 2005).

Embora não seja medida de carcinogenicidade, a mutagenicidade é freqüentemente associada ao aparecimento do câncer (AZEVEDO et al., 2003). O aumento de danos no DNA, quebras ou perdas cromossômicas são importantes fatores para o desenvolvimento de muitos tipos de câncer (HAGMAR et al., 1998; BONASSI et al., 2000).

O processo de carcinogênese é resultante do acúmulo de lesões genéticas (DIAZ, 2005). As mais importantes alterações genéticas no câncer ocorrem nos genes que controlam a proliferação celular (proto-oncogenes e genes supressores tumorais), resultando em um crescimento descontrolado característico de uma célula maligna. Além disso, há ainda o descontrole dos genes associados ao processo de reparo de danos no DNA, os quais, quando inativos, podem elevar a taxa de mutação das células, acelerando ainda mais o acúmulo de alterações moleculares importantes (OJOP e NETO, 2004).

1.2 Plantas medicinais

A natureza é uma fonte atrativa de compostos farmacologicamente ativos, candidatos ao tratamento de diversas patologias, devido à ampla diversidade química encontrada nas milhões de espécies de plantas, animais e microorganismos (ROCHA et al., 2001).

Atualmente, muitos estudos de produtos naturais são contínuos e focalizados em descobrir drogas para as mais diversas áreas terapêuticas (VAN ELSWIJK; IRTH, 2003). Segundo Newman et al. (2003), das drogas desenvolvidas a partir de produtos naturais, 60 e 70% são utilizadas, respectivamente, no combate ao câncer e a doenças infecciosas.

Produtos naturais, sobretudo os de origem vegetal, tem sido consistentemente uma importante fonte de agentes terapêuticos para o desenvolvimento de novos fármacos (NEWMAN et al., 2003; CALIXTO, 2005). Estima-se que aproximadamente 25% dos fármacos modernos são desenvolvidos direta ou indiretamente, de fontes naturais, especialmente de plantas (CALIXTO, 2001a; LIU e WANG, 2008).

Uma planta medicinal pode ser definida como qualquer vegetal que produza, em quantidade considerável, substâncias biologicamente ativas utilizadas direta ou indiretamente como medicamento (CASTRO et al., 2004). A utilização de plantas medicinais ou seus componentes ativos para tratamento, cura e prevenção de doenças, tem sido extensamente relatado ao longo dos anos (RIBEIRO e SALVADORI, 2003; SIMÕES et al., 2004; NEWMAN, 2006). Segundo a OMS, 80% da população mundial fizeram ou fazem uso de medicamentos derivados de plantas medicinais (NICOLETTI et al., 2007).

A importância das plantas medicinais deve-se à sua contribuição como fonte natural de fármacos e por proporcionar grandes chances de obter-se uma molécula protótipo devido à diversidade de constituintes presentes nestas (YUNES e CALIXTO, 2001).

Segundo Cechinel Filho e Yunes (1998) a avaliação do potencial terapêutico de plantas medicinais e de alguns de seus componentes ativos, tais como flavonóides, alcalóides, triterpenos, taninos, lignanas, entre outros, tem sido objeto de estudos na busca de novos fármacos. Muitas destas substâncias têm grandes possibilidades de futuramente virem a ser aproveitadas como agentes medicinais.

Nesse sentido, as plantas medicinais constituem uma fonte renovável de onde podem ser obtidos novos e eficazes medicamentos. A indicação de plantas medicinais aumenta a opção terapêutica, ofertando medicamentos equivalentes, com espectros de ação mais adequados e, talvez, com indicações terapêuticas complementares às medicações existentes,

sem substituir os medicamentos já comercializados e registrados (DI STASI, 1995; SIMÕES et al., 2004).

Erroneamente acredita-se que uma planta medicinal, ou produtos derivados de plantas medicinais, por serem naturais, são livres de efeitos colaterais ou adversos. Isto porque a maioria das informações disponíveis sobre o consumo dessas plantas não tem nenhum suporte científico e seu uso é baseado somente na cultura popular (LOPES et al., 2000).

A detecção e avaliação de efeitos citotóxicos, mutagênicos e carcinogênicos de compostos de origem vegetal e o entendimento dos benefícios e/ou toxicidade potencial dessas plantas à saúde, são de fundamental importância para reduzir os possíveis riscos desses agentes. Assim, o desenvolvimento de novas drogas bioativas necessita de modelos apropriados para a identificação de alvos moleculares que sejam fundamentais no crescimento celular, seja *in vitro* ou *in vivo* (LODISH et al., 1999).

Deste modo, estudos de mutagenicidade e genotoxicidade ajudam na avaliação da segurança e efetividade dos produtos naturais (BAST et al., 2002). Tanto nos modelos *in vivo* como *in vitro* podemos listar vantagens e desvantagens, porém, ambos são necessários quando se pretende comprovar a ação farmacológica de um determinado extrato e/ou princípio ativo e, sobretudo, para elucidar o mecanismo de ação de extratos totais ou de princípios ativos isolados de plantas (CALIXTO, 2001b).

1.3 Alcalóides de plantas medicinais

Os alcalóides constituem um vasto grupo de metabólitos secundários com grande diversidade estrutural, representando cerca de 20% das substâncias naturais descritas. Podem ser definidos como compostos nitrogenados cíclicos de origem natural, contendo um nitrogênio em um estado de oxidação negativo. São farmacologicamente ativos e, na sua grande maioria, possuem caráter alcalino (HENRIQUES et al., 2004).

As fontes mais reconhecidas de alcalóides são os vegetais (CASTRO et al., 2004). Aproximadamente 20% das espécies vegetais possuem alcalóides, muitos dos quais fazem parte do arsenal químico das plantas contra estresse biótico ou abiótico, atuando como protetores das mesmas (KUTCHAN, 1995). São encontrados em representantes de todos os grupos vegetais, apresentando distribuição restrita nas talófitas, pteridófitas e nas gimnospermas. Sua maior ocorrência é verificada nas angiospermas (HENRIQUES et al., 2004).

Muitos alcalóides são reconhecidos por seu potencial mutagênico (BELJANSKI e BELJANSKI, 1982; HENRIQUES et al., 1991; TAIRA et al., 1997; BOEIRA et al., 2001; ANSAH et al., 2005), porém, muitos deles possuem uma série de propriedades farmacológicas de grande interesse, apresentando atividades antimicrobiana, antiplasmodial, antidepressiva, anti-hipertensiva e antitumoral (FREDERICH et al., 1999; HENRIQUES et al., 2004).

Devido ao elevado número de atividades biológicas atribuídas aos alcalóides originados de plantas, estes são utilizados há séculos na medicina popular e, nos dias atuais, como matéria-prima para a síntese de fármacos e cosméticos (YUNES e CECHINEL FILHO, 2001; AMARAL et al., 2006).

1.3.1 Alcalóide Isatina

A isatina (1H-indole-2,3-dione) e seus derivados são compostos heterocíclicos com considerável versatilidade sintética (Figura 1). São matérias primas importantes na síntese de fármacos e corantes, podendo ser utilizadas na obtenção de diversos sistemas heterocíclicos, como derivados indólicos e quinolínicos. A isatina foi descoberta primeiramente por Erdmann e Laurent em 1840, como um produto formado a partir da oxidação do índigo, usando ácidos nítrico e crômico (SILVA et al., 2001).

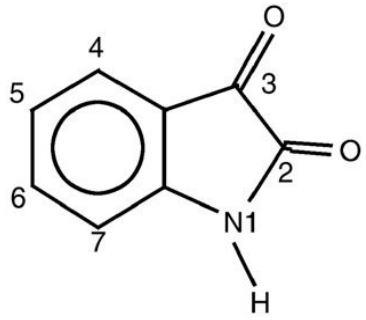


Figura 1: Estrutura química da isatina (MATHEUS et al., 2007).

Esta classe de alcalóides possui interessantes atividades biológicas e farmacológicas (MEDVEDEV et al., 2005; MEDVEDEV et al., 2007). A isatina e seus derivados apresentam uma série de propriedades biológicas, incluindo anticolinesterase, anticonvulsiva, anti-inflamatória, anti-hipertensiva, anti-hipoxia, antimicrobiana, anti-viral e antineoplásica. Também foram descritas atividades mutagênica e tóxica para estas substâncias (SILVA et al., 2001).

Em artigos de revisão, Silva et al. (2001) e Vine et al. (2009) citam diversos trabalhos mostrando que, na natureza, a isatina é encontrada em plantas do Gênero *Isatis,* em *Couroupita guianensis* Aubl (Figura 2) e como componente da secreção da glândula parótida do sapo Bufo. Vários derivados da isatina também foram identificados em espécies de plantas, fungos e moluscos marinhos, como por exemplo, o alcalóide melosatin (metoxi phenylpentyl isatins) obtido a partir da espécie *Melochia tomentosa* encontrada no Caribe. De acordo com Maugard et al. (2001), a isatina também pode ser encontrada nas espécies de plantas *Polygonum tinctoria* (na China e Coréia), *Isatis tinctoria* (encontrada na Europa) (Figura 3) e espécies pertencentes ao gênero *Indigofera* (espécies tropicais encontradas na África, Ásia e América do Sul) (Figura 4).

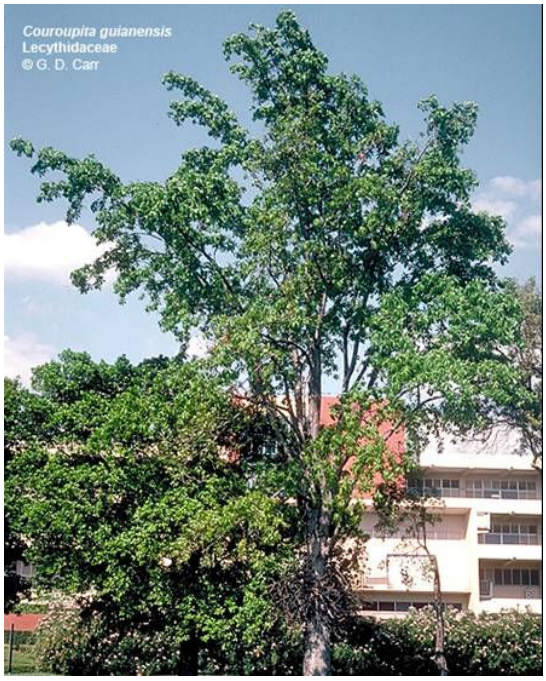


Figura 2: *Couroupita guianensis* Aubl (Nome popular: Bola-de-canhão).
(http://images.google.com.br/).

Figura 3: *Isatis tinctoria* (Nome popular: Pastel-dos-tintureiros).
(http://upload.wikimedia.org).

Figura 4: *Indigofera suffruticosa* (Nome popular: Anileira).
(www.luminousgreen.net).

No Brasil, a triagem fitoquímica dos diferentes extratos das espécies pertencentes ao gênero *Indigofera* foi realizada no projeto "*Uso sustentável da biodiversidade brasileira: prospecção químico-farmacológica em plantas superiores*" (Biota/FAPESP) e indicou este gênero como portador de diversificadas classes de substâncias (flavonóides, ácidos fenólicos, terpenos e alcalóides). Dentre as substâncias que podem ser isoladas das espécies do gênero *Indigofera* destacam-se a isatina e seus derivados, como os alcalóides bis-indólicos (indigóides) indirubina e índigo (CALVO, 2007).

A Figura 5 mostra os passos da biossíntese dos pigmentos indigóides em plantas. Quando as folhas envelhecem os precursores "isatan" e "indican" são hidrolisados. O indoxil liberado neste processo é oxidado espontaneamente pelo oxigênio, gerando o índigo. A isatina é gerada paralelamente ao indoxil. A condensação de indoxil com a isatina produz a indirubina (MAUGARD et al., 2001).

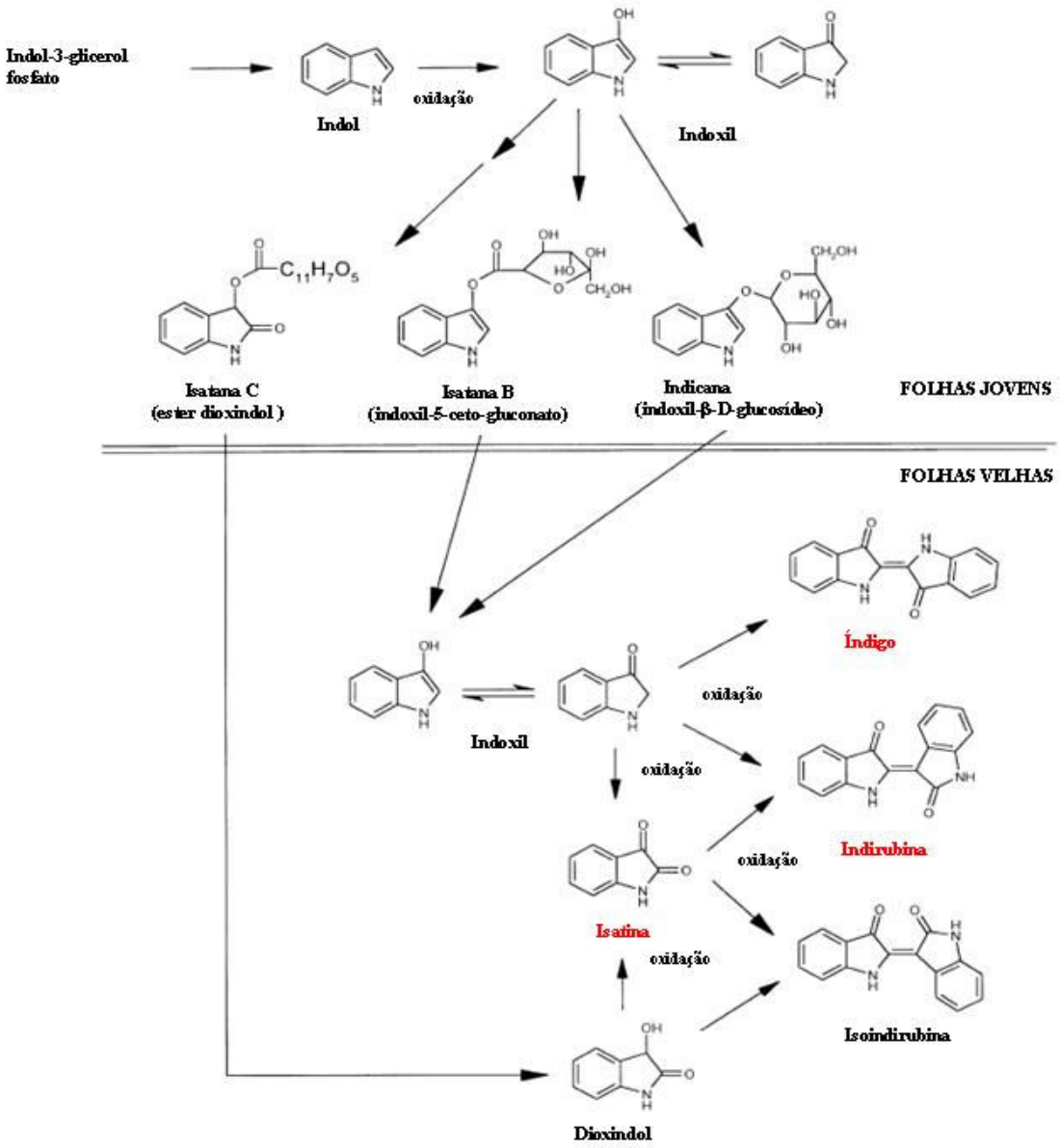


Figura 5: Biossíntese dos pigmentos indigóides em plantas (MAUGARD et al., 2001).

1.4 Sistemas-teste *in vivo* e *in vitro*

1.4.1 Teste do micronúcleo em células da medula óssea e sangue periférico de camundongos

Os testes *in vivo* são importantes na avaliação dos riscos de genotoxicidade de substâncias químicas porque permitem a consideração de aspectos envolvidos na metabolização da substância em estudo, parâmetros farmacocinéticos (ativação, detoxificação e distribuição aos tecidos) e também o processo de reparo do DNA. São muito utilizados na investigação de substâncias que tiveram efeitos mutagênicos detectados nos testes *in vitro* (KRISHNA e HAYASHI, 2000). Dessa maneira, o metabolismo, incluindo a detoxificação, é uma característica fundamental na determinação da genotoxicidade das substâncias testadas e a ativação metabólica pode ser um parâmetro diferencial nos experimentos *in vivo* e *in vitro* (TWEATS et al., 2007).

Dentre os métodos utilizados para avaliar a mutagenicidade de compostos *in vivo*, os testes do micronúcleo em células da medula óssea e sangue periférico de camundongos tem sido amplamente utilizados e aceitos pelas agências reguladoras e comunidade científica (STOPPER e MULLER, 1997; MATEUCA et al., 2006) para a avaliação e o registro de novos produtos químicos e farmacêuticos que entram anualmente no mercado mundial (CHOY, 2001).

Segundo Fenech (2000), o teste do micronúcleo em células da medula óssea e do sangue periférico é um dos mais bem estabelecidos testes citogenéticos na área da genética toxicológica. Ambos detectam alterações genômicas e/ou danos no aparato mitótico, sendo os micronúcleos originados, indicativos de perdas irreversíveis de material genético (VALADARES et al., 2007).

O micronúcleo (MN) se constitui em pequena massa de cromatina separada do núcleo principal, formada durante a telófase da mitose ou meiose; é resultante de fragmentos de cromossomos acêntricos, que podem ser originados de quebras cromatídicas, ou de cromossomos inteiros que não foram incluídos no núcleo principal (FENECH et al., 1997; FENECH et al., 1999).

Embora os micronúcleos possam ser originados espontaneamente, a sua indução é comumente usada para se detectar danos clastogênicos (quebras cromossômicas) ou aneugênicos (aneuploidia ou segregação cromossômica anormal) resultantes de exposição a

agentes mutagênicos (Figuras 6 e 7), (FENECH, 2000; AARDEMA e KIRSCH-VOLDERS, 2001).

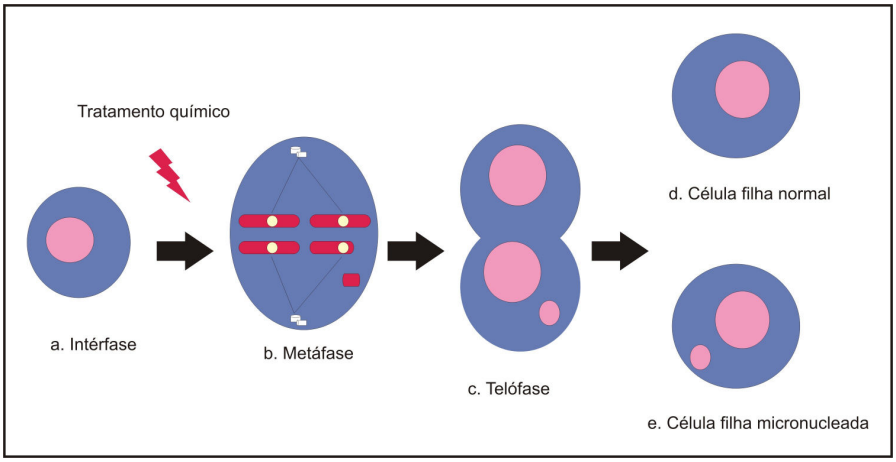


Figura 6: Formação de uma célula micronucleada por clastogênese, contendo um fragmento cromatídico acêntrico (AARDEMA e KIRSCH-VOLDERS, 2001 modificado por CÂNDIDO-BACANI, 2009).

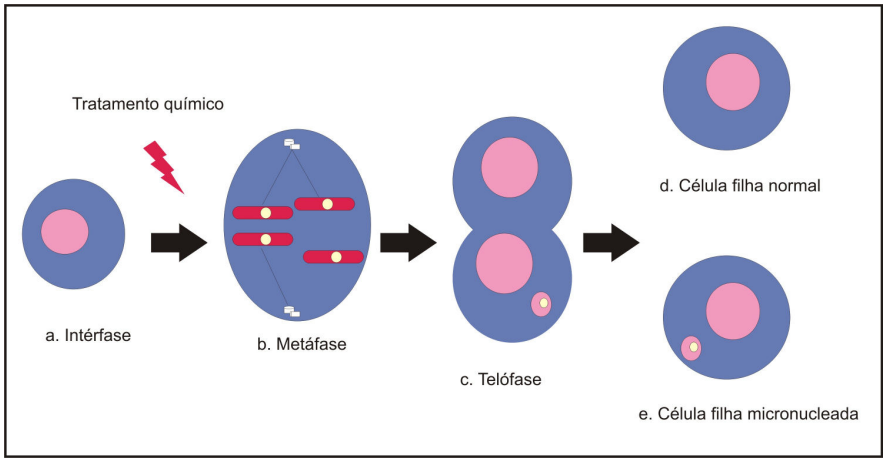


Figura 7: Formação de uma célula micronucleada por aneugênese, contendo um cromossomo inteiro (AARDEMA e KIRSCH-VOLDERS, 2001 modificado por CÂNDIDO-BACANI, 2009).

O teste do Micronúcleo foi inicialmente desenvolvido na década de 70 por Schmid em eritrócitos policromáticos da medula óssea de camundongos (Figura 8). Os eritrócitos policromáticos (eritrócitos jovens) são células alvo para a avaliação da freqüência de micronúcleos, pois quando os eritroblastos expelem seus núcleos e se transformam em

eritrócitos, estes permanecem no citoplasma, onde são facilmente identificáveis, devido à sua morfologia arredondada e coloração características (SCHMID, 1975). Essas células apresentam um tempo de vida relativamente curto, de modo que qualquer micronúcleo que ela contenha deve ter sido gerado como resultado de danos cromossômicos induzidos recentemente (RIBEIRO, 2003).

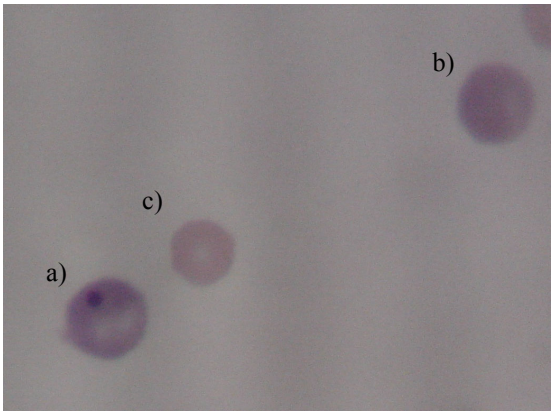


Figura 8: (a) eritrócito policromático com micronúcleo (b) eritrócito policromático sem micronúcleo (c) eritrócito normocromático normal. Coloração: Leishman eosin-blue. Microscopia óptica, aumento de 1000x. FOTO: CÂNDIDO-BACANI, 2009.

Em 1990 Hayashi et al. modificaram a técnica original e criaram um método mais prático e rápido para detecção de micronúcleos usando eritrócitos do sangue periférico de roedores, os reticulócitos. Neste método as células são coradas com laranja de acridina, o que permite a fácil distinção dos reticulócitos com micronúcleos, os quais coram-se de verde devido à presença de DNA em seu interior, enquanto que os reticulócitos sem micronúcleo coram-se de vermelho por serem ricos em RNA (Figura 9). Esta técnica apresenta algumas vantagens quando comparada com o ensaio do micronúcleo em células da medula óssea de camundongos. A população celular no sangue periférico é mais uniforme e mais fácil de ser analisada; a simplicidade da preparação das amostras de sangue que podem ser retiradas repetidamente da cauda dos animais, permitindo assim, que um mesmo animal possa fornecer várias amostras de material, sem a necessidade de eutanásia (HAYASHI et al., 1990).

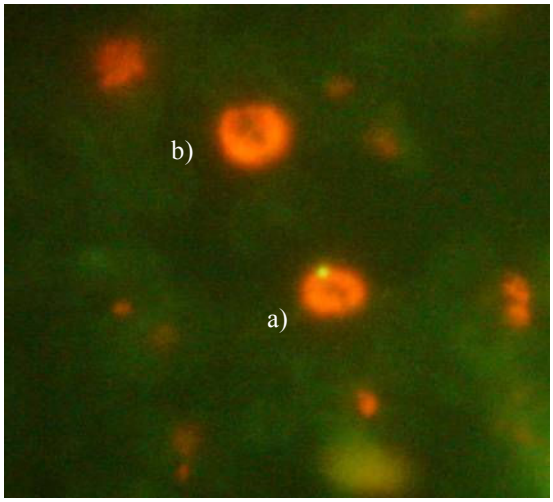


Figura 9: (a) reticulócito com micronúcleo (b) reticulócito sem micronúcleo. Coloração: Laranja de acridina. Microscopia de fluorescência, aumento 1000x. FOTO: CÂNDIDO-BACANI, 2009.

1.4.2 Teste do micronúcleo *in vitro* com Bloqueio de Citocinese (MNCtB)

Os testes citogenéticos *in vitro*, juntamente com os testes de mutação gênica em bactérias (teste de Ames) e em células de mamíferos, tem fornecido informações importantes na determinação do potencial mutagênico de agentes químicos e físicos (SALVADORI et al., 2003).

O teste do micronúcleo *in vitro* é amplamente utilizado na pesquisa, indústria e laboratório para a detecção do potencial mutagênico de compostos aneugênicos e clastogênicos e tem se tornado uma alternativa aos testes de aberrações cromossômicas *in vitro* (KIRSCH-VOLDERS, 1997). Segundo Kirsch-Volders et al. (2003), o teste do micronúcleo *in vitro* tem se mostrado eficiente em diferentes linhagens celulares. Como a maioria das linhagens celulares não tem capacidade de metabolização de drogas (PRESTON, 1997), um resultado positivo encontrado neste sistema-teste indica um composto mutagênico de ação direta.

A versão *in vitro* do teste do micronúcleo foi proposta inicialmente por HEDDLE (1976) para detectar agentes carcinogênicos, utilizando cultura de linfócitos periféricos humanos. Em 1985, Fenech e Morley desenvolveram um método simples e eficaz para detecção de micronúcleos *in vitro*, com bloqueio da citocinese, utilizando citocalasina B (CtB). Esta substância é isolada do fungo *Helminthosporium dermatioideum*, que bloqueia a citocinese, por inibir a polimerização de filamentos de actina durante a mitose, requerida para a formação do anel de microfilamentos que induzem a contração do citoplasma e clivagem da célula em duas células filhas (FALCK et al., 1997).

O uso da CtB leva o bloqueio da citocinese, mas não da divisão nuclear, resultando em um acúmulo de células binucleadas a partir de células que sofreram apenas uma divisão celular, independente do grau de sincronia e da proporção de células em divisão (SALVADORI et al., 2003). Vale ressaltar que a CtB pode levar até seis horas para exercer sua funcionalidade (FENECH, 2000).

Segundo Fenech (2000), para analisar a freqüência de micronúcleos em células binucleadas, algumas características devem ser levadas em consideração no momento da análise, para que uma estrutura seja caracterizada como um micronúcleo. Os micronúcleos devem possuir morfologia e coloração semelhantes à do núcleo principal, não serem birrefringentes, devem estar no mesmo plano do núcleo principal, não devem estar ligados a um dos núcleos principais e apresentar diâmetro de até 1/3 do núcleo principal (Figura 10).

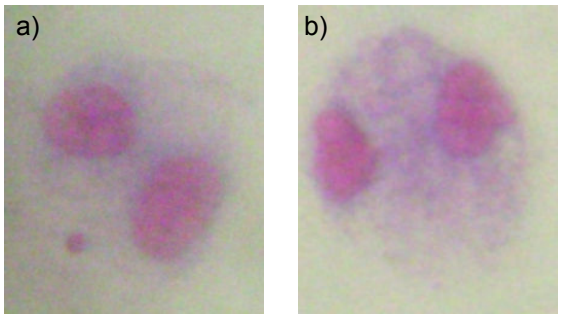


Figura 10: (a) célula CHO-K1 binucleada com micronúcleo, (b) célula CHO-K1 binucleada sem micronúcleo. Coloração Giemsa 5%. Microscopia óptica, aumento de 1000x. FOTO: CÂNDIDO-BACANI, 2008.

O ensaio do micronúcleo *in vitro* com bloqueio da citocinese foi originalmente desenvolvido como um sistema teste ideal para detecção de micronúcleos em células binucleadas. No entanto, também pode ser usado para avaliar pontes nucleoplásmáticas, brotamentos nucleares, morte celular (necrose ou apoptose) e taxa de divisão nuclear, através da contagem de células mono, bi e multinucleada (FENECH, 2006) (Figura 11).

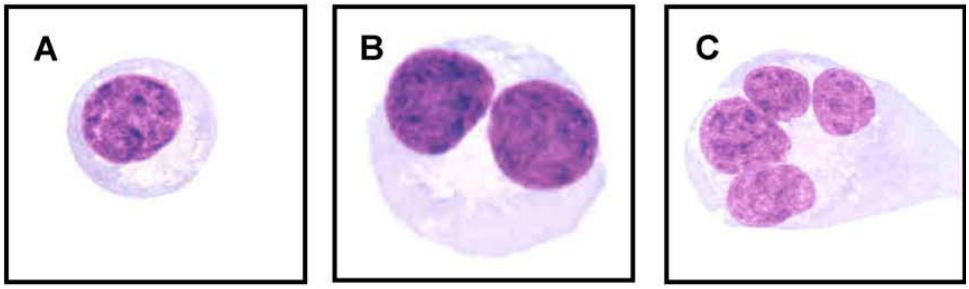


Figura 11: (a) célula mononucleada, (b) célula binucleada, (c) célula tetranucleada. (FENECH, 2006).

Este teste apresenta algumas vantagens e desvantagens. As vantagens incluem sensibilidade e precisão, baixo custo e detecção de perda cromossômica (FENECH, 1997). As desvantagens se referem à necessidade de divisão celular para a análise de MN, já que esta técnica só é efetiva em populações de células em constante divisão; a citocalasina-B pode causar interferência na detecção de compostos que podem inibir a citocinese ou a polimerização dos filamentos de actina (SALVADORI et al., 2003), além do ensaio não fornecer informações sobre os tipos de aberrações cromossômicas estruturais ocorridas (CORVI et al., 2008).

1.4.3 Ensaio do Cometa

O teste do cometa, também conhecido como SCGE ("single cell gel electrophoresis"), foi inicialmente descrito por Östling e Johanson em 1984 como uma técnica capaz de visualizar danos ocasionados no DNA em células individuais, sob condições eletroforéticas em pH neutro (FAIRBAIRN et al*.,* 1995; COLLINS et al., 2008). Poucos anos depois, Singh et al. (1988) desenvolveram um método similar, mas utilizando condições alcalinas.

A versão alcalina (pH>13 do tampão de eletroforese) combina a simplicidade de técnicas bioquímicas para detectar quebras de fita simples e dupla do DNA, sítios álcali lábeis (sítios apurínicos e apirimidínicos), pontes entre DNA-DNA, DNA-proteína (PFUHLER e WOLF, 1996; MERK e SPEIT, 1999; HARTMANN et al*.,* 2003; MORLEY et al., 2006).

O princípio básico do teste do cometa é a migração do DNA em uma matriz de agarose sob condições eletroforéticas. Quando observadas em microscópio de fluorescência, as células têm a aparência de um cometa, com cabeça (a região nuclear) e uma cauda contendo os fragmentos de DNA que migram em direção ao pólo positivo (HARTMANN et al*.,* 2003). Os cometas podem ser corados com qualquer corante DNA-específico, sendo os mais utilizados os fluorescentes (HARTMANN et al., 2001).

Segundo Kobayashi et al. (1995) os cometas podem ser analisados visualmente e classificados em quatro classes, de acordo com o tamanho da cauda: classe O - nucleóide sem dano, não apresenta cauda; classe 1 – dano pequeno, nucleóide com cauda menor que o diâmetro do nucleóide; classe 2 – dano médio, nucleóide com cauda de tamanho entre 1 a 2 vezes o diâmetro do nucleóide; classe 3 – dano máximo, nucleóide com cauda 2 vezes maior que o diâmetro do nucleóide (Figura 12).

O teste do Cometa é utilizado para detectar lesões genômicas que, após serem processadas, podem resultar em mutação. Diferente das mutações, as lesões detectadas pelo teste do cometa são passíveis de reparo. Uma vez que danos no DNA são freqüentemente célula e tecido específicos, uma metodologia como o teste do cometa que permite a detecção de danos e seu reparo em uma única célula, e conseqüentemente, em determinada subpopulação celular, é amplamente utilizado para avaliar o potencial genotóxico de diversos compostos (GONTIJO e TICE, 2003).

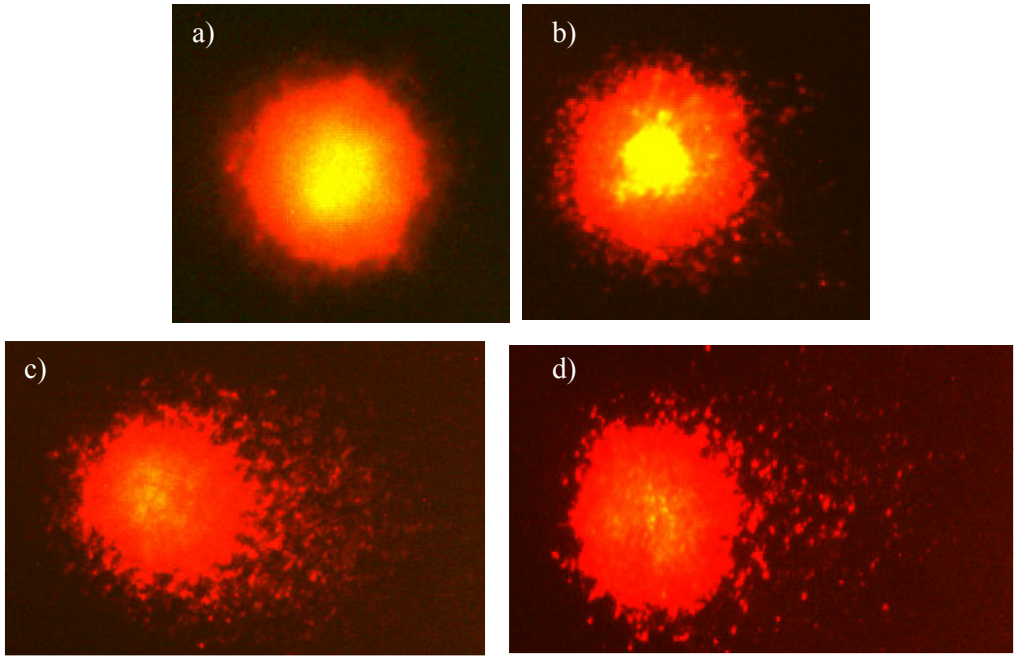


Figura 12: Critérios de classificação dos danos no DNA em células do sangue periférico de camundongos no teste do cometa. (a) classe 0, (b) classe1, (c) classe 2, d) classe 3. Coloração: Brometo de Etídio. Microscopia de fluorescência, aumento 1000x. FOTO: FOGAÇA, 2009.

O ensaio cometa é um método rápido e sensível para a detecção de danos primários no DNA em células individuais (FAIRBAIRN et al., 1995; COLLINS et al., 1997). É amplamente empregado em estudos de genética toxicológica, biomonitoramento ambiental e de populações humanas expostas a agentes genotóxicos. Este teste se constitui em uma ferramenta valiosa para investigar danos e reparo no DNA em diferentes tipos celulares em sistemas *in vivo* e *in vitro* em resposta a agentes que causam lesões no DNA (COLLINS, 2004; DUSINSKA e COLLINS, 2008).

O teste possui vantagens como simplicidade, rapidez e baixo custo. Pode ser utilizado para distinguir entre danos genotóxicos ou citotóxicos *in vitro* ou entre carcinógenos de ação genotóxica ou não genotóxica *in vivo* (TICE et al*.,* 2000).

A versão *in vivo* do teste tem sido cada vez mais utilizada em ensaios genotoxicidade. As vantagens do teste do cometa *in vivo* incluem a aplicabilidade a diferentes tecidos e/ou células específicas, sensibilidade para a detecção de baixos níveis de dano ao DNA, uso de pequeno número de células por amostra, fácil aplicação, curto período de tempo para concluir o experimento, simplicidade e baixo custo (TICE et al., 2000; BRENDLER-SCHWAAB et al., 2005).

1.4.4 Ensaio de citotoxicidade – MTT

De acordo com o *guideline* da OECD (2004) (Organization for Economic Co-operation and Development) para testar substâncias químicas usando o teste do micronúcleo *in vitro*, devem ser avaliadas, no mínimo, três concentrações diferentes da droga. Estas deverão ser escolhidas dos dados obtidos de estudos preliminares sobre citotoxicidade.

Os testes de citotoxicidade *in vitro* são importantes para se verificar a toxicidade de novos compostos, principalmente quando se está verificando sua aplicabilidade como agente terapêutico (MELO et al., 2000). Entre os diferentes testes que podem ser utilizados na avaliação da citotoxicidade de um composto, destaca-se o ensaio MTT proposto por Mosmann em 1983. O ensaio MTT tem sido amplamente utilizado para determinar a citotoxicidade, viabilidade celular e proliferação de células vivas, após a exposição a substâncias tóxicas. Pode ser também utilizado para caracterização da toxicidade de novos fármacos (MOSMANN, 1983; BERRIDGE e TAN, 1993; LIU et al., 1997; BERRIDGE et al., 2005).

No ensaio do MTT, o sal de tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) é facilmente incorporado por células viáveis, que reduzem este composto em suas mitocôndrias pela ação da enzima succinato desidrogenase, uma enzima do ciclo de Krebs. O método envolve a conversão, pelas células, do MTT de coloração amarela em cristais de formazan de coloração roxa. Após a adição de dimetilsulfóxido (DMSO), os cristais são solubilizados, liberados e quantificados colorimetricamente, através de espectrofotometria tipo ELISA (COLLIER e PRITSOS, 2003; VELLONEN et al., 2004).

A capacidade das células em reduzirem o MTT fornece uma indicação da atividade e da integridade mitocondrial, que são interpretadas como medidas da viabilidade celular. Ao contrário de outros ensaios de citotoxicidade, o ensaio do MTT apresenta algumas vantagens, como rapidez e precisão (MOSMANN, 1983).

1.4.5 Apoptose em Cultura de Células de Mamíferos *in vitro*: Análise morfológica com coloração diferencial por Laranja de Acridina / Brometo de Etídio

Apoptose ou morte celular programada é um processo essencial para a manutenção do desenvolvimento dos seres vivos, que envolve ativação de genes específicos e a subseqüente síntese de novas proteínas, as quais participam da execução do programa de morte. A apoptose possui importante atuação durante os processos de embriogênese, na involução dos órgãos e no envelhecimento e na manutenção da homeostase de tecidos normais no organismo (THOMPSON, 1995; BOLD et al., 1997).

Durante a apoptose a célula sofre alterações morfológicas características, que são observadas em conseqüência de uma cascata de eventos moleculares e bioquímicos específicos e geneticamente regulados por um conjunto de genes. Entre estas alterações podemos destacar a retração da célula, alteração da integridade da membrana plasmática, perda de aderência com a matriz extracelular e células vizinhas, condensação da cromatina, fragmentação internucleossômica do DNA e formação dos corpos apoptóticos (GRIVICICH et al., 2007).

Diferentemente, a necrose é um tipo de morte na qual as células sofrem alterações na forma e função das mitocôndrias e rapidamente tornam a célula incapaz de manter a homeostase celular interna, resultando no aumento do volume celular, agregação da cromatina, desorganização do citoplasma, perda da integridade da membrana plasmática e conseqüente ruptura celular (WYLLIE, 1993; GRIVICICH et al., 2007). Na necrose, a degradação do DNA, quando presente, é um fenômeno tardio e a cromatina é digerida por proteases e endonucleases, ocorrendo destruição das histonas e expondo toda a extensão do DNA às nucleases (WYLLIE, 1993).

De acordo com Eisenbrand et al. (2002), a indução de apoptose pode ser investigada levando-se em consideração diferentes parâmetros, que incluem alterações na morfologia celular, rearranjos na membrana, fragmentação do DNA, ativação de caspases, liberação do citocromo c da mitocôndria, entre outros.

A identificação de células apoptóticas por análise das alterações morfológicas, utilizando corantes como Hoechst 33342 ou Laranja de acridina/Brometo de etídio, podem ser consideradas metodologias padrões, devido à sua simplicidade, baixo custo e precisão (WYLLIE, 2008).

A análise morfológica com coloração diferencial por Laranja de Acridina/Brometo de Etídio, foi descrita por McGahon et al. (1995) como uma técnica capaz de quantificar a

viabilidade celular e o índice de apoptose. Este método permite diferenciar células viáveis daquelas em processo de morte por apoptose ou necrose através da contagem de uma dada população de células observadas em microscópio de fluorescência.

Esta técnica pode ser utilizada para identificar células apoptóticas, tanto no estágio inicial como final, levando em consideração a integridade da membrana plasmática. O laranja de acridina intercala-se no DNA, conferindo uma coloração verde. Este pode se ligar ao RNA, mas como não se intercala, o RNA é corado em vermelho-alaranjado. O brometo de etídio é incorporado somente por células não viáveis, intercalando-se ao DNA corando-o de laranja, ligando-se fracamente ao RNA, o qual pode aparecer ligeiramente vermelho (MCGAHON et al., 1995).

Desse modo, as células viáveis com membrana intacta apresentam o núcleo uniformemente corado de verde. Células em apoptose inicial apresentam a membrana ainda intacta, mas com seu DNA fragmentado, mostram uma coloração verde no núcleo e citoplasma, sendo visível uma marginalização do seu conteúdo nuclear. Células em apoptose final apresentam áreas coradas em laranja tanto no citoplasma como nos locais onde a cromatina está condensada no núcleo, o que as distingue de células necróticas, que têm um padrão de coloração uniforme alaranjado no núcleo (Figura 13) (MCGAHON et al., 1995).

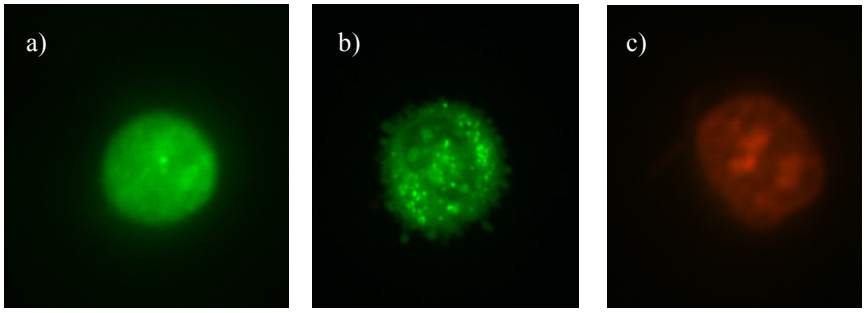


Figura 13: (a) célula normal; (b) célula em apoptose; (c) célula em necrose. Coloração: Laranja de Acridina / Brometo de Etídio. Microscopia de florescência, aumento 1000x. FOTO: CÂNDIDO-BACANI, 2009.

2 OBJETIVOS

2.1 Objetivo Geral:

Avaliar os potenciais citotóxico, apoptótico, genotóxico e mutagênico da isatina em células de mamíferos.

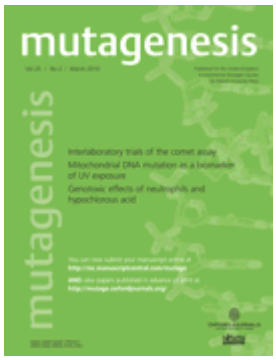
2.2 Objetivos Específicos:

- avaliar, através do teste de toxicidade oral aguda, o efeito da isatina em camundongos Swiss albino e determinar as doses que serão utilizadas nos experimentos subsequentes;
- avaliar os potenciais genotóxico, mutagênico e citotóxico da isatina em células de camundongos Swiss albino *in vivo*, sob tratamentos agudo e de doses repetidas.
- avaliar a citotoxicidade da isatina em células de ovário de hamster Chinês (linhagem CHO-K1) e em células epiteliais de carcinoma do colo uterino humano (linhagem HeLa) e determinar as concentrações que serão utilizadas nos experimentos subsequentes;
- investigar os potenciais apoptótico, antiproliferativo e mutagênico da isatina em células CHO-K1 e em células HeLa sob tratamento agudo *in vitro*;

3 EXPERIMENTOS *IN VIVO*

3.1 ARTIGO 1

Mutagenicity and genotoxicity of isatin in mammalian cells *in vivo*



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Mutagenicity and genotoxicity of isatin in mammalian cells *in vivo*

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Abstract

Isatin (1H-indole-2,3-dione) is a synthetically versatile substrate, that can be used for the synthesis of a large variety of heterocyclic compounds and as a raw material for drug synthesis. Isatin and its derivatives demonstrate diverse biological and pharmacological activities, including anticonvulsant, antibacterial, antifungal, antiviral and anticancer properties. The aim of this paper was to evaluate, *in vivo*, the genotoxic and mutagenic potentials of isatin in acute test (24 hours) and repeated doses test (14 days), using the comet assay and the micronucleus test. For genotoxic and mutagenic evaluation, three different doses were tested: 50, 100 and 150 mg/kg b.w., via gavage. These doses were selected according to the LD_{50} of isatin (1000 mg/kg b.w.), estimated in a preliminary test. For the comparison of the results it were used parametric (ANOVA/Tukey) and non parametric tests (Kruskal-Wallis/Dunn's *post hoc* test), according to the nature of the data distribution. For all tested doses (50, 100 and 150mg/kg b.w.) it was not observed alterations in DNA migration (comet assay) and no mutagenic effect was found in the micronucleus test in peripheral blood cells after acute treatment with isatin. Nevertheless, after repeated doses treatment, the results obtained in the 3 assays showed clearly that only the highest dose of the isatin (150 mg/kg b.w.) was able to induced alterations in DNA giving rise to micronuclei in the bone marrow and peripheral blood cells of the treated mice. According to the experimental conditions used in this work, it may be observed that the mutagenic and genotoxic effects of isatin depend on the alkaloid concentration and period of exposition used.

Key Words: isatin, mice, micronucleus test, comet assay.

Introduction

Studies have demonstrated that natural products, including medicinal plants play a dominant role in the discovery of new compounds for the development of drugs for the treatment of human diseases (1), especially in developing countries (2). However, medicinal plants are consumed with few or no evidence of its pharmacologic properties. Many of medicinal plants have substances potentially aggressive among their secondary metabolites and, for this reason, must be used with care, respecting their toxicological risks (3).

Among the secondary metabolites most known in the chemistry pharmaceutical, the alkaloids are a group of natural products of great interest in medicine (4,5).

Isatin (1H-indole-2,3-dione) is a synthetically versatile substrate, that can be used for the synthesis of a large variety of heterocyclic compounds, such as indoles and quinolines, and as a raw material for drug synthesis. Isatin was first discovered by Erdmann and Laurent in 1840 as a product from the oxidation of indigo using nitric and chromic acids (6).

In review articles, Silva et al. (6) and Vine et al. (7) reported that in nature, isatin is found in plants of the *Isatis* genus, in *Couroupita guianensis* Aubl., in *Calanthe discolor* Lindl., and also in secretion from the parotid gland of *Bufo* frogs. Various derivatives of the isatin have also been identified in plants, for example, the melosatin alkaloids (methoxy phenylpentyl isatins) obtained from the Caribbean tumorigenic plant *Melochia tomentosa*, as well as from fungi and marine mollusks.

Isatin and its derivatives demonstrate diverse biological and pharmacological activities, including anticonvulsant, antineoplasic, antimicrobial and antiviral (6). The most recent review of the isatin is focused on its citotoxic and anticancer properties (7). However, *in vivo* studies evaluating the genotoxic and/or mutagenic potentials of isatin are still lacking. As a result, the aim of this paper was to evaluate the genotoxic and mutagenic potentials of

isatin in acute and repeated doses tests *in vivo*, using the comet assay and the micronucleus test.

Materials and methods

Test compound

Isatin P.A., ≥99.0% was purchased from Fluka (CAS: 91-56-5) and diluted in distilled water. Three different doses of isatin (50, 100 and 150 mg/kg b.w.) were administered by gavage in groups of eight animals for each treatment. These doses were selected according to the LD_{50} of isatin (1000 mg/kg b.w.), estimated in a preliminary test (8).

Chemical

Cyclophosphamide (CPA) is an alkylating agent, able to induce chromosomal damage in bone marrow and peripheral blood cells (9). The CPA (CAS:50-18-0) was purchased from Sigma Chemical Co. (St. Louis, MO), diluted in distilled water and used as positive control.

Animals

Male and female albino Swiss mice (*Mus musculus*), aged 7–8 weeks old and weighing ~30 g at the beginning of the experiments, were obtained from the breeding colonies of the State University of Londrina (Paraná, Brazil). The animals were kept individually in polypropylene cages with *ad libitum* access to diet and water throughout the treatment period. All experiments and activities were carried out according to the *Canadian Council on Animal Care* (10). To perform the acute and repeated doses tests it were used two groups of 40 animals to each test.

Experimental designs

The LD_{50} of the isatin was estimated using the Acute Oral Toxicity Test according to OECD (8) and after this it were definite the doses to be used in genotoxicity and mutagenicity tests.

In order to investigate the genotoxic and mutagenic effects in mice after acute and repeated doses exposure with isatin, the mice were divided into groups with 8 mice (four males and four females) for each treatment.

The mice were treated with 0.1ml of each solution of the isatin for every 10g of body weight (b.w.) at three different doses: 50, 100 and 150 mg/kg b.w. After treatments, the animals were anesthetized and killed with a application of a mix 1:1 of Xylazine Hydrochloride 2% and Ketamine Hydrochloride 10%. Animals of all the groups were weighed at the beginning and at the end of each treatment to evaluate possible variations in body weight caused by the administration of isatin.

Acute treatment: To the assessment of the genotoxic and mutagenic effects of isatin, the animals were treated with a unique dose of each of the three concentrations, by gavage, by 24 hours. The negative control group received only water by gavage and positive control group was treated with one dose of CPA (50mg/kg b.w.) intraperitoneally (i.p.), by 24 hours.

In the micronuclei test in peripheral blood cell, the blood samples were obtained from the tail vein of mice before the treatment via gavage (T0) and after 36 (T1), 48 (T2) and 72 hours (T3) from treatment. For the comet assay, blood samples from animal's tail vein were obtained 4 (T1) and 24 hours (T2) after the treatment via gavage with isatin, according, respectively to OECD (11) and Tice et al. (12).

Repeated doses treatment (14 days): For the evaluation of the genotoxic, citotoxic and mutagenic effects of isatin, the animals received, via gavage, during two weeks with 24 hours intervals, the same doses of this alkaloid used in the acute treatment. The negative control group received daily drinking water for 2 weeks, via gavage, but the mice of the positive control group were treated intraperitoneally on 14th day with CPA (50 mg/kg b.w.).

For the micronucleus test in bone marrow cells, the animals were killed 24 hours after the last treatment. For the analysis of the comet and micronucleus in peripheral blood, samples of blood were obtained, respectively, 4 and 36 hours after the last treatment of the animals, according, respectively to Tice et al. (12) and OECD (11).

Micronucleus test in bone marrow cells

The micronucleus test in bone marrow cells was performed using the method of Schmid (13) with some modifications. Animals were euthanized 24 hours after the treatments and immediately after this, both femurs of the mice were removed and the femur bone was freed from the extra muscles. The epiphyses were cut and the bone marrow was flushed out with fetal calf serum (Gibco). The cell suspension was centrifuged at 900 rpm for 10 min and the supernatant was discarded. A small drop of the resuspended cell pellet was spread on to clean glass slides and air-dried and fixed in absolute methanol for 10 minutes. The smears were stained with Leishman eosin-blue for detecting micronucleated polychromatic erythrocytes (MNPCE). For each animal, three slides were prepared and 2000 polychromatic erythrocytes (PCE) were counted to determine the frequency of MNPCE. To evaluate bone marrow toxicity, the ratio the PCE/PCE + NCE (normochromatic erythrocytes) was calculated by counting the total of 200 erythrocytes using these slides.

According to the Bartlett test the data were not homogeneous with regard to variance, so the Kruskal-Wallis test followed by Dunn's *post hoc* test was used to analyze the results.

Micronucleus test in peripheral blood cells

The micronucleus test on peripheral blood cells was performed as described by Hayashi et al. (14), who used slides pre-stained with acridine orange (CAS: 494-38-2). Glass slides were heated to about 70ºC on a hot-plate and a 10µL drop of an aqueous solution of the dye (1 mg/mL) was placed on each slide and spread evenly over the surface with the end of a second well-cleaned slide. Once dry, the slides were kept in the dark at room temperature for at least 24 h.

An internal control was established for each animal by preparing a test slide with a drop of blood taken from its tail before the first treatment (time T_0). After the treatment with the different compounds, the blood was sampled by perforating the tail vein of the mice with a needle and collecting 5 µL drops, each of which was placed at the centre of a pre-stained slide and covered with a cover-slip (24 x 60 mm). These slides were then kept in the dark at –20ºC for a minimum of 24 h, before the cytological examination of the blood cells was performed.

The cell preparations were examined under a fluorescence microscope (Nikon) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter, using an immersion objective. One thousand reticulocytes per treated animal were analyzed and the proportion of micronucleated cells was counted.

For the comparison of the results it were used parametric (ANOVA/Tukey) and non parametric tests (Kruskal-Wallis/Dunn's *post hoc* test), according to the nature of the data distribution.

Comet assay

The alkaline version of the comet assay was performed essentially as described by Singh et al. (15), with a slight modification (16). Four and twenty-four hours after the treatment, 20 µl of heparinized periphery blood was mixed with 120 µl of 0.5% low-melting-

temperature agarose in phosphate buffered saline (PBS) and applied to microscope slides pre-coated with 1.5% normal-melting-temperature agarose in PBS. The slides were covered with a microscope coverslip and refrigerated for 5 min to gel. This was followed by immersion in ice-cold alkaline lysing solution (2.5M NaCl, 10 mM Tris, 100 mM ethylenediaminetetraacetic acid (EDTA), 10% dimethyl sulphoxide, 1% Triton X-100, final pH 10.0) for at least 1 h. The slides were then incubated for 20 min in ice-cold electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13), followed by electrophoresis at 25 V:300 mA (1.25 V/cm) for 20 min (17). All of the above steps (preparation of slides, lysis and electrophoresis) were conducted without direct light in order to prevent additional DNA damage. After electrophoresis, the slides were then neutralized (Tris 0.4 M, pH 7.5) and stained with ethidium bromide (20 μg/mL). One hundred cells per animal (two slides of 50 cells each) were analyzed using a fluorescence microscope (Nikon-Brasil) with a blue (488nm) excitation filter and yellow (515nm) emission (barrier) filter.

Quantification of DNA breakage was realized by visual scoring and the cells were classified into four classes, according to tail size (class 0, undamaged cells) to the maximum length comet [class 3, maximally damaged cells] (18).

The frequency of damaged cells (DC) was obtained by totaling the number of undamaged (class 0) and DC from classes 1, 2 and 3 and dividing by the total number of cells analyzed in each treatment. The total score for 100 nucleoids was obtained by multiplying the number of cells in each class by the damage class, according to the formula modified from Manoharan and Banerjee (19). Total score = $(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3)$, where n = number of cells in each class analyzed. Thus, the total score could range from 0 (100 cells presenting no damage) to 300 (all cells presenting damage class 3).

For the comparison of the results it were used parametric (ANOVA/Tukey) and non parametric tests (Kruskal-Wallis/Dunn's *post hoc* test), according to the nature of the data distribution.

Results

Acute tests

In the comet assay, the lesions observed in the DNA at 4 and 24 hours after acute treatment with isatin were not statistically significant when compared to data from the negative control group (Table I).

For all tested doses (50, 100 and 150 mg/kg b.w.) in different times of evaluation (36, 48 and 72 hours) it was not observed a significant increase of micronucleus frequency on peripheral blood cells compared to the negative control group ($P > 0.05$) (Table II).

→ Table I

→ Table II

Repeated doses tests (14 days)

Variance analysis showed no significant differences among the median body weight of the groups of mice treated with isatin and the negative control group ($p > 0.05$) during the repeated doses tests (data not shown).

Table III shows the results of the comet assay for all three doses of isatin. The average values of the scores and frequency of DC obtained for the doses evaluated, show that only the higher concentration tested (150 mg/kg b.w.) was able to induce damage in the DNA and was statistically different from the negative control, indicating genotoxic activity. The most of the analyzed cells were classified as class 1, with few cells showing damage class 2 and no cells were observed damage class 3.

The results of mutagenic evaluation of isatin by micronucleus test in bone marrow and peripheral blood cells showed that only the dose of 150 mg/kg b. w. of the isatin presented frequencies of micronucleated cells significantly larger than that found in the negative control. The lower doses of isatin did not increase the micronucleus frequency in relation to the negative control group (Tables IV and V).

To evaluate the cytotoxicity of this substance in the repeated doses treatment, the PCE/NCE ratio was calculated. PCE/PCE + NCE ratio showed that the isatin even at higher doses and the positive control did no presented cytotoxic effect in mice bone marrow cells (Table IV).

→ Table III

→ Table IV

→ Table V

Discussion

The worldwide use of natural products including medicinal plants has become more and more important in primary health care. Many pharmacognostical and pharmacological investigations are carried out to identify new drugs and structures for the development of novel therapeutic agents for the treatment of human diseases such as cancer and infectious diseases (1).

The evaluation of the genotoxic and mutagenic potentials of new drugs is fundamentally relevant for the introduction in the market of safer compounds to therapeutic human use (20,21). A balance between therapeutic versus toxicological effects of a compound is an important parameter to evaluate its usefulness as a pharmacological drug (22).

Although many studies have been done on the pharmacological properties and chemical composition of isatin, there are few *in vivo* studies about the evaluation of the

genotoxic and/or mutagenic potentials of this compound. This boarding is important, once that the use of chemotherapics in the clinic shows adverse effects related mostly with toxicity in not target tissues. The comet assay and the micronucleus test used in the present study to evaluate the genotoxic and mutagenic properties of isatin are considered effective tests for this kind of evaluation (23-27).

Tests *in vivo* are important in the evaluation of chemical substance genotoxicity risk since they allow the consideration of aspects involved in the metabolization of the studied substance, pharmacokinetics parameters (activation, detoxification and distribution to tissues) and also DNA repair (28). In this way, metabolism, including detoxification is a fundamental characteristic in the genotoxicity determination of the substance tested and the metabolic activation may be a differential parameter in the experiments *in vivo* and *in vitro* (29).

Since the mice present metabolic patterns similar to humans (30), the administration via chosen for the tested substance in this study was by gavage. The administration via and the regime of treatment used (acute and repeated doses exposition) are the most suitable, because it expose the animals to similar conditions by those the humans are in contact with a chemotherapic.

In the present study was observed absence of primary DNA damage by comet assay after acute treatment with isatin (4 and 24 hours). These sampling times make possible the verification of repair occurrence of possible DNA damage caused by the isatin alkaloid and are in agreement with the protocol suggested by Tice et al. (12). The absence of any mutagenic effect of this compound after 36, 48 and 72 hours of treatment also was demonstrated by the micronucleus assay in peripheral blood cells. These results may be related with the DNA damage repair system by the cells, preventing the primary DNA damage and micronucleus formation.

It has been suggested that alkaloids can be considered potentially mutagenic, since the therapeutic action of some of them is related to their interaction with DNA (4,31-33). This fact was corroborated in the present study only when the mice were submitted by 14 consecutive days the higher dose of isatin (150 mg/kg b.w.). The results obtained in the micronucleus (RET and PCE) and comet assays showed clearly that the isatin was able to induce primary damage in the DNA molecule and breaks and/or loss of entire chromosomes, giving rise to micronuclei in the treated animals.

Foureman et al. (34) also demonstrated that isatin was mutagenic using the sex-linked recessive lethal (SLRL) assay in germ cells of male *Drosophila melanogaster*. Nevertheless, using Ames test the absence of mutagenic effect of isatin was demonstrated by Rannung et al. (35) in concentrations up to 8μM/plate and by Bacchi et al. (36) that used derivatives of isatin. The mutagenic effect observed in our study only in the highest dose tested (150 mg/kg b.w.) and the absence of mutagenicity in the lowest doses, added with the negatives results for the Ames test and positive result for the SRLR test, indicate differences in the sensibility of each test and complexity of each organism used.

According Miranda et al. (37) a mutagenic agent, can weaken an animal promoting cachexia and result in weight loss. In our experimental conditions there was no influence of isatin in the body weight of animals when compared with data from negative control group after exposure for 14 days, indicating absence of toxicity of this substance *in vivo.*

The cytotoxic effect of isatin in mice bone marrow cells after repeated doses treatment was determined by PCE/NCE relationship in the micronuclei test. When normal proliferation of the bone marrow cells is affected by a toxic agent, the number of immature erythrocytes (PCE) is prejudiced in relation to mature erythrocytes (NCE) (38). In the present study, the isatin had not affected the cellular proliferation of the bone marrow cells in the experimental conditions used. These results are important since the isatin did not interfered in proliferation

normal cells, but several studies have demonstrated the ability of isatin to inhibit the proliferation of several tumoral lines *in vitro* (39-43). Sathisha et al. (44) also related that bis-isatin thiocarbohydrazone metal complexes in the dose of 50 mg/kg b.w. showed good activity when tested in Ehrlich ascites carcinoma model in Swiss albino mice.

The genotoxic and mutagenic effects observed in the repeated doses tests and the absence of such effects after acute treatment with isatin showed that induction of DNA damage is dependent of dose and of the time of animal exposition. Thus, the two different periods of exposition used in the present study are important and useful in the evaluation of security of this compound. In addition, we observed that the isatin (150mg/kg w.b.) was genotoxic and mutagenic after repeated doses treatment. This type of treatment is needed, since the human medicinal treatments usually make use of repeated doses for long periods and thus the indiscriminate use of higher dose of this compound can be dangerous to health.

In conclusion, the concentrations of isatin used in the present study should be used in future studies to evaluate its properties in terms of pharmacological potential and determine the safe dose-limit this alkaloid in mammal cells. This type of study associated with the data available in the literature about the therapeutic potential of isatin may contribute for future record of this alkaloid as a chemotherapic.

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Table I. Detection of DNA damage in 100 cells per mouse (n = 8 mice per group) exposed to water, cyclophosphamide (CPA) and isatin after 4 and 24 hours from treatment.

Exposure time (hours)	TREATMENTS (mg/kg b.w.)		Levels of Damage 0	1	2	3	Score (Mean ± SD)	Frequency of DC (Mean ± SD)
4	Water		93.6 ± 2.06	6.37 ± 2.06	0.00 ± 0.00	0.00 ± 0.00	6.37 ± 2.20[a]	0.06 ± 0.02 [a]
	CPA	50	45.2 ± 2.62	34.4 ± 3.42	16.6 ± 2.39	3.75 ± 0.66	78.9 ± 5.05 [b]	0.53 ± 0.06 [b]
	Isatin	50	91.4 ± 2.73	8.62 ± 2.73	0.00 ± 0.00	0.00 ± 0.00	8.62 ± 2.92 [a]	0.09 ± 0.03 [a]
		100	89.5 ± 2.60	10.0 ± 2.34	0.50 ± 0.50	0.00 ± 0.00	11.0 ± 3.12 [a]	0.10 ± 0.03 [a]
		150	89.5 ± 3.53	9.62 ± 2.87	0.87 ± 0.78	0.00 ± 0.00	11.4 ± 4.53 [a]	0.10± 0.04 [a]
24	Water		93.4 ± 1.93	6.62 ± 1.93	0.00 ± 0.00	0.00± 0.00	6.62 ± 2.06 [a]	0.07 ± 0.02 [a]
	CPA	50	32.4 ± 3.15	42.0 ± 4.61	20.5 ± 3.43	5.12 ± 1.16	97.6 ± 5.18 [b]	0.67 ± 0.03 [b]
	Isatin	50	93.4 ± 1.65	6.62 ± 1.65	0.00 ± 0.00	0.00 ± 0.00	6.62 ± 1.77 [a]	0.07 ± 0.02 [a]
		100	91.4 ± 2.50	7.87 ± 2.03	0.75 ± 0.66	0.00 ± 0.00	9.37 ± 3.25 [a]	0.09 ± 0.03 [a]
		150	90.5 ± 2.69	9.00 ± 2.40	0.50 ± 0.50	0.00 ± 0.00	9.87 ± 3.14 [a]	0.09 ± 0.03 [a]

Mean ± SD: mean ± standard deviation; water: negative control; CPA: positive control. DC: damage cells.
Means with the same letter do not differ statistically (P>0.05).

Table II. Frequency of micronucleated reticulocytes (MNRETs) for a total of 1000 analyzed cells per mouse (n = 8 mice per group) in different acute treatments to evaluate the mutagenicity of three different doses of isatin and their positive and negative control groups.

TREATMENTS (mg/kg b.w.)		T0 (0 hour)		T1 (36 hours)		T2 (48 hours)		T3 (72 hours)	
		MNRETs	Mean ± SD/animal	MNRETs	Mean ± SD/animal	MNRETs	Mean ± SD/animal	MNRETs	Mean ± SD/animal
Water		21	2.6 ± 0.52	15	1.9 ± 0.64[a]	17	2.1 ± 0.64 [a]	19	2.1 ± 0.83 [a]
CPA	**50**	15	1.9 ± 0.64	180	22.5 ± 2,07[b]	107	13,4 ± 1,68 [b]	41	5.1 ± 1.64 [b]
Isatin	**50**	17	2.1 ± 0.83	13	1.6 ± 0.92 [a]	14	1.7 ± 0.46 [a]	14	1.7 ± 0.70 [a]
	100	13	1.6 ± 0.74	15	1.9 ± 0.64 [a]	16	2.0 ± 0.75 [a]	14	1.7 ± 0.89 [a]
	150	13	1.6 ± 0.74	27	3.4 ± 1.19 [a]	13	1.6 ± 0.74 [a]	11	1.4 ± 0.74 [a]

Mean ± SD: mean ± standard deviation; water: negative control; cyclophosphamide (CPA): positive control.
Means with the same letter do not differ statistically (P>0.05).

Table III. Detection of DNA damage in 100 cells per mouse (n = 8 mice per group) after repeated doses treatment with three different doses of isatin. The blood samples were obtained 4 hours after the last treatment.

TREATMENTS		Levels of Damage					
(mg/kg b.w.)		0	1	2	3	Score (Mean ± SD)	Frequency of DC (Mean ± SD)
Water		91.1 ± 2.20	8.87 ± 2.20	0.00 ± 0.00	0.00± 0.00	8.87 ± 2.36[a]	0.09 ± 0.02[a]
CPA	**50**	24.5 ± 5.15	55.7 ± 3.53	15.1 ± 2.26	4.62 ± 1.41	99.9 ± 7.85[b]	0.75 ± 0.05[b]
Isatin	**50**	89.6 ± 2.17	8.62 ± 1.93	1.75 ± 0.97	0.00 ± 0.00	12.12 ± 2.95[a]	0.10 ± 0.02[a]
	100	84.5 ± 2.18	13.12 ± 1.45	2.25 ± 1.09	0.00 ± 0.00	17.62 ± 3.42[a,c]	0.15 ± 0.02[a,c]
	150	71.9 ± 3.82	26.6 ± 2.82	2.50 ± 1.00	0.00 ± 0.00	31.62 ± 2.33[b,c]	0.29 ± 0.02[b,c]

Mean ± SD: mean ± standard deviation; water: negative control; CPA: positive control. DC: damage cells. Means with the same letter do not differ statistically (P>0.05).

Table IV. Frequency of polychromatic erythrocytes micronucleated (MNPCEs) for a total of 2000 analyzed cells per mouse (n = 8 mice per group) after repeated doses treatment to evaluate the mutagenicity of three different doses of isatin. The blood samples were obtained 24 hours after the last treatment.

TREATMENTS (mg/kg b.w.)		MNPCEs	Mean ± SD	PCEs/ PCEs + NCEs (Mean ± SD)
Water		34	4.2 ± 2.12[a]	9
CPA	**50**	341	42.6 ± 6.02[b]	193
Isatin	**50**	80	10.0 ± 2.14[a,c]	10
	100	81	10.1 ± 1.25[a,c]	18
	150	128	16.0 ± 1.93[b,c]	26

Mean ± SD: mean ± standard deviation; water: negative control; cyclophosphamide (CPA): positive control. Means with the same letter do not differ statistically (P> 0.05).

Table V. Frequency of micronucleated reticulocytes (MNRETs) for a total of 1000 analyzed cells per mouse (n = 8 mice per group) after repeated doses treatment to evaluate the mutagenicity of three different doses of isatin. The blood samples were obtained before treatment (0 hour) and 36 hours after the last treatment.

TREATMENTS (mg/kg b.w.)		T0 (0 hour)		T1 (36 hours)	
		MNRETs	Mean ± SD	MNRETs	Mean ± SD
Water		13	1,6 ± 0,74	9	1,1 ± 0,99[a]
CPA	**50**	11	1,4 ± 0,92	193	24,1 ± 1,13[b]
Isatin	**50**	8	1,0 ± 0,53	10	1,2 ± 0,46[a]
	100	14	1,7 ± 0,89	18	2,2 ± 0,89[a]
	150	12	1,5 ± 0,75	26	3,2 ± 0,71[c]

Mean ± SD: mean ± standard deviation; water: negative control; cyclophosphamide (CPA): positive control. Means with the same letter do not differ statistically (P>0.05).

4 EXPERIMENTOS *IN VITRO*

4.1 ARTIGO 2

In vitro assessment of the cytotoxic, apoptotic and mutagenic potentials of isatin

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In vitro assessment of the cytotoxic, apoptotic and mutagenic potentials of isatin

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Abstract

Isatin (1H-indole-2,3-dione) is a synthetically versatile molecule, found in various medicinal plants species. Several pharmacological and biological properties that may be beneficial to the health of humans have been described to isatin, including anticholinesterase, anticonvulsant, anti-inflammatory, anti-hypertensive, antihipoxia, antimicrobial, antiviral and anticancer. The aim of the present study was to determine *in vitro* the possible cytotoxic, mutagenic, apoptotic and antiproliferative effects of isatin in CHO-K1 and HeLa cells using the cytotoxicity assay MTT, micronucleus test (MN), apoptosis index and nuclear division index. The five concentrations evaluated in the mutagenicity and apoptosis tests (0.5, 1.0, 5.0, 10. and 50.0μM) were chosen through MTT assay. Positive (DXR) and negative (PBS) control groups were also included in the analysis. Isatin did not show mutagenic effect in CHO-K1 and HeLa cells in all times evaluated. Nevertheless, the concentrations (10 and 50 μM) were able of the inhibit the cell proliferation and promote apoptosis both CHO-K1 and HeLa cells. According to the experimental conditions used in this work, it may be observed that the cytotoxic, apoptotic and antiproliferative effects of isatin were concentration-dependent and cell line independent. These data suggest that isatin should be further explored as a possible therapeutic agent for the treatment of cancer.

Key Words: isatin, CHO-K1, HeLa, apoptosis.

1 Introduction

Chemoprevention is considered one of the most promising propositions for prevention of human cancers (Turini and Dubois, 2002). Organic molecules which interact with DNA, RNA and/or proteins, have the potential to inhibit the activity of structurally and functionally important molecules in the tumoral cells (Marczi et al., 2008). Based on this strategy, many probable compounds have been undergoing clinical trials for prevention of various sites of malignancy (Turini and Dubois, 2002).

Isatin is a synthetically versatile molecule, found in various medicinal plants species (Vine et al., 2009). Several pharmacological and biological properties that may be beneficial to the health of humans have been described to isatin, including anticholinesterase, anticonvulsant, anti-inflammatory, anti-hypertensive, antihipoxia, antimicrobial and antiviral (Silva et al., 2001).

Recently it has been shown that isatin can be a potential anticancer agent to a variety of cancer cell lines (Cane et al., 2000; Igosheva et al., 2005; Matheus et al., 2007; Vine et al., 2007; Hou et al., 2008). Studies exploring its mechanism of action indicated that isatin inhibit cancer cell proliferation *via* interaction with extracellular signal related protein kinases (ERKs), thereby promoting apoptosis (Vine et al., 2009). Compounds that interfere with the cell cycle have become a major interest in cancer research because they inhibit the proliferation of tumoral cell lines derived from various organs (Jordan and Wilson, 1998).

In view of the potential therapeutic of isatin, the Chinese hamster ovary cells (CHO-K1) and human cervical cancer cells (HeLa) were utilized in the study to determine the possible mutagenic, apoptotic, cytotoxic and antiproliferative activities of isatin utilizing micronucleus test (MN), apoptosis index, cytotoxicity assay MTT and nuclear division index.

2 Materials and methods

2.1 Chemical compounds

2.1.1 Test compound

Isatin P.A., ≥99.0% was purchased from Fluka (CAS: 91-56-5) and diluted in PBS (Phosphate Buffer Saline). Ten different concentrations of isatin were applied to cell cultures in the cytotoxicity evaluation: 0.1; 0.5; 1.0; 5.0; 10; 50; 100; 200; 500 e 1000μM. The mutagenic and apoptotic activities were evaluated using five concentrations defined from the cytotoxicity test: 0.5; 1.0; 5.0; 10; 50μM.

2.1.2 Doxorubicin (DXR)

Chemotherapeutic doxorubicin (DXR) is an anthracycline type antibiotic and is highly effective against a wide variety of cancers, as it is capable of generating breaks in DNA strands and countless free radical species, promoting DNA adducts and blocking the replication of genetic material (Quiles et al., 2002). The DXR was used in commercial formula: Adriblastin® RD (Pharmacia & Upjohn, Milan – Italy), at the concentration of 0.3 μM in culture medium.

2.1.3 Cytochalasin B

Cytochalasin B (C29H37NO5 – CAS: 14930-96-2, Sigma) was diluted in dimethyl sulfoxide (DMSO, CAS: 67-68-5, Mallinckrodt) to obtain a stock solution of 2 mg/mL, which was kept at 4ºC in the dark until use. This stock solution was used to prepare a working solution with PBS (0.3 mg/mL) which was kept at 4ºC in the dark and utilized for the experiments at a final concentration of 3 μg/mL in culture medium.

2.2 Cell line and culture conditions

Chinese hamster ovary cells (CHO-K1) and human cervical cancer cells (HeLa), originated from the Faculty of Medicine at Leiden University (Netherlands), were kindly furnished for our laboratory by Dr. E.T. Sakamoto-Hojo from the Faculty of Medicine of Ribeirão Preto-USP (Brazil). The cells were grown at 37º C in 10 mL DMEM/Ham-F-10 (1:1) medium (Sigma) supplemented with 10% fetal calf serum (Gibco), antibiotics (penicillin 0.06 g/L and streptomycin 0.12 g/L – Sigma) and HEPES (2.38 g/L – Sigma) in 25 cm^2 culture flasks (Nunc) at 37º C in a B.O.D. incubator (Fanem).

2.3 Cytotoxicity assay – MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

The viability of the cells was measured using MTT assay as described by Mosmann (1983) with some modifications. The cells were harvested from culture flasks by trypsinization and seeded independently in a 96-well plate with the final volume 200μL of complete culture medium containing $2x10^4$ cells per well (cell line CHO-K1) and $1x10^4$ cells per well (cell line HeLa). Medium was aspirated and cells were exposed to 10 concentrations of the isatin (7 wells for each concentration) and incubated for 3 and 24 hours at 37 ºC (cell line CHO-K1) and 24 hours at 37 ºC (cell line HeLa). After treatments 100μL of MTT solution (5mg/mL) was added to each well and incubated at 37 ºC for 4 hours. After incubation, MTT-containing medium was removed, and 200 μL of DMSO was added to each well to dissolve formazan crystals formed. The absorbance of the samples was measured at 550nm with a spectrophotometer (Uniscience). The percentage of cell growth inhibition was calculated as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance treatment}}{\text{Absorbance control}} \right) \times 100$$

where the negative control group was considered as 100% of viable cells.

2.4 Cytokinesis Block Micronucleus (CBMN) assay

Analysis of micronucleus (MN) was performed with the CBMN assay according to the method described by Fenech and Morley (1985) with some modifications. A total of 10^6 cells were seeded in culture flasks containing 5mL of complete culture medium. All experiments were carried out, in triplicate, using CHO-K1 and HeLa cells between the 3rd and 8th culture passage after thawing. Cells were treated with five different concentrations of isatin (0.5; 1.0; 5.0; 10; 50μM). Positive (DXR) and negative (PBS) control groups were also included in the analysis. The cultures were treated for 3 and 24 hours (cell line CHO-K1) and 24 hours (cell line HeLa). After the treatments, the cultures received cytochalasin B for 20 hours (cells CHO-K1) and 24 hours (cells HeLa) to obtainment binucleated cells.

The cells were rinsed twice with 5 mL PBS, trypsinized and centrifuged for 5 min at 900 rpm. The pellet was resuspended in chilled hypotonic solution (1% sodium citrate), together with one drop of 1% formaldehyde and fixed with a 3:1 methanol/acetic acid solution (CHO-K1 cells). However, the pellet of the HeLa cells was resuspended in chilled hypotonic solution (KCl 0.4%), together with one drop of 1% formaldehyde and fixed with a 4:1 methanol/acetic acid solution. Cells were dropped onto cooled, clean slides and air-dried. Slides were stained in 3% Giemsa dissolved in phosphate buffer (Na_2HPO_4 0.06 M and KH_2PO_4 0.06 M – pH 6.8) for 5 min, washed with water, dried and kept at 4°C until the realization of microscopic analysis.

To determine MN frequencies were analyzed 3000 binucleated cells with well preserved cytoplasm (1000 cells for repetition). The criterion for the identification of MN was according to Fenech (1993) and Titenko-Holland et al. (1997).

2.4.1 Nuclear division index (NDI)

The nuclear division index (NDI) was determined in 500 cells analyzed by repetition, in a blind test. Cells with well preserved cytoplasm, containing 1–4 nucleus, were scored using a Nikon microscope. The NDI was calculated according to Eastmond and Tucker (1989) using the following formula:

$$NDI = \left[\frac{M1 + 2(M2) + 3(M3) + 4(M4)}{N} \right]$$

where *M1, M2, M3 and M4* corresponds to the number of cells with one, two, three and four nuclei, respectively, and *N* is the total number of viable cells.

2.5 Apoptosis Index

The Apoptosis Index was performed using the method of McGahon et al. (1995) that use in a cellular population a differential staining with acridine orange and ethidium bromide to identify normal cells, early apoptotic cells (EA), late apoptotic cells (LA) and necrotic cells. The slides were prepared using 25 μL of cellular suspension obtained from *CBMN* test (approximately $1.5x10^3$ cells) before hypotonization and 10μL of stain (100 μg/mL acridine orange and 100 μg/mL ethidium bromide).Two hundred cells per repetition were analyzed using a Nikon microscope with excitation filter of 515–560 nm and barrier filter of 590 nm. The percentage of apoptosis was calculated according the following formula:

$$\%apoptosis = \left[\frac{(EA + LA)}{\text{total of cells analyzed}} \right] \times 100\,\%$$

2.6 Statistical analysis

All data are expressed as the mean ± SD. Statistical analysis was performed using two-ways ANOVA followed by Tukey's test. A level of $p \leq 0.05$ was accepted as indicating statistical significance.

3 Results

3.1 Cytotoxicity assay (*MTT)*

Figure 1 shows the results obtained for the cytotoxicity test (MTT) in CHO-K1 cells after exposure for 3 and 24 hours to different concentrations of isatin. There was a decrease in mitochondrial activity in the highest concentrations evaluated (200, 500 and 1000μM) for both exposure times.

There was a reduction in HeLa cell viability only at concentrations of 500 and 1000μM after 24 hours of treatment, compared with to negative control (Figure 2). These results demonstrated that the two cell lines showed different responses when treated for 24 hours with the same concentrations of isatin. Also non-cytotoxic concentrations were established to be used in the micronucleus and apoptosis tests: 0.5, 1.0, 5.0, 10 and 50μM.

3.2 Cytokinesis Block Micronucleus (CBMN) assay

Isatin did not show mutagenic effect in CHO-K1 and HeLa cells in all time evaluated, since the frequency of micronuclei observed after treatment of cells with different concentrations of isatin was not statistically different of the negative control (Tables 1, 2 and 3).

3.2.1 NDI

The nuclear division index (NDI) obtained in CHO-K1 cells after 3 hours of exposure to different concentrations of isatin no was statistically significant compared to negative control group (Table 1). However, after 24 hours of treatment with the highest concentrations of isatin (10 and 50 μM) both CHO-K1 and HeLa cells, showed a reduction in IDN compared to negative control group, indicating a delaying effect on the cell cycle (Tables 2 and 3).

3.3 Apoptosis Index

Table 1 show the apoptosis index obtained to CHO-K1 cells after 3 hours of exposure with different concentrations of isatin, in which can be observed that no concentration induced apoptosis, compared to negative control group. When the CHO-K1 and HeLa cells were treated during 24 h with isatin, the apoptosis index of the concentrations 10 and 50μM were statistically superior than in the negative control group (Tables 2 and 3).

Discussion

In the last years the synthesis and characterization of novel anticancer compounds have represented a field of research that has aroused expectations for more specific and less toxic therapies (Wilson and Jordan, 2004). The treatment of cancer may be benefited from the introduction of novel therapies derived from natural products. Natural products have served to provide a basis for many of the pharmaceutical agents in current use in cancer therapy (Pietras and Weinberg, 2005). Although there is a wide variety of active compounds from natural origin that may be used in future cancer treatment, the challenges are focused mainly in the developing of drugs that act selectively in the tumoral cells.

Mutagenicity studies help in the evaluation of safety and effectiveness of natural products (Bast et al., 2002). The detection and evaluation of the cytotoxic, apoptotic and mutagenic potentials of futures chemotherapic in not tumoral and tumoral cell lines is an important boarding, once in the clinical practice the chemotherapics present adverse effects of toxicity in not target tissues. In the present study the evaluation of isatin, an alkaloid with therapeutic potential, was accomplished in not tumoral (CHO-K1) and in a tumoral (HeLa) cell line.

The MTT assay is one of the most used and most sensitive tests to detect *in vitro* cytotoxicity (Fotakis and Timbrell, 2006). In our study this cytotoxicity assay was performing

to determine the cell viability of the cultures (CHO-K1 and HeLa) treated with different concentrations of isatin and define the concentrations to be used in posterior experiments *in vitro*. The concentrations of isatin assessed in the present study (0.1 a 1000μM) are comparable with those used in other *in vitro* studies. Igosheva et al. (2005) demonstrated cytotoxic activity of concentrations 200 and 400 μM of isatin in human neuroblastom cell line (SH SY5Y) after 24 hours of exposition. The capacity of reduce the cell viability after 24 hours of treatment with isatin (300μM), was also demonstrated by Matheus et al. (2007) using RAW264.7 cells (mouse monocyte-macrophages). The results obtained in the present study from cytotoxicity assay MTT indicate differences between the two cell lines concerning their sensitivity to isatin. The results reveals that CHO-K1 cells are more sensitive than HeLa cells, because when both are submitted to the same concentrations of isatin for 24 hours, the tumoral cell line HeLa showed higher resistance to induced cytotoxicity by observing a reduction in the cell viability in the concentration of 500 and 1000μM. For the CHO-K1 cell, concentrations of 200, 500 and 1000μM were cytotoxic. The absence of cytotoxicity of isatin in the concentration of 0.1 to 100 μM demonstrated in the present study is in agreement with the literature studies (Igosheva et al., 2005; Matheus et al., 2007).

Cytotoxicity and genotoxicity of anticancer drugs to the normal cells are major problem in cancer therapy and engender the risk of inducing secondary malignancy (Aydemir and Bilaloglu, 2003). Therefore, assessment of mutagenic potential of the isatin is necessary to ensure a relatively safe use of this compound. Isatin did not showed mutagenic effect in CHO-K1 and HeLa cells in all time evaluated, since the frequencies of micronuclei observed after treatment of cells with different concentrations of isatin were not statistically different of the negative control.

The evaluation of isatin (Rannung et al., 1992) and of some derivatives (Bacchi et al., 2005) as possible inductors of genetic mutation was accomplished, respectively, in strains

TA98 and TA100 and in TA100 and TA102 of *Salmonella typhimurium*. Both authors did not observed mutagenic effect of these substances. Therefore, the negative results obtained in the present study in the *in vitro* mutagenicity evaluation of isatin, added with the negatives results for the Ames test suggest that this compound do not induces chromosomic or genic mutations in test systems *in vitro*. Model *in vivo* and *in vitro* are necessary when is intended to prove the pharmacologic action of determinate extract and/or active principle and especially, to elucidate the action mechanism of active principles isolated of plants (Calixto, 2001). Evaluation of isatin *in vivo* was done by our group (data in preparation) and indicated genotoxic and mutagenic effects in mice bone marrow and peripheral blood cells after treatment for 14 days consecutives. Our negative results obtained *in vitro* and the positive results *in vivo* suggest an indirect mutagenic action of this compound.

Isatin and its derivatives present high synthetic versatility and demonstrate diverse biological and pharmacological activities (Silva et al., 2001; Pandeya et al., 2005; Vine et al., 2009). Several studies have demonstrated the ability of isatin to inhibit the proliferation and induce programmed cell death of several tumoral lines (Cane et al., 2000; Igosheva et al., 2005; Matheus et al., 2007; Vine et al., 2007; Hou et al., 2008), an effect of high interest in the search for compounds with purpose chemotherapic. Our results showed that the treatment of 24 hours with isatin in the concentration of 10 and 50μM inhibited the cellular proliferation and induced apoptosis in both cell lines evaluated. These results were concentration-dependent and cell line independent and corroborate those obtained by Cane et al. (2000) and Igosheva et al. (2005). Cane et al. (2000) observed antiproliferative and apoptotic effects of isatin in tumoral cells (HL60-human promyelocytic leukemia, PC12-rat adrenal pheochromocytoma and N1E-115-neuroblastoma cells), and in normal cells (BALB/c3T3-mouse fibroblast and BBC-cells bovine brain capillary) in concentrations of 15 to 100μM. Igosheva et al. (2005) have shown that concentration of 50μM of isatin induced apoptosis in

human neuroblastom SH-SY5Y cells after exposure for 48 h. Vine et al. (2007) showed that a derivative of isatin, the 5,6,7-tribromoisatin, induced apoptosis at low concentrations (4μM) in histiocytic lymphoma cell line (U937). According the authors this result indicates that the cytotoxic effect of derivatives may vary with structural modification of compound.

In the present study, the low frequency of micronucleated cells and the high frequency of apoptotic cells observed after treatment of the cells with 10 and 50μM of isatin may indicate, according Vukicevic et al. (2004), that could be occurring others genetic damages that do not result in the formation of micronucleus, and in this way do not elevate its frequency, but it may increase the frequency of apoptotic cells.

Compounds that interfere with the cell cycle have become a major interest in cancer research because they inhibit the proliferation of tumoral cell lines derived from various organs (Jordan and Wilson, 1998; Sun et al., 1998). Although the precise mechanism of isatin-induced cell death remains uncertain, recent studies suggest that isatin induced cytotoxicity might be related to its ability to inhibit cell growth (Igosheva et al., 2005). In terms of its mode of action, isatin itself is proposed to inhibit cancer cell proliferation *via* interaction with extracellular signal related protein kinases (ERKs), thereby promoting apoptosis (Cane et al., 2000; Vine et al. 2009). Studies have been showed that the effect of isatin on apoptosis is related with the inhibition of the ERKs phosphorylation (Cane et al., 2000; Hou et al., 2008). This enzyme ERK plays a central role in signaling of proliferation and cell growth (Liu et al., 1996; Gille and Downward, 1999) and is required for sequential transition of cell cycle phases (particularly G1/S and G2/M transitions (Chambard et al., 2007). The antiproliferative effect and induction of apoptosis found in the present study may be related with interaction of isatin with ERK enzymes and subsequent activation of several signaling cascades to apoptosis, although the role of ERK signaling in this study was not investigated.

In conclusion, we observed that the isatin was cytotoxic to two cell types used, but the not tumoral cell line (CHO-K1) was more sensible compared to tumoral cell (HeLa). However, the isatin inhibited cell proliferation and also promoted apoptosis in both CHO-K1 and HeLa cells *in vitro*. These data suggested that isatin should be further explored as a possible therapeutic agent for the treatment of cancer. Thus, further studies are warranted to better characterize the biological effects of this compound in normal and tumoral cells.

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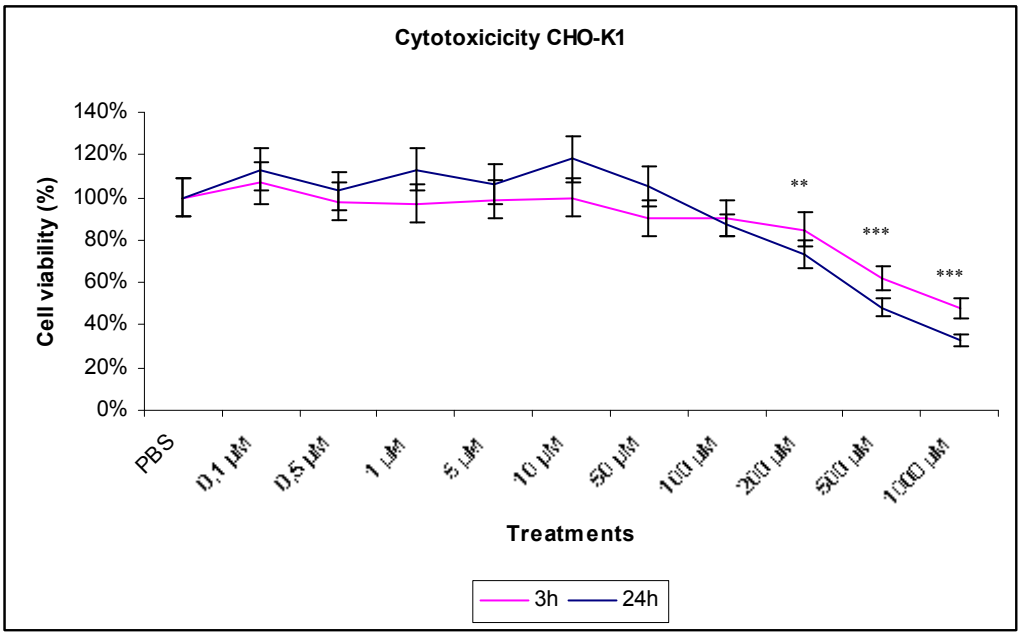

Figure 1. Cell viability (%) observed for CHO-K1 cell after 3 and 24 hours of treatment with isatin.
**Statistically significant difference compared to negative control (P<0.01).
***Statistically significant difference compared to negative control (P<0.001).

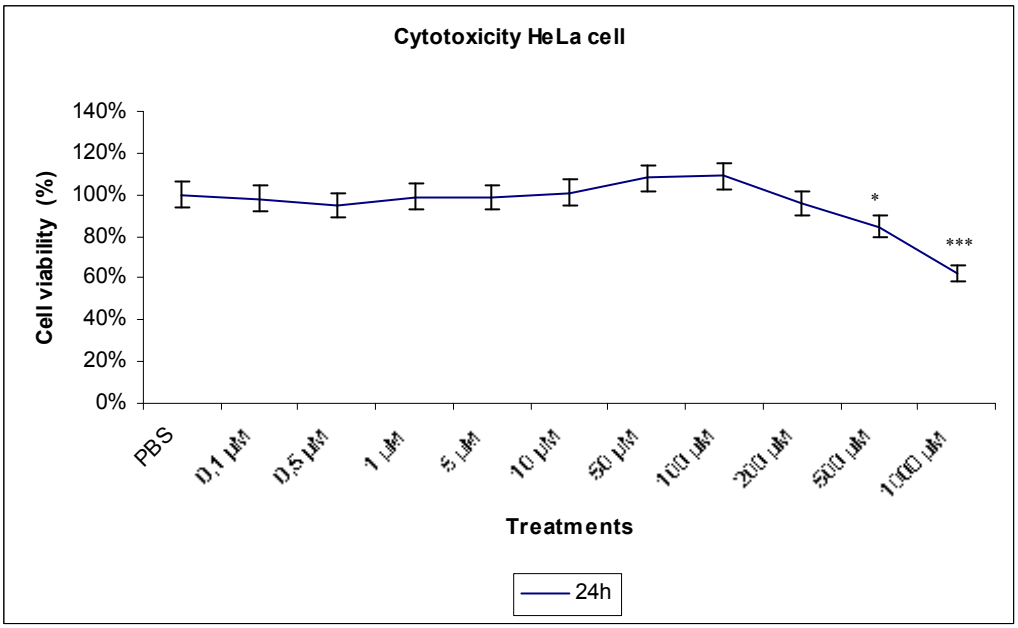

Figure 2. Cell viability (%) observed for HeLa cell after 24 hours of treatment with isatin.
*Statistically significant difference compared to negative control (P<0.05).
***Statistically significant difference compared to negative control (P<0.001).

Table 1. Frequency of micronucleated cells (MN cells), nuclear division index (NDI) and apoptosis index (%) obtained in the determination of mutagenicity for five concentrations of isatin and respective controls in CHO-K1 cells after 3 hours from treatment.

Treatments (in culture)		Frequency of MN cells (1000 binucleated cells/experiment) Experiments				Nuclear Division Index (1000 cells/experiment) Experiments				Apoptosis (%) (200 cells/experiment) Experiments			
		I	II	III	X±SD	I	II	III	X±SD	I	II	III	X±SD
PBS (μL)	50	7	12	13	10.67 ± 3,21[a]	1.82	1.81	1.78	1.81 ± 0.021[a]	16.0	16.0	18.0	16.7 ± 1.15[a]
DXR (μM)	0.3	24	26	22	24.00 ± 2.00[b]	1.74	1.66	1.68	1.69 ± 0.04[b]	45.0	41.0	48.0	44.7 ± 3.51[b]
Isatin (μM)	0.5	6	4	8	6.00 ± 2.00[a]	1.79	1.80	1.75	1.78 ± 0.028[a]	17.0	20.0	17.5	18.2 ± 1.61[a]
	1.0	5	5	10	6.67 ± 2.89[a]	1.83	1.81	1.78	1.81 ± 0.028[a]	17.5	18.5	19.0	18.3 ± 0.76[a]
	5.0	4	5	11	6.67 ± 3.79[a]	1.79	1.80	1.81	1.80 ± 0.009[a]	15.5	19.5	18.0	17.7 ± 2.02[a]
	10	7	4	7	6.00 ± 1.73[a]	1.81	1.81	1.77	1.80 ± 0.022[a]	23.0	16.5	18.0	19.2 ± 3.40[a]
	50	9	11	6	8.67 ± 2.51[a]	1.82	1.82	1.82	1.82 ± 0.003[a]	22.0	19.5	23.0	21.5 ± 1.80[a]

X ± SD: mean ± standard deviation; PBS: negative control; doxorubicin (DXR): positive control.
Values with same letters do not differ statistically (P > 0.05).

Table 2. Frequency of micronucleated cells (MN cells), nuclear division index (NDI) and apoptosis index (%) obtained in the determination of mutagenicity for five concentrations of isatin and respective controls in CHO-K1 cells after 24 hours from treatment.

Treatments (in culture)		Frequency of MN cells (1000 binucleated cells/experiment) Experiments				Nuclear Division Index (1000 cells/experiment) Experiments				Apoptosis (%) (200 cells/experiment) Experiments			
		I	II	III	X±SD	I	II	III	X±SD	I	II	III	X±SD
PBS (μL)	50	6	8	8	7.33 ± 1.15^{a}	1.79	1.79	1.78	1.79 ± 0.006^{a}	20.5	23.0	18.5	20.7 ± 2.25^{a}
DXR (μM)	0.3	24	26	26	25.3 ± 1.15^{b}	1.65	1.63	1.67	1.65 ± 0.023^{b}	47.0	49.5	48.0	48.2 ± 1.25^{b}
Isatin (μM)	0,5	7	8	8	7.67 ± 0.58^{a}	1.79	1.82	1.77	1.79 ± 0.026^{a}	20.5	22.0	21.0	21.2 ± 0.76^{a}
	1.0	9	9	10	9.33 ± 0.58^{a}	1.80	178	1.76	1.78 ± 0.022^{a}	25.0	21.5	24.0	24.5 ± 0.50^{a}
	5.0	10	11	9	10.0 ± 1.00^{a}	1.80	1.79	1.78	1.79 ± 0.010^{a}	21.0	19.5	26.0	22.2 ± 3.40^{a}
	10	10	10	12	10.7 ± 1.15^{a}	1.66	1.67	1.67	1.67 ± 0.004^{b}	30.0	33.0	30.0	31.0 ± 1.73^{b}
	50	8	10	13	10.3 ± 2.52^{a}	1.70	1.68	1.68	1.69 ± 0.013^{b}	29.0	31.0	24.0	31.3 ± 2.52^{b}

X ± SD: mean ± standard deviation; PBS: negative control; doxorubicin (DXR): positive control.
Values with same letters do not differ statistically ($P > 0.05$).

Table 3. Frequency of micronucleated cells (MN cells), nuclear division index (NDI) and apoptosis index (%) obtained in the determination of mutagenicity for five concentrations of isatin and respective controls in HeLa cells after 24 hours from treatment.

Treatments (in culture)		Frequency of MN cells (1000 binucleated cells/experiment) Experiments				Nuclear Division Index (1000 cells/experiment) Experiments				Apoptosis (%) (200 cells/experiment) Experiments			
		I	II	III	X±SD	I	II	III	X±SD	I	II	III	X±SD
PBS (μL)	50	18	20	18	18.7 ± 1.15^{a}	1.92	1.87	1.89	1.89 ± 0.023^{a}	20.5	22.0	19.0	20.5 ± 1.50^{a}
DXR (μM)	0.3	36	39	31	35.3 ± 4.04^{b}	1.85	1.85	1.85	1.85 ± 0.002^{b}	48.0	47.5	47.0	47.5 ± 0.50^{b}
Isatin (μM)	0.5	20	21	22	21.0 ± 1.00^{a}	1.86	1.87	1.86	$1.86 \pm 0.003^{a,b}$	21.0	21.5	23.0	21.8 ± 1.04^{a}
	1.0	23	24	23	23.3 ± 0.58^{a}	1.90	1.90	1.87	1.89 ± 0.016^{a}	23.0	22.5	20.0	21.8 ± 1.61^{a}
	5.0	26	22	23	23.7 ± 2.08^{a}	1.86	1.88	1.86	$1.86 \pm 0.010^{a,b}$	23.5	24.0	23.0	23.5 ± 0.50^{a}
	10	25	20	24	23.0 ± 2.65^{a}	1.85	1.85	1.84	1.85 ± 0.006^{b}	30.0	33.5	30.0	31.2 ± 2.02^{c}
	50	24	24	25	24.3 ± 0.58^{a}	1.85	1.85	1.84	1.85 ± 0.004^{b}	35.5	35.5	33.0	34.7 ± 1.44^{c}

X ± SD: mean ± standard deviation; PBS: negative control; doxorubicin (DXR): positive control.
Values with same letters do not differ statistically ($P > 0.05$).

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CONSIDERAÇÕES FINAIS

A partir dos resultados obtidos com o ensaio do cometa e o teste do micronúcleo em sistema-teste *in vivo* realizados com três doses diferentes da isatina, após tratamento agudo (24 horas) e de doses repetidas (14 dias), podemos concluir que:

1) Todas as concentrações da isatina avaliadas nos testes agudo não apresentaram atividade genotóxica e mutagênica para células do sangue periférico dos camundongos.

2) Após tratamento de doses repetidas, a maior concentração da isatina (150mg/kg p.c.) apresentou genotoxicidade e mutagenicidade, tanto em células da medula óssea quanto do sangue periférico dos camundongos.

3) Os efeitos genotóxico e mutagênico da isatina foram concentração e período de exposição dependentes.

A partir dos resultados obtidos no ensaio do MTT, teste do micronúcleo e índice de apoptose em cultura de células de mamíferos *in vitro* submetidos a cinco concentrações diferentes da isatina, podemos concluir que:

1) No ensaio de citotoxicidade MTT as células normais (CHO-K1) apresentaram maior sensibilidade à isatina do que a linhagem tumoral (HeLa).

2) Todas as concentrações avaliadas no teste do micronúcleo não apresentaram atividade mutagênica para as duas linhagens utilizadas (CHO-K1 e HeLa).

3) As concentrações de 10 e 50μM da isatina induziram a apoptose e inibiram a proliferação celular nas duas linhagens estudadas.

4) Os resultados obtidos nos experimentos *in vivo* e *in vitro* indicam uma potencialidade terapêutica da isatina a ser explorada para fins farmacológicos.

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