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### PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA


# Atividade biológica da Biflorina, uma ɵ-naftoquinona isolada das raízes da *Capraria biflora L.*, em células tumorais e não tumorais


**Gabrielle Gianna Nunes de Souza Wisintainer**



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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção de grau de Mestre em Biotecnologia.

**Orientadora:** Profa. Dra. Mariana Roesch Ely

**Co-orientador:** Prof. Dr. João A. P. Henriques

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# ÍNDICE

## LISTA DE ABREVIATURAS

| | |
|---|---|
| DMEM | *Dulbecco's Modified Eagle Medium* |
| DMSO | Dimetilsulfóxido |
| DNA | Ácido desoxirribonucleico |
| EGFR | Epidermal Growth Factor Receptor |
| EUA | Estados Unidos da América |
| $H_2O_2$ | Peróxido de Hidrogênio |
| HR-DIMS | High-resolution direct-infusion mass spectrometry |
| $IC_{50}$ | Concentração inibitória de 50% do crescimento celular |
| INCA | Instituto Nacional do Câncer |
| MTT | 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio |
| PBS | Tampão fosfato salina |
| ERO | Espécies reativas de oxigênio |
| SFB | Soro fetal bovino |
| 5-FU | 5 - Fluoracil |
| TLC | Thin-layer chromatographic |

## RESUMO

A biflorina é uma *o*-naftoquinona que apresenta uma variedade de atividades biológicas, entre as quais pode-se destacar a atividade antitumoral. Esta molécula é obtida a partir das raízes da planta *Capraria biflora L.*(Schrophulariaceae) originária das Antilhas e América do Sul e habita zonas temperada e tropical. No continente europeu é muito usada como ornamento. No Brasil, pode ser encontrada nos Estados de Goiás, Minas Gerais e na faixa litorânea entre o Piauí até o Espírito Santo. O presente trabalho traz informações referentes aos primeiros estudos de caracterização estrutural até os mais recentes relatos sobre a atividade biológica desta molécula. Neste trabalho avaliamos os efeitos citotóxicos e detecção de apoptose tardia por análise *in situ* de células tratadas com a biflorina. Para isso utilizamos a linhagem tumoral HeLa e a não tumoral HEK-293 tratadas com biflorina nas concentrações de 5 - 50 µg/mL por 24h, 48h e 72h. A citotoxicidade foi avaliada exclusivamente para o tratamento de 48h em seis linhagens celulares diferentes sendo elas: Hep-2, HeLa, HT-29, A-375, A-549 e HEK-293. Os resultados mostraram citotoxicidade seletiva da biflorina contra a linhagem não tumoral HEK-293 ($IC_{50}$ = 56,01 ± 1,17 µg/mL) comparada com todas as linhagens de células tumorais analisadas, com $IC_{50}$ variando de 29,44 ± 1,32 µg/mL até 47,37 ± 3,21 µg/mL. Modificações morfológicas em células HeLa foram observadas após o tratamento de 48 horas com biflorina de acordo com o aumento da concentração (5-50 µg/mL). Além disso, na maior parte dos casos, observou-se um incremento do estágio de apoptose tardia, na análise *in situ* da imunocoloração de anexina V de todas as linhagens (Hep-2, HeLa, HT-29, A-375, A-549 e HEK-293) após tratamento com a biflorina. A apoptose tardia para HEK-293 (77,69 ± 6,68%) foi mais evidente em concentrações mais elevadas de biflorina em comparação com as linhagens tumorais testadas. Os resultados indicam que a biflorina mostrou uma importante citotoxicidade contra linhagens de células tumorais. No entanto, mais estudos são necessários para entender melhor os mecanismos envolvidos na citotoxicidade e morte celular programada.



**Palavras-chave:** *Capraria biflora*, Biflorina, Atividade biológica, Citotoxicidade, Anexina V, Apoptose.

## ABSTRACT

Biflorin is an *o*-naphthoquinone with proven cytotoxic effects on tumor cells with antimicrobial, antitumor and antimutagenic activities. Biflorin is an isolated compound taken from the roots of the plant *Capraria biflora L.* (Schrophulariaceae), indigenous of the West Indies and South America, that inhabits temperate and tropical areas. In the European continent, this plant is used as an ornament. In Brazil, it can be found in the states of Goiás, Minas Gerais and the coastal strip between Piauí in the Northeast, and it extends to the states of Ceará, Pernambuco, Pará and Espírito Santo. In this work, we report the information related to the first structural characterization studies, as well as the latest reports concerning the biological activity of this molecule. It was work verified the cytotoxic effects of biflorin and detection of late apoptosis by *in situ* analysis. Initially, tumor HeLa and non-tumor HEK-293 cells were treated with biflorin for 24h, 48h and 72h at a range of 5-50 µg/mL. The cytotoxicity was further evaluated exclusively for 48h treatment on six different cell lines Hep-2, HeLa, HT-29, A-375, A-549 and HEK-293. The results indicate that biflorin showed selective cytotoxicity against non-tumor line HEK-293 ($IC_{50}$ = 56,01 ± 1,17 µg/mL) compared to all tumor cells analyzed in a concentration dependent manner, with $IC_{50}$ ranging from 29.44 ± 1.32 µg/mL to 47,37 ± 3,21 µg/mL. Substantial morphological changes in HeLa cells were observed after 48h treatment with biflorin with increased concentrations (5-50 µg/mL) of extract. In addition, *in situ* immunostaining of annexin V showed that all lines were majority seen at late apoptotic stages in a dose-dependent manner. Late apoptosis for HEK-293 was more evident (77.69 ± 6.68%) at higher extract concentrations compared to all tumor lines tested. The data here presented indicate that biflorin showed an important cytotoxicity against tumor cell lines. However, more studies are needed to better understand the pathways involved in programmed cell death.



**Key words:** *Capraria biflora*, Biflorin, Biological activity, Cytotoxicity, Annexin V, Apoptosis.

# 1 INTRODUÇÃO

Os produtos naturais tem sido empregados na prevenção e tratamento de doenças humanas, sendo que mais de 60% dos medicamentos utilizados tem em algum momento sua origem relacionada a uma fonte natural. Dentre as fontes encontradas na natureza, a maior contribuição para o desenvolvimento de substâncias terapêuticas deve-se as plantas (Kirkpatrick *et al*., 2002; Silva, 2006; Costa-Lotufo *et al*., 2010). Entre 1981 e 2002, por exemplo, das novas drogas lançadas no mercado farmacêutico, 28% são de produtos naturais e 24% são derivados sintéticos destes, o que dá ênfase a importância dos estudos nessa área (Gullo *et al*., 2006). A presença de fármacos derivados de produtos naturais tem destaque entre os anticancerígenos, antimicrobianos e antivirais (Silva, 2006). Atualmente, mais de uma centena de compostos derivados de produtos naturais estão em fase de testes clínicos (Harvey, 2008).

Estudos com plantas medicinais possibilitaram a descoberta de vários fármacos para o tratamento do câncer, como exemplo os alcalóides vimblastina e vincristina, ambos isolados de *Catharanthus roseus* G., utilizadas respectivamente para linfomas e leucemias agudas, e ainda seus derivados semi-sintéticos vindesina, utilizada para leucemia linfoblástica aguda, e vinorelbina, usada para câncer de pulmão e mama (Cragg & Newman, 2005; Newman & Cragg, 2007; Silva, 2006). Compostos naturais e sintéticos que apresentam quinonas em suas estruturas são reconhecidos por possuírem potentes atividades biológicas, entre elas antitumorais (Silva *et al.*, 2003, Asche, 2005), moluscicidas (Santos *et al.* 2000, Barbosa *et al.*, 2005), leishmanicidas (Teixeira *et al.*, 2001), anti-inflamatórias (Almeida *et al.*, 1990), antifúngicas (Gafner *et al.*, 1996), tripanocidas (Pinto *et al.*, 2000, De Moura *et al.*, 2001), antiprotozoárias (Zani *et al.*, 1997) e anti-viral (Alves *et al.*, 2008; Stagliano *et al.*, 2006).

Desta forma, o interesse por produtos naturais foi intensificado nos últimos anos devido à sua importância farmacológica. O lapachol e a β-lapachona podem ser consideradas as naftoquinonas que mais influenciaram os grupos brasileiros de pesquisa em química e farmacologia de quinonas. Apesar de ainda não ser um fármaco, a β-lapachona é uma substância muito importante do ponto de vista da pesquisa científica. A β-lapachona é facilmente obtida a partir do lapachol e é

encontrada como constituinte minoritário do cerne de árvores da família Bignoniaceae, conhecidas no Brasil como ipês (Ferreira *et al.*, 2010). A β-lapachona é um potente tripanocida e mostrou-se citotóxica para células tumorais. Parte do mecanismo de ação das quinonas envolve a geração de espécies reativas de oxigênio (ERO), as quais são tóxicas para o *Trypanossoma cruzi*, por exemplo, e induzem apoptoses e interagem com topoisomerases das células tumorais (Ferreira *et al.*, 2010). A β-lapachona tem-se mostrado eficaz em modelos *in vitro*, contra diversas linhagens de células malignas humanas, incluindo melanoma, leucemia, câncer colorretal, câncer de pulmão, de mama e de próstata (Ferreira *et al.*, 2010). Esta naftoquinona é o grande destaque desta classe de substâncias, tanto que se encontra em testes clínicos de fase II para o tratamento do câncer de pâncreas (Ferreira *et al.*, 2010).

A *Capraria biflora L.* é uma planta que pertence à família Schrophulariaceae, originária das Antilhas e América do Sul, que habita zonas temperadas ou áreas de clima tropical (Aquino *et al.*, 2006). Por se tratar de uma planta invasora, é encontrada em terrenos baldios, preferindo locais úmidos, próximos ao litoral, riachos ou lagoas (Aquino *et al.*, 2001). A espécie é amplamente distribuída na América do Sul (Venezuela, Peru, Brasil e Guiana Francesa); na América Central (El Salvador, Trinidad-Tobago, Bahamas, Panamá, Curaçao, Guatemala e Porto Rico); na América do Norte (Estados Unidos e México) e na Ásia (Índia e China) (Correia, 1984). No Brasil, está localizada nos estados de Minas Gerais, Goiás e na faixa litorânea entre o Piauí e Espírito Santo (Aquino *et al.*, 2006). Trata-se de uma planta herbácea ou arbustiva, cujo caule é ramoso, chegando até 1,5m de altura (Aquino *et al.*, 2001).

A biflorina (6,9-dimetil-3-(4-metil-3-pentenil)nafta[1,8-bc]-piran-7,8-diona) é uma o-naftoquinona de origem natural que pode ser encontrada nas raízes da *Capraria biflora* L. Os primeiros estudos ocorreram em 1999 onde foram realizados ensaios demonstrando atividade antimicrobiana da biflorina, utilizando-se principalmente cepas de *Candida albicans* (Lyra Júnior *et al.*, 1999), além de outras bactérias já estudadas anteriormente (Gonçalves de Lima *et al.*, 1961), com potencial antimicrobiano frente a bactérias gram positivo e em concentrações mais altas de biflorina frente a bactérias gram negativo.

A atividade antitumoral da biflorina, por sua vez, tem sido estudada como novo recurso terapêutico frente à alta incidência de diferentes tipos de tumores que acometem a população. O câncer é a segunda causa de morte registrada no mundo, precedida

apenas de doenças de origem vascular. Desta forma, buscam-se um melhor entendimento quanto à etiopatogenia das neoplasias e o desenvolvimento de novas estratégias preventivas e terapêuticas no seu combate.

Em face ao exposto, este trabalho teve como objetivo caracterizar quimicamente a molécula da biflorina por espectrometria de massas, avaliar a atividade antitumoral *in vitro* através da citotoxicidade da biflorina em cultura de células tumorais e analisar a morte celular por apoptose através de ensaios *in situ* utilizando coloração Anexina-V com células tratadas com biflorina.

# 2 REVISÃO BIBLIOGRÁFICA

A revisão bibliográfica deste trabalho esta apresentada na forma de um artigo científico de revisão. Este artigo é intitulado "**Biflorin: an o-naphthoquinone of clinical significance**," o qual foi submetido para a revista *Phytochemistry Letters.*

## 2.1 CAPÍTULO 1

# Biflorin: an o-naphthoquinone of clinical significance

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## Abstract

Biflorin is an *o*-naphthoquinone with proven cytotoxic effects on tumor cells with antimicrobial, antitumor and antimutagenic activities. Biflorin is an isolated compound taken from the roots of the plant *Capraria biflora L.* (Schrophulariaceae), indigenous of the West Indies and South America, that inhabits temperate or tropical areas. In the European continent, this plant is used as an ornament.. This compound has shown to be strongly active against gram-positive and alcohol-acid-resistant bacteria. Biflorin also inhibit the proliferation on five tumor cell lines CEM and HL-60, B16, HCT-8 and MCF-7 in a dose dependent manner. Recently, SK-Br3 cell line was treated with biflorin showing important cytotoxic effects. In this article, we report the information related to the first structural characterization studies, as well as the latest reports concerning the biological activity of this molecule.

**Keywords:** *Capraria biflora*, Biflorin, Biological activity

## 1. Introduction

Natural products are the largest contributor to the production of activity metabolites, and many of them are used as drugs, cosmetics and pesticides (Mann, 2002). Natural products do not always contribute in an integral manner to the synthesis of drugs; however, they occasionally serve as models for the synthesis of new drugs, such as procaine, chloroquine, vincristine, vinblastine and camptothecin (Newman *et al*., 2003; Ferreira *et al*., 2010).

Natural products play an important role in the treatment and prevention of human diseases, and over 60% of drugs used have natural origins. Among the sources of such drugs found in nature, plants provide the largest contribution to the development of therapeutic substances (Kirkpatrick *et al*., 2002; Silva, 2006; Costa-Lotufo *et al*., 2010). Among new drugs launched between 1981 and 2002 in the pharmaceutical market, 28% are made from natural products, and 24% are synthetic derivatives of

natural origin, which emphasizes the importance of studies in this area (Gullo *et al.*, 2006). Drugs derived from natural sources have anti-cancer, anti-microbial and antiviral activity (Silva, 2006). Currently, more than 100 compounds derived from natural products are undergoing clinical trials (Harvey, 2008). The study of medicinal plants allowed the discovery of several drugs for the treatment of cancer, such as the alkaloids vinblastine and vincristine, both isolated from *Catharanthus roseus G*., which are used for lymphomas and acute leukemias, respectively. These drugs also served as the basis for the semisynthetic derivatives vindesine, which is used for acute lymphoblastic leukemia, and vinorelbine, which is used for lung and breast cancer (Cragg & Newman, 2005; Newman & Cragg, 2007; Silva, 2006).

## 2. Chemical structure and biological activity of quinones

The quinones are a large and important class of substances present in nature and can be obtained as a synthetic product with a great variety of applications (Thomson, 1987). The quinone structure was identified as an important pharmacophore group and is characterized by a significant cytotoxic effect through several drugs used in treating diseases (Driscoll *et al*. 1974; Liu, 2004), thus constituting a class of highly representative antineoplastic agents (Asche, 2005).

Quinones are organic compounds produced by the oxidation of phenols, and reduction of quinones also gives the corresponding phenols. Their hallmark is the presence of two carbonyl groups that form a conjugated system with at least two CC double bonds. Thus, according to the cycle in which the system of conjugated double and ketones is inserted, three major groups of quinones exist: benzoquinones have a benzene ring, naphthoquinones have a naphthalene ring and anthraquinones have an anthracene ring (Figure 1) (Simoes *et al.*, 2001).

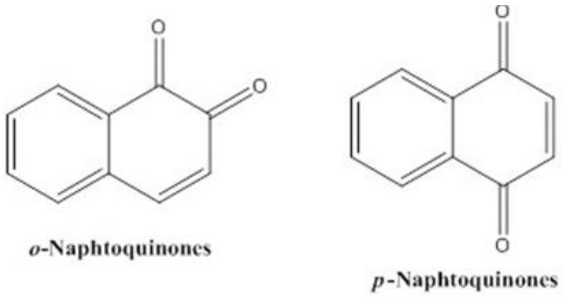


**Figure 1.** Isomeric forms of ortho-and para-quinonone.

Natural and synthetic compounds that have quinones in their structures are known to possess potent biological activities such as anti-cancer (Silva *et al.*, 2003, Asche 2005), anti-mollusc (Santos *et al.*, 2000; Barbosa *et al.*, 2005), anti-leishmanial (Teixeira *et al.*, 2001), anti-inflammatory (Almeida *et al.*, 1990), anti-fungal (Gafner *et al.*, 1996), trypanocidal (Pinto *et al.*, 2000; Moura *et al.*, 2001), anti-protozoal (Zani *et al.*, 1997), and anti-viral (Stagliano *et al.*, 2006) activity. Anti-tumor agents are effective inhibitors of DNA topoisomerase I (Asche, 2005). Thus, the interest in these substances has intensified in recent years because of their pharmacological importance. Some pharmacological activities are known for compounds such as β-lapachone, a natural substance that is easily obtained from lapachol, which is found as a minor constituent of the heartwood of trees of the family Bignoniaceae, known in Brazil as Ipe (Ferreira *et al.* 2010) (Figure 2). β-lapachoneis a potent trypanocidal agent and has shown cytotoxicity against tumor cells. Part of the mechanism of action of quinones involves the generation of reactive oxygen species (ROS), which have been shown to be toxic to *Trypanosoma cruzi* and induce apoptosis and / or interact with topoisomerase of tumor cells (Ferreira *et al.* 2010). Naphthoquinone is the most notable member of this class of substances because it is has been applied to phase II studies for the treatment of pancreatic cancer (Ferreira *et al.*, 2010). β-lapachone is effective *in vitro* against various human malignant cell lines, including melanoma, leukemia, colorectal, lung, breast and prostate cancer cells (Ferreira *et al.*, 2010).

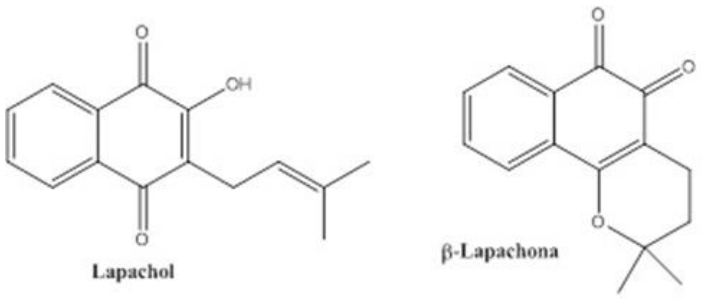


**Figure 2.** Chemical structure of lapachol and β- lapachone.

## 3. General information regarding the species *Capraria biflora L.*

*Capraria biflora L.* is a plant that belongs to the Schrophulariaceae family, originates from the Antilles and South America and inhabits temperate or tropical areas (Aquino *et al.*, 2006). As it is an invasive plant, it is found on wasteland, preferring humid, near-shore streams or ponds (Aquino *et al*., 2001). It is widely distributed in South America (Venezuela, Peru and Guyana), Central America (El Salvador, Trinidad and Tobago, Bahamas, Panama, Curacao, Guatemala and Puerto Rico), North America (United States and Mexico) and Asia (India and China) (Correia, 1984). In Brazil, it is located in the states of Minas Gerais and Goiás and in the coastal strip between Espirito Santo and Piauí (Aquino *et al.*, 2006). *Capraria biflora L.* is an herbaceous plant or shrub whose stem is twiggy, reaching a height of 1.5 m (Aquino *et al.*, 2001). It has been used to treat fever and for their diuretic, stimulant and digestive properties and also is regarded as a tonic and beneficial to digestion (Matos Brito *et al.*, 1994). The aqueous extract demonstrated both peripheral and central analgesic effects (Acosta *et al*., 2003). The roots of *Capraria biflora L.* yield biflorin, an o-naphthoquinone that to date has been the only substance isolated from this plant with proven medicinal activity (Aquino *et al*., 2006).

## 4. Biflorin characterization

The first studies with phytochemicals from *C. biflora L* were performed by Gonçalves de Lima *et al.* (1954), who isolated, from the roots of the plant, an active ingredient that showed antimicrobial activity against Gram-positive, acid-resistant bacteria and some fungi (Gonçalves de Lima *et al.*, 1954). This substance was termed isolated biflorin, 6,9-dimethyl-3-(4-methyl-3-pentenyl)naphtha[1,8-bc]pyran-7,8-dione. In the same year, the spectrum of activity of biflorin was determined by Eli Lilly (Mc Cowen & Stone, 1953). In the following year, a method for obtaining pure crystals of naphthoquinone was developed. These crystals had higher activity against Gram-positive bacteria than the naphthoquinone used in the initial studies and showed activity against other microorganisms (dermatophytes and yeasts) that had not been previously tested (Gonçalves de Lima *et al*., 1954; Gonçalves de Lima *et al.,* 1958). In 1958, a method was developed with an even higher yield than the methods previously described. In the same year, the physical and chemical characteristics of biflorin were elucidated using the methods of UV (ultraviolet) and IR (infrared) spectroscopy and elemental analysis, in which the presence of a naftoquinonica structure was confirmed (Prelog *et al*., 1958; Comin *et al*., 1963; Grant *et al.*, 1963).

The earliest studies on the chemical structure biflorin include the work of Hoppe (1960), who used crystallographic analysis with X-ray diffraction (Hoppe, 1960) to determine the crystalline structure and structural formula $C_{20}H_{20}O_3$. Recently, Fonseca *et al.* (2003) indicated, using $^{1}H$ and $^{13}C$ nuclear magnetic resonance in one- and two-dimension experiments, that biflorin naturally exists as a dimer that was named Bis-biflorin (Fonseca *et al.,* 2003), as shown in Figure 3.

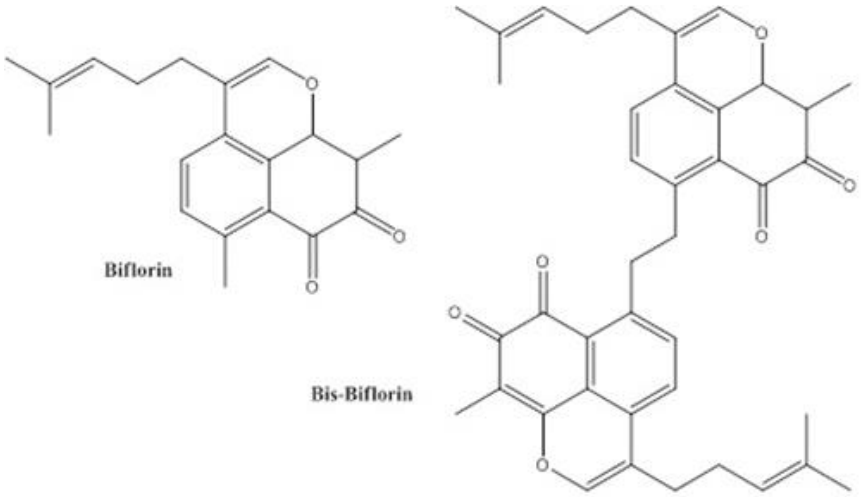


**Figure 3.** Chemical structures of biflorin and bis-biflorin (Fonseca *et al.*, 2003).

Gonçalves de Lima *et al*. (1961) demonstrated the photosensitivity of biflorin to natural light (Gonçalves de Lima *et al.*, 1961; Gonçalves de Lima *et al.*, 1962).

In more recent studies, new methods of quantification and qualification of the biflorin through liquid chromatography coupled to mass detection, were evaluated and found to be fast and accurate (Lyra Jr. *et al.*, 2007).

## 4.1. Chemical synthesis

Since the early nineteenth century, when the chemical structures of the first naphthoquinones were identified, researchers have been attempting the synthesis or semi-synthesis of these compounds (Ehrlich & Herter, 1904). Recently, synthesis procedures as metathesis became easier for the formation of the condensed cycles that are part of the basic structure of these compounds (Song *et al*., 2009). The search for the synthesis of new structures target the enhancement of biological activity, and compounds such as fluoro-1,4-naphthoquinones were synthesized by Zakharova *et al*. (2011). Later, several natural naphthoquinones such as β-lapachone were also synthesized (Zakharova *et al*., 2011). The condensation reactions to produce a series of

compounds were further tested according to their anti-inflammatory activity (Tseng *et al.*, 2013).

Interestingly, only one total synthesis procedure for biflorin has been presented in the literature (Best *et al.*, 1986), most likely because of the complex chemical structure of is compound. In this procedure, an addition-type intramolecular Diels-Alder reaction was the key step for obtaining the final product, as shown in Figure 4.

Previously, Comin *et al.* (1963) obtained differentiated structures of biflorin through hydrogenation reactions. In a typical case of semi-synthesis, these authors described physicochemical properties of the products obtained by reaction of 1 mol of natural biflorin extracted from *C. biflora* with one and two equivalents of hydrogen. The hydrogenation products were subsequently acetylated and oxidized to yield the diacetate and prenitico acid, respectively (Comin *et al.*, 1963).

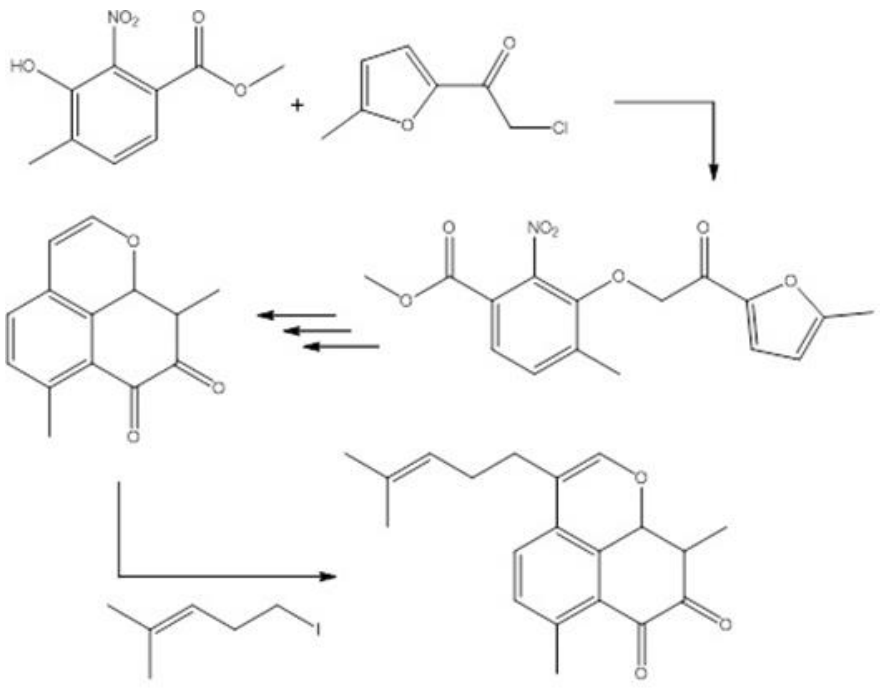


**Figure 4.** Schematic representation of the chemical synthesis of biflorin presented by Wayne *et al*. (1986).

## 4.2 Obtaining biflorin from other natural sources

Biflorin has been found in other plan*ts*. In 2007, Ndi *et al.* reported the presence of biflorin in extracts of an Australian desert plant known as *Eremophila neglecta*. The family Myoporaceae is characterized by its large amount of potential secondary metabolites. To obtain compounds including biflorin, the authors performed a series of extractions with varying solvents (Ndi *et al*., 2007). The products were identified by $^{1}$H- and $^{13}$C NMR. Previously, Forster *et al.* (1986) indicated the presence of naphthoquinone in plants of the same family, *Eremophila latrobei* (Forster *et al*., 1986).

## 4.3 Biological activity of biflorin

Biflorin is an o-naphthoquinone of natural origin that can be found in the roots of *Capraria biflora* L. Studies related to o-naphthoquinone began in 1999 and showed antimicrobial activity, mostly in strains of *Candida albicans* (Lyra Jr. *et al.*, 1999). Other bacteria were studied previously, and biflorin showed antimicrobial activity against gram-positive and gram-negative bacteria (Gonçalves de Lima *et al*., 1961).

The only clinical case reporting the use of biflorin was in 1958, where 1% biflorin crystallized paste was used in a 14-year-old male patient with an erythematous perioral lesion covering the outside area of the lip. After sample collection in different areas, it was possible to isolate a fungus identified as *Trichosporon margaritipherum*. The patient began using 1% biflorin with lanolin and petrolatum, and after two days, the lesion showed a pink color with no exudation and the skin returned to normal after four days of treatment. The paste vehicle was later replaced by polyethylene glycol, which provided better results, and after a week, the erythema had completely recovered (Aquino *et al.*, 2006).

Biflorin has *in vivo* and *in vitro* antitumor effects (Table 1), but its exact mechanism of action is not yet known (Vasconcellos *et al*., 2007; Vasconcellos *et al*., 2005). It has been shown that biflorin has a concentration-dependent cytotoxic effect in different eukaryotic cells but is not mutagenic in bacteria and yeast (Vasconcellos *et al*., 2010).

**Table 1.** Different activities shown in biflorin studies.

| Organisms | Type of activity | Reference |
|---|---|---|
| *Trichosporon margaritipherum* | Antifungical | Aquino *et al.*, 2006 |
| Gram-positive bacteria, Bacteria alcohol-acid-resistent | Antimicrobial | Gonçalves de Lima *et al.*, 1953 |
| *Candida albicans* | Antimicrobial | Lyra Junior *et al.*, 1999 |
| Yeast *S. cereviseae and V79* | Antimutagenic | Vasconcellos *et al.*, 2010 |
| Cells V79 | Antigenotoxic | Vasconcellos *et al.*, 2010 |
| Tumor cell lines CEM, HL-60, B16, HCT-8 and MCF-7 | Antitumor | Vasconcellos *et al*., 2007<br>Vasconcellos *et al*., 2005 |

The protective effects of biflorin against mutagenesis in yeasts and V79 cells occurred only at low concentrations; high concentration of biflorin (concentrations higher than 250 μg/ml in haploid yeast and 10 μg/ml in V79 cells) was cytotoxic, inducing breaks in the DNA chain as evidenced by the comet test (Vasconcellos *et al*., 2010). Another mechanism that leads to breaks in the DNA chain is the interaction of the particles with DNA topoisomerase I (Vasconcellos *et al*., 2010). The induction of DNA damage by biflorin underlies its anti-proliferative potential (Vasconcellos *et al*., 2007). In the same study, it was demonstrated that biflorin does not induce frameshift mutations in yeast and bacteria; therefore, the authors assumed that biflorin is not a good DNA intercalating agent. However, other studies have shown that biflorin causes no changes in the cell cycle; rather, it inhibits the synthesis of DNA and interacts directly with single-stranded DNA and double-stranded DNA to induce cell death by apoptosis (Vasconcellos *et al*., 2007).

Antitumor activity *in vivo* was evaluated in animals transplanted with sarcoma 180 and Ehrlich carcinoma cells, showing promising antitumor activity with low toxicity on histopathological examination of the spleen, kidney and liver. Moreover, when coupled with 5-fluoracil chemotherapy (5-FU), biflorin increased the effectiveness in addition to reducing systemic toxicity (Vasconcellos *et al*., 2007). In experiments with animals transplanted with B16 melanoma cells, biflorin was

administered at 25 mg/day for 10 days, and there was a significant inhibition of tumor growth with no systemic toxicity (Vasconcellos *et al*., 2011). In an experimental metastasis assay performed by intravenous injection of B16-F10 cells (a cell line with high metastatic potential) in mice, a dose-dependent inhibitory effect of biflorin was observed against the formation of metastatic nodules in the lung tissue (Carvalho *et al*., 2009).

In a recent study, the SK-Br3 cell line (breast cancer) was treated with biflorin, and the results showed cytotoxicity. Moreover, biflorin produced a decrease in the expression of EGFR (epidermal growth factor receptor), where inhibition of proliferation in biflorin-treated cells was shown to be mediated in part by down-regulation of the EGFR signaling pathway (Montenegro *et al.*, 2013).

Studies have also indicated immunoadjuvant activity for biflorin. Carvalho *et al*. (2009) investigated Swiss mice treated with ovalbumin in the presence and absence of biflorin to investigate the production of total anti-OVA. An increase in the total production of anti-OVA antibodies (IgG, IgA and IgM) was observed in mice immunized with ovalbumin in combination with biflorin when compared to the group treated with ovalbumin, indicating humoral immunoadjuvant activity for this compound (Carvalho *et al*., 2009).

Biflorin has protective capacity against oxidative stress induced by $H_2O_2$ (hydrogen peroxide) as confirmed by the reduction of lipid peroxidation in V79 cells (Vasconcellos *et al*., 2010). Hydrogen peroxide is an important compound for ROS that, in combination with metals such as reduced iron or copper, is transformed by a Fenton reaction with the highly reactive hydroxyl radical (OH •), which causes damage to practically all macromolecules (Halliwell & Gutteridge, 2000). Thus, biflorin reduces DNA damage and mutation triggered by $H_2O_2$ in yeast *S. cerevisiae* and V79 cells, suggesting an anti-oxidant effect (Vasconcellos *et al*., 2010).

The study suggests a mechanism of action for biflorin in MDA-MB-435 melanoma cells, most likely by the inhibition of N-cadherin. Inhibitors of N-cadherin function have been demonstrated to cause apoptosis and regulate the AKT-1 pathway (Montenegro *et al.*, 2013).

## 5. Conclusions

The chemical characteristics of biflorin were elucidated using the methods of UV (ultraviolet) and IR (infrared) spectroscopy and elemental analysis, in which the presence of a naphtoquinonic structure was confirmed. In different concentrations, the scavenging of hydroxyl radicals by biflorin may contribute to antioxidant activity and protective effects against cytotoxicity, genotoxicity, mutagenicity and intracellular lipid peroxidation induced by $H_2O_2$. Higher biflorin concentrations, however, are cytotoxic, possibly because of its ability to induce DNA strand breaks (Vasconcellos *et al.,* 2010). Biflorin has numerous biological activities related to the regulation of apoptosis, the cell cycle and cell differentiation. Studies indicate that biflorin presents antioxidant activity and cytotoxic potential against tumor cells *in vitro* and *in vivo* models. These cellular effects of biflorin are dependent on its concentration. In addition to these activities, biflorin has been shown to be efficacious and safe, as it has no mutagenic potential. Biflorin can thus be considered a new prototype substance with potential anticancer activity. Further studies of this substance isolated from the roots of *C. biflora L*. are necessary.

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# 3 RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de artigo científico. O artigo intitulado ***O*-naphthoquinone isolated from *Capraria biflora* L. presents selective cytotoxicity against tumor cell lines** será submetido a revista *Clinical Biochemistry*.

## 3.1 CAPÍTULO 2

# *O*-naphthoquinone isolated from *Capraria biflora* L. presents selective cytotoxicity against tumor cell lines

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**Abstract**

Biflorin is an *o*-naphthoquinone isolated from the roots of the plant *Capraria biflora* L. (Schrophulariaceae) collected in the northeast region of Brazil. The present work verified the cytotoxic effects of biflorin and detection of late apoptosis by *in situ* analysis. Initially, tumor HeLa and non-tumor HEK-293 cells were treated with biflorin for 24h, 48h and 72h at a range of 5-50 µg/mL. The cytotoxicity was further evaluated exclusively for 48h treatment on six different cell lines Hep-2, HeLa, HT-29, A-375, A-549 and HEK-293. The results indicate that biflorin showed selective cytotoxicity against all tumor cells analyzed, with $IC_{50}$ ranging from 29.44 ± 1.32 µg/mL to 47.37 ± 3.21 µg/mL compared non-tumor line Hek-293 ($IC_{50}$ = 56.01 ± 1.17 µg/mL). Given the increased susceptibility of HeLa to biflorin ($IC_{50}$ = 29.44 ± 1.32 µg/mL), this line was selected for further morphological analysis with giemsa staining. Substantial morphological changes in HeLa cells were observed after 48h treatment with biflorin with increased concentrations (5-50 µg/mL) of extract. Late apoptosis/necrosis events by *in situ* immunostaining of annexin V varied according to each cell lines and presented an increase in late apoptotic events with higher doses of biflorin. From the tumor lines, Hep-2 cells showed greater values when treated with higher concentrations of biflorin - 40 µg/mL (69.63 ± 2.28%). The non tumor HEK-293 line showed more resistance to apoptosis and late events for the this line was more evident (77.69 ± 6.68%) at higher extract concentrations compared to the tumor lines tested. The data here presented indicate that biflorin showed an important cytotoxicity against tumor cell lines; however, more studies are needed to better understand the pathways involved in programmed cell death.

**Key words**: *Capraria biflora*, Biflorin, Cytotoxicity, Annexin V, Apoptosis.

## 1. Introduction

*Capraria biflora* L. (Schrophulariaceae) is a perennial shrub distributed in North and South America (Correia, 1984). Phytochemical investigations of this plant species led to the isolation and characterization of biflorin [6,9-dimethyl-3-(4-methyl-3-pentenyl)naphtha[1,8-bc]pyran-7,8-dione] from its roots (Gonçalves de Lima *et al.*, 1958; Fonseca *et al.*, 2003).

This compound has shown to be strongly active against gram-positive and alcohol-acid-resistant bacteria (Gonçalves de Lima *et al.*, 1958). Biflorin also inhibit the proliferation on five tumor cell lines CEM and HL-60, B16, HCT-8 and MCF-7 in a dose dependent manner, with concentrations ranging from (0.39 to 25 µg/mL) (Vasconcellos *et al.*, 2005). Recently, SK-Br3 cell line was treated with biflorin (1-20µM) showing important cytotoxic effects (Montenegro *et al.*, 2013). The study suggests a mechanism of action for biflorin in MDA-MB-435 melanoma cells, most likely by the inhibition of N-cadherin. Inhibitors of N-cadherin function have been demonstrated to cause apoptosis and regulate the AKT-1 pathway (Montenegro *et al.*, 2013).

The antitumor activity *in vivo* has also been evaluated in animals transplanted with sarcoma 180 and Ehrlich carcinoma, presenting promising antitumor effects with low toxicity in the histopathology exam of the spleen, kidney and liver (Vasconcellos *et al*., 2007). *In vivo* studies in mouse also revealed that biflorin coupled with 5-Fluoracil chemotherapy (5-FU) was able to increase its effectiveness and reduce systemic toxicity (Vasconcellos *et al*., 2007). In experiments with animals transplanted with B16 melanoma, 25mg/day of biflorin was administered for ten days and showed significant inhibition of tumor growth with no systemic toxicity (Vasconcellos *et al*., 2011).

Despite that previous studies from the same group using *in vitro* and *in vivo* models indicate that biflorin presents a potential antitumor activity, so far there are no reports on selective cytotoxicity and effects of this drug on morphological changes and induction of apoptosis with annexin V on tumor cell lines. In this study we evaluated the cytotoxic activity of biflorin on different tumor lines Hep-2 (human larynx carcinoma), HeLa (human cervical adenocarcinoma), HT-29 (human colon cancer), A-375 (human melanoma), A-549 (human alveolar epithelial) and non-tumor line HEK-293 (human embryonic kidney). Morphological aspects were investigated and data is

presented exclusively for HeLa cells. This work is the first to show apoptosis by in situ analysis after treatment with biflorin.

## 2. Materials and Methods

### 2.1. Isolation of Biflorin

*Capraria biflora* was collected at a plantation located in Fortaleza, Ceará, Brazil, in April 2005 and identified by Dr. Edson Nunes. A voucher specimen (No.30848) was deposited in the Herbarium of the Federal University of Ceará.

Air-dried powdered roots (6 kg) were extracted with light petroleum (4:l) for 2 days. The extract was partially evaporated at room temperature until the formation of a solid material was observed. The later was filtered under vacuum and yielded purple solid compost (2 g). The purple solid material was chromatographed on Si gel and isocratic elution using a binary mixture of light petroleum/EtOAc 9:1 (v/v). Fractions were pooled together according to thin-layer chromatographic (TLC) analysis. Combined fractions having the purified biflorin yielded 1.5g.

### 2.2. Mass analysis – Chemical Identification

The dry extract was dissolved in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid. The solution was infused directly with HPLC (Shymadzu) assistance into the ESI source at a flow rate of 100 μL.min$^{-1}$. ESI(+)-MS and ESI(-)-MS were acquired using a hybrid high-resolution and high accuracy (5 μL/L) microTof (Q-TOF) mass spectrometer (Bruker Scientific®) under the following conditions: capillary and cone voltages were set to + 3500 V and +40 V, respectively, with a de-solvation temperature of 180◦C. Diagnostic ions in where identified by the comparison with theoretical results given the difference in ppm. For data acquisition and processing, TOF control software (Bruker Scientific®) was used. The data were collected in the *m/z* range of 70–800 at the speed of two scans per second, providing the resolution of 50,000 (FWHM) at *m/z* 200. No important ions were observed below *m/z* 100 or above *m/z* 600, therefore ESI(+)-MS data is shown in the *m/z* 295−350 range.

### 2.3. Cytotoxic assay

The Hep-2 (human larynx carcinoma), HeLa (human cervical adenocarcinoma), HT-29 (human colon cancer), A-375 (human melanoma), A-549 (human alveolar epitelial) and HEK-293 (human embryonic kidney) cell lines were cultured in Dulbecco's Modified Eagle Medium supplemented with antibiotics and 10% fetal bovine serum (Gibco BRL; Life Technologies), at 5% $CO_2$ and 37°C. For the assessment of the cytotoxic activity, cells were seeded in 96-well flat-bottom microplates at a density of approximately $5\times10^4$ cells/well in 10% fetal bovine serum Dulbecco's Modified Eagle Medium. After cell attachment, serial dilutions of biflorin in culture medium were prepared and HeLa and HEK-293 cells were incubated at 24h, 48h and 72h. Because cytotoxic curve profiles for different time cultivation with biflorin were similar, 48h incubation was elected and performed for all tumor cell lines here investigated.

Cell viability was determined by the tetrazolium salt method using MTT (Denizot & Lang, 1986). Briefly, $5 \times 10^4$ cells/well were cultured in 96-well plates and treated for 48 hours with biflorin, dissolved in dimethyl sulfoxide (DMSO) at increasing concentrations (5 µg/mL – 50 µg/mL). After incubation with MTT solution at room temperature for 2h, dimethyl sulfoxide was added, the cells were harvested, and absorption was determined at 540 nm (SpectraMAX M2/M2e - Molecular Devices, USA). At least 3 independent experiments were performed for each experimental cell line, and $IC_{50}$ (%) values were determined (Monks *et al.*, 1991). For all treatments, biflorin was prepared immediately prior to use. The negative control was exposed to an equivalent concentration of DMSO solvent.

### 2.4. Detection of late apoptosis by in situ analysis

Apoptotic cell death was detected by *in situ* analysis using Annexin-V staining and visualized by fluorescence microscopy. The Hep-2, HeLa, HT-29, A-375, A-549 and HEK-293 cells were seeded ($1.0 \times 10^5$ cells/mL) into coverslips in 24-well plates at with 500 µL of supplemented culture media. After 24 h, cells were treated with 5, 10 and 40 µg/mL of biflorin and negative controls was exposed to an equivalent concentration of DMSO, incubated at 37ºC, in 5% $CO_2$ for 48 hours. Next, the medium was removed and the cells were incubated with 2.5 µg/mL of Annexin V (abcam® - ab14196) primary antibody diluted in biding buffer (Hepes 10 mM, NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1mM, $CaCl_2$ 1.8 mM pH 7.4) for 30 minutes. They were washed once

with binding buffer, then incubate with 2.5 µg/mL Dylight® 488 (abcam® - ab115637) secondary antibody. Cells in the late apoptotic stages or necrosis were stained also with propidium iodate (Sigma-Aldrich®- P4170). The percentage of cells undergoing early (Annexin-V positive) or late (Annexin-V positive/ PI positive) apoptosis was estimated by counting at least 500 cells in 10 different fields in triplicates and expressed as a percentage of the total number of cells.

### 2.5. Statistical analyses

The results are expressed as the means ± standard deviation (SD) of each group. Analysis of variance followed by the Tukey post hoc test was used to evaluate differences among the treatment groups in triplicate. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS 19.0). The level of significance was uniformly set at $p < 0.05$. All analyses were carried out using the GRAPHPAD program (Intuitive Software for Science, SanDiego, California, USA).

## 3. Results

### 3.1 Isolation and chemical characterization of Biflorin

In this work, ESI(+)-MS and tandem ESI(+)-MS/MS were acquired using a microTOF-Q II (Q-TOF) and the spectra from biflorin is represented in **Figure 1.**

Accordingly, the *m/z* 309.1483 $[M + H]^+$ and 331.1286 $[M + Na]^+$ matched with molecular formula $C_{20}H_{21}O_3$ and $C_{20}H_{21}O_3Na$ with experimental difference 2.49 and 7.29 ppm respectively (**Figure 1)**. The isotopic ratio of $[M + H]^+$ is confirmatory with *m/z* 310.1511 (22.85%) and 311.1530 (2.67%). There are no ions in the mass spectra in positive mode ESI(+) beyond those reported (**Figure 1).** In negative mode ESI(-), using ammonia hydroxide (0.1%) in solution, there is no answer which is expected because the poor ionization of this functional groups. These set of results contributes to confirm the purity of the compound.

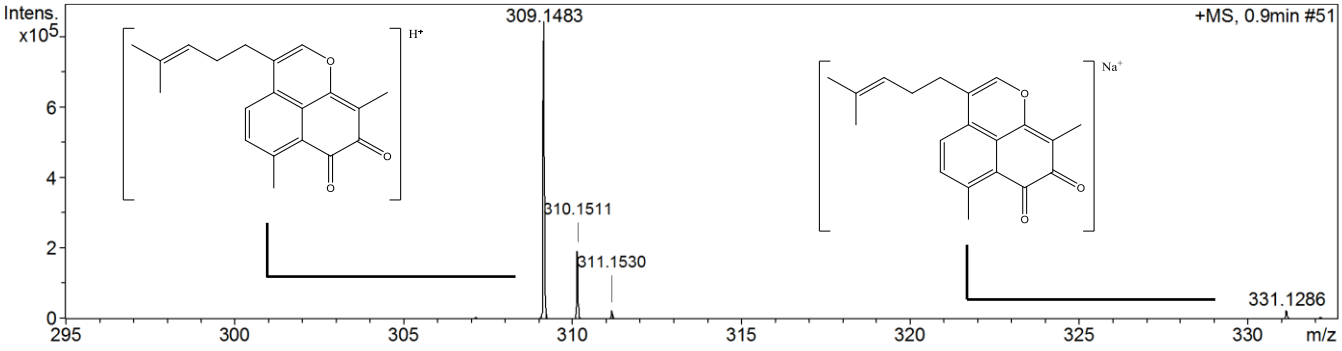
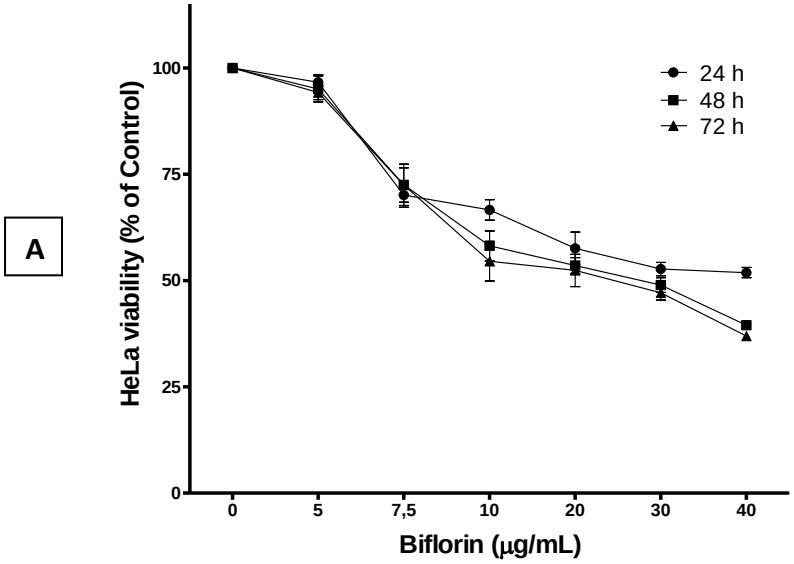



**Figure 1.** ESI(+) mass spectra of Biflorin (6,9-Dimethyl-3-(4-methyl-3-pentenyl) naphtha[1,8-*bc*]-pyran-7,8-dione), highlighting *adducts* chemical structure $[M + H]^+$ and $[M + Na]^+$ with *m/z* 309.1483 and 331.1286 respectively.

### 3.2 Cytotoxicity assay

For all tumor lines analyzed, cell viability was determined by the tetrazolium salt method using MTT was decreased in a concentration dependent manner. Initially, tumor line HeLa and non-tumor cell line HEK-293 were treated with biflorin for 24h, 48h and 72h at a range of 5-50 µg/mL (**Figure 2)**. A selective cytotoxicity was found for HeLa (**Figure 2A**) compared to HEK-293 (**Figure 2B**).



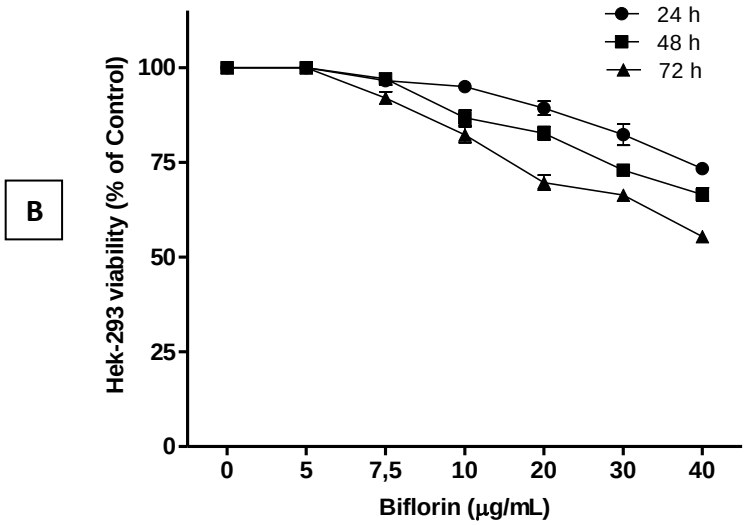



**Figure 2.** Cytotoxic effect of biflorin extracts on the viability of HeLa (A) and HEK-293 (B) cells lines. Cells were treated for 24, 48 and 72 h with 5; 7.5; 10; 20; 30; 40 µg/mL. Bars represent mean ± SD.

The cytotoxicity was further evaluated exclusively for 48h treatment on six different cell lines Hep-2, HeLa, HT-29, A-375, A-549 and HEK-293 and the half maximal inhibitory concentration ($IC_{50}$) obtained showed in **Figure 3**. HeLa cells were more susceptible to biflorin, followed by HT-29, A-549, A-375 and Hep-2 for all concentration ranges (5-50 µg/mL), and HEK-293 presented the highest value for $IC_{50}$ (56.01 ± 1.17).

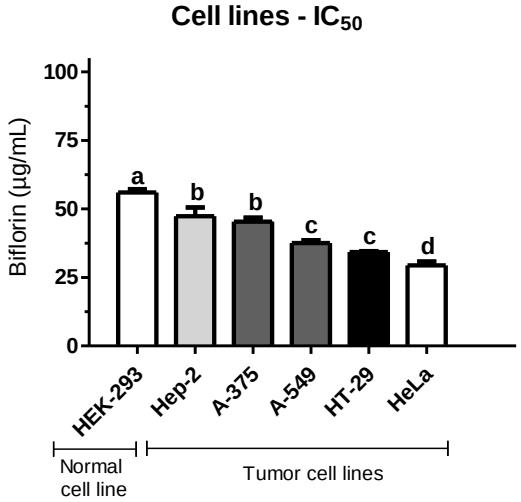



**Figure 3.** $IC_{50}$ after 48h treatment with biflorin in different cell lines *$IC_{50}$ (µg/mL) presented as mean ± SD. Different letters indicate the level of significance as tested by a one-way ANOVA (Tukey test): represents $p < 0.05$ when compared of the normal cell line (Hek-293).

Given the increased susceptibility of HeLa to biflorin, this line was selected for further morphological analysis with giemsa staining. Substantial morphological changes in HeLa cells were observed after 48h treatment with biflorin with increased concentrations (5-50 µg/mL) of extract (**Figure 4**).

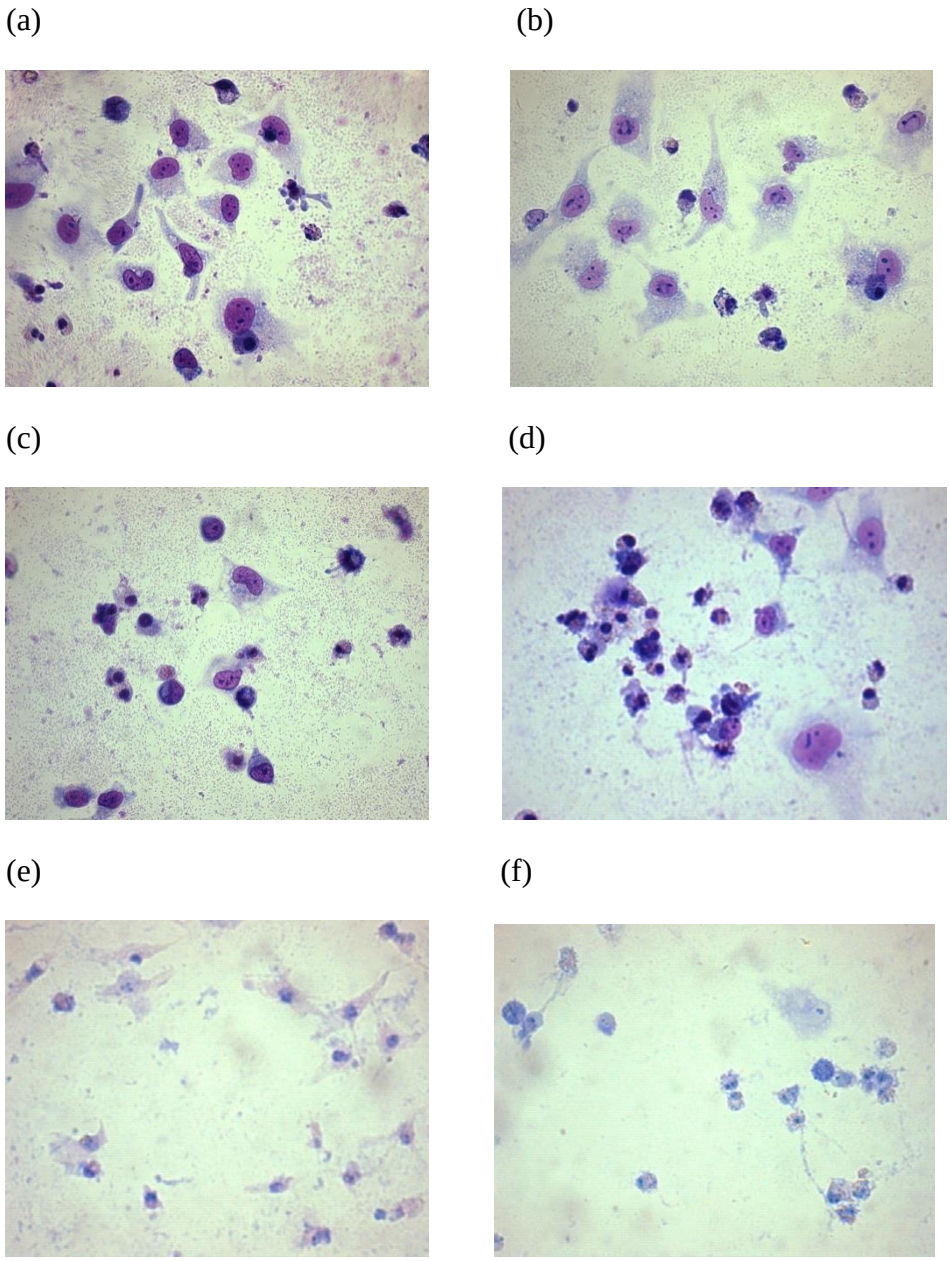
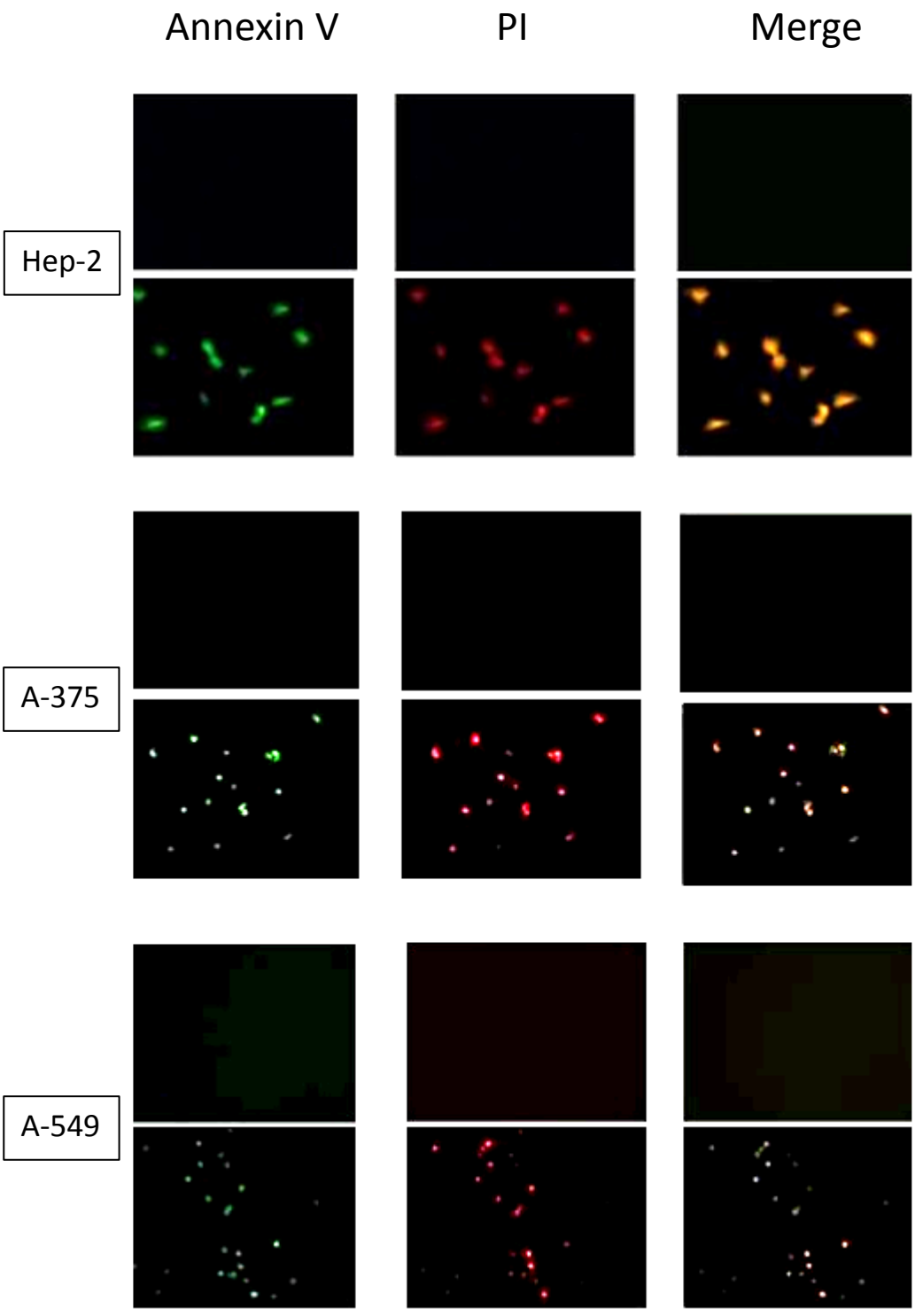



**Figure 4.** Morphologically changes of HeLa cells after 48h treatment with Biflorin (5-50µg/mL). Photomicrographs were taken under a light microscopy (200×). Treated cells were stained with Giemsa at different concentrations of biflorin: a) 5µg/mL; b) 7,5µg/mL; c) 10µg/mL; d) 20 µg/mL; e) 30µg/mL; and f) 40µg/mL.

### 3.2 Detection of late apoptosis by *in situ* analysis

Biflorin induced apoptosis in Hep-2, A-375, A-549, HT-29, HeLa cancer cells and Hek-293 non tumor cells. *In situ* immunostaining of annexin V showed that all lines were majority seen at late apoptotic stages in a dose-dependent manner (**Figures 5 and 6**). Annexin-V labeling (green fluorescence) without PI uptake indicates phosphatidylserine detection on the external surface of an intact plasma membrane. PI staining (red fluorescence) in conjunction with Annexin-V labeling (Merge) indicates a compromised membrane that may result from either late apoptosis or necrosis.

Late apoptosis/necrosis events varied according to the lines analyzed. In general, all cells presented an increase in late apoptotic events with higher doses of biflorin. From the tumor lines, Hep-2 cells showed greater values when treated with higher concentrations of biflorin - 40 µg/mL (69.63 ± 2.28%). Late apoptosis event profile started at lower levels of biflorin concentration (10 µg/mL) for all tumor lines. The non tumor HEK-293 line showed more resistance to apoptosis and late events for the this line was more evident (77.69 ± 6.68%) at higher extract concentrations compared to the tumor lines tested **(Figure 6).**


Annexin V
PI
Merge
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A-549

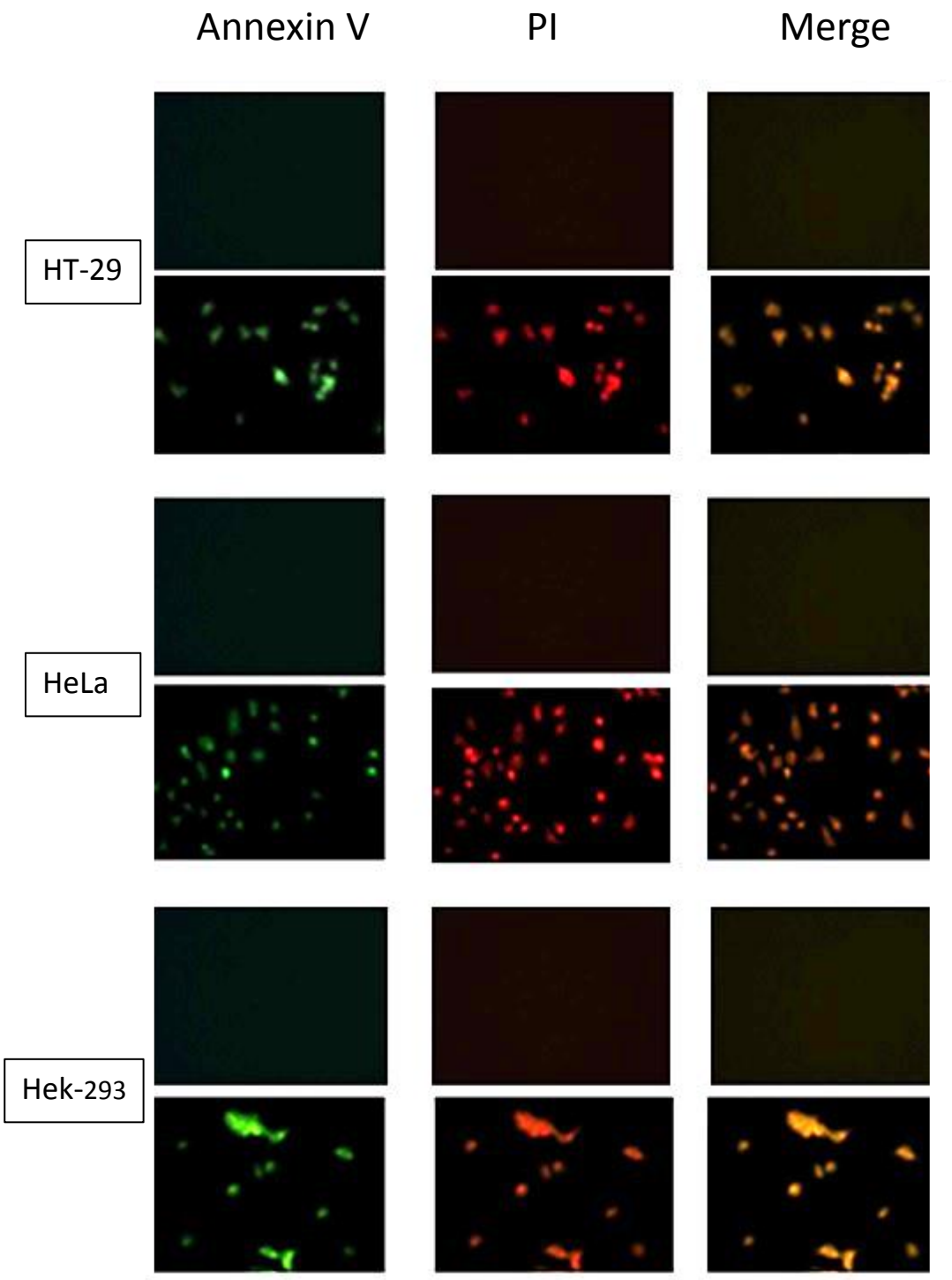


**Figure 5:** *In situ* immunostaining of annexin V detection of late apoptosis by *in situ* analysis with PS-binding Annexin V. Tumor cells were grown on glass slides and treated for 48h with Biflorin at 40 µg/mL compared to untreated cells. Images are representative taken from triplicates under fluorescent microscopy at x 400: A- Hep-2 cell line; B- A-375 cell line; C- A-549 cell line; D- HT-29 cell line; E- HeLa cell line, and F- Hek-293 cell line. First lines are control groups and second lines are represented as treated groups.

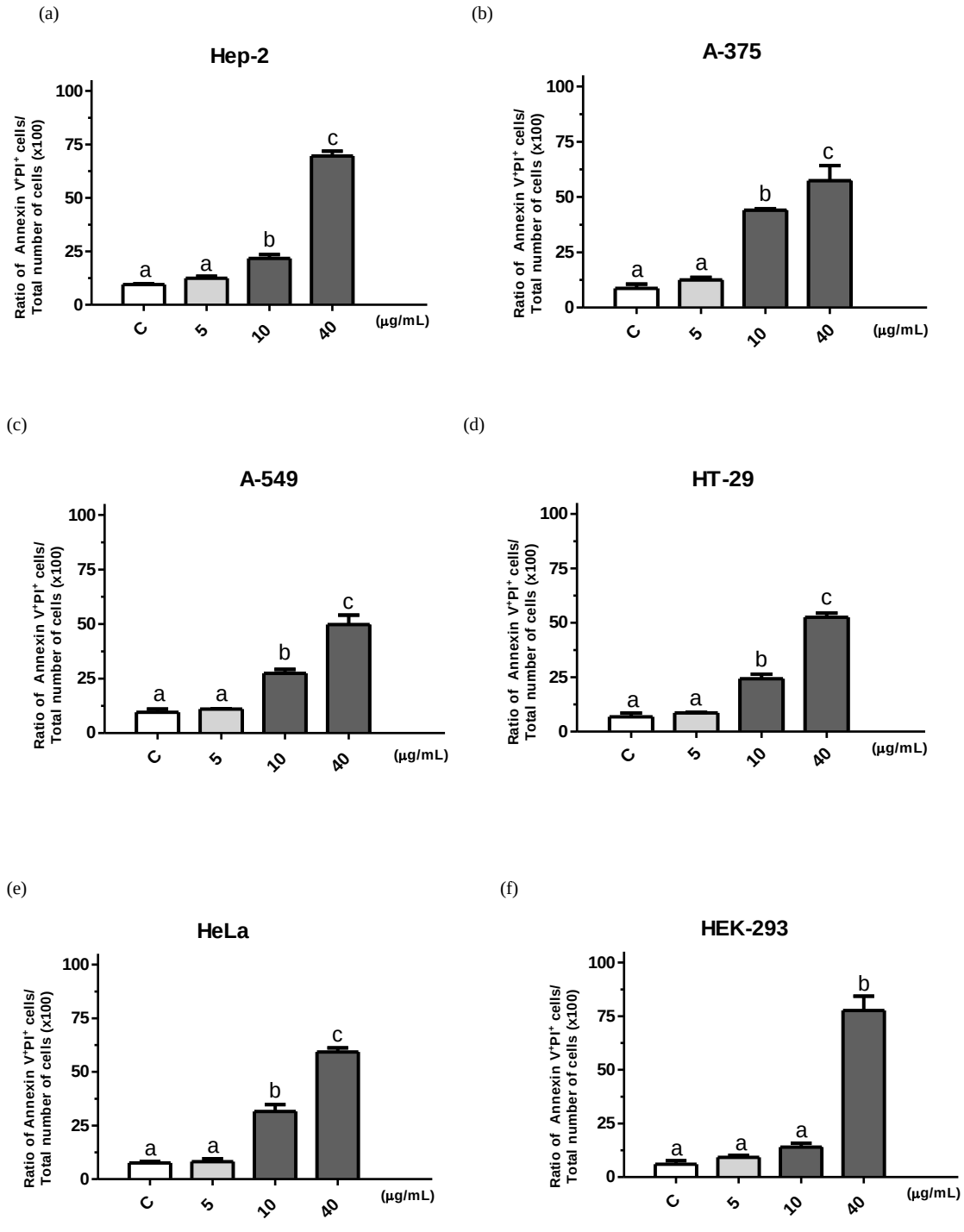


**Figure 6.** Detection of apoptosis by *in situ* analysis with PS-binding Annexin V after 48h biflorin treatment on Hep-2 (a), A-375 (b), A-549 (c), HT-29 (d), HeLa (e) and HEK-293 (f). C – negative solvent control. Different letters indicate the level of significance as tested by a one-way ANOVA (Tukey test): represents $p < 0.05$ when compared of the control.

## 4. Discussion

High-resolution direct-infusion mass spectrometry (HR-DIMS) was used for extract chemical characterization, which are important tools to characterize and identify natural metabolites. To response in positive mode, 0.1% of formic acid was introduced into the sample solution. The instruments accurate mass measurement and the isotopic ratio give the elemental composition.

Despite that previous studies using *in vitro* and *in vivo* models indicate that biflorin presents a potential antitumor activity, to date there are no reports on selective cytotoxicity and effects of this drug on morphological changes and induction of late apoptosis pathways on tumor cell lines. In this study we evaluated the cytotoxic activity of biflorin in several tumor lines Hep-2, HeLa, HT-29, A-375, A-549 and compared to the non tumor Hek-293 line. Morphological aspects were also investigated for HeLa after biflorin treatment and induction of apoptosis by *in situ* analysis was evaluated in all cell lines. Here, the same concentration of biflorin was able to inhibit cell proliferation more effectively in tumor cells (5-50 µg/mL) rather than in the Hek-293 (56,01 µg/mL). Previous studies have screened a group of tumor cell lines different form the ones here analysed for biflorin using a range of concentration (0,39 to 25 µg/mL) very similar to the ones applied here (5-50 µg/mL) (Vasconcellos et al., 2005; Vasconcellos *et al.*, 2007, Vasconcellos *et al.*, 2011). Recently, Montenegro *et al.* (2013) showed cytotoxic effects on breast cancer SK-Be3 cell line treated with biflorin (1- 20 µM). The study revealed that SK-Br3 cell treated with biflorin displayed selective citotoxicity against cancer cell, in relation to normal cell, as observed in the present report. Authors also demonstrate that inhibition of cell proliferation by biflorin could be mediated through the down-regulation of EGFR signaling pathway, once biflorin decreases the expression levels of total EGFR.

It is known that selective induction of cell death is very important in chemotherapy in order to decrease possible side effects (Parkinson et al., 2013; Reichert & Wenger 2008). The relation between cancer and apoptosis has being emphasized for a long time, suggesting that tumor progression involves the inibition of apoptosis stages in tumor cells (Vasconcellos *et al.*, 2011; Yang *et al.*, 2006). Apoptosis, autophagy and necrosis are the three main forms of cell death, each one presenting set of biochemical and morphological changes that occur within the dying cell (Elmore, 2007; Ouyang *et al.*, 2012). Apoptosis is a programmed form of cell death observed in tissues during

development and maintains tissue homeostasis through elimination of excessive or injured cells (Ouyang *et al*., 2012; Solá *et al*., 2013).

The giemsa staining analyzes cell morphology using a simple colorimetric technique that gives a good high-contrast polychromatic stain (Dolan, 2011; Nersesyan *et al.,* 2006). Here, substantial morphological changes in HeLa cells were observed after 48h biflorin treatment upon increased concentrations (5-50 µg/mL) of extract.

*In situ* immunostaining with annexin V showed that cells were seen mainly at late apoptotic stages. Late apoptosis for the non tumor line HEK-293 was even more evident (77.69 ± 6.68%) at higher extract concentrations compared to the tumor cell lines tested, which may indicate a resistance of non tumor cells in achieving late stages of apoptosis. Exposure of PS anionic phospholipids at the outer leaflet of the plasma membrane is related to loss of asymmetry of cell membrane and plays a physiological role in the recognition and subsequent removal of the dying cell by means of phagocytosis (Poon *et al.,* 2010). This phenomenon permits detection and differentiation of cell death apoptosis from necrosis. Staining cells by membrane impermeable dyes such PI allows differentiation between early apoptosis of late apoptosis/necrosis (Elmore, 2007).

Evidence suggests that tumor cells with apoptotic changes in function may diverge to another way of cell death like necrosis and therefore promoting an alternative therapeutic approach (Zong & Thompson, 2006; Degenhardt *et al.,* 2006 , Zong *et al.*, 2004). Necrosis may rise due to errors in apoptotic function generating alterations also in the glucose metabolism. Although necrosis can be considered a less efficient mechanism of cell death compared to apoptosis, defective apoptosis tumor cells may be more susceptible targets for therapy despite possible consequences with inflammation (Degenhardt *et al.,* 2006).

Many therapeutic agents for cancer, such as cisplatin, paclitaxel, isothiocyanate and adriamycin, have been reported to eliminate tumor cells by inducing apoptotic cell death (Hanahan & Weinberg 2011; Tan *et al.,* 2009). It is known that tumor growth is determined not only by the progression of the malignant cells, but also by the speed which these cells are taken to death and eliminated (Mester & Redeuilh, 2008). Therefore, inhibiting tumor growth and inducing tumor cell death are elective therapeutic strategies to control tumor progression (Mester & Redeuilh, 2008; Yang *et al.,* 2006). The activation of programmed cell death should be focused on the tumor

cells, minimizing the side effects by inappropriate apoptosis induction in normal cells. Thus, the development of cancer new treatment strategies is recommended, and understanding of the multiple pathways of cell death and molecular mechanisms involved in the carcinogenesis process is required.

Natural Products have played an important role in the world for treating and preventing human diseases, since over 60% of the drugs used have their origin related to a natural sources. Among the sources found in nature, the largest contribution to the development of therapeutic substances is taken from plants (Kirkpatrick *et al.*, 2002; Silva, 2006; Costa-Lotufo *et al.*, 2010). Quinones are secondary metabolites found in several living plant cells and present a variety of biological effects, like anticancer, antibacterial, or antimalarial. In particular, naphthoquinones, in which biflorin is included, is a promising group of compounds with antitumor properties (Asche, 2005). Quinone moieties are present in many drugs, such as anthracyclines, daunorubicin, doxorubicin, mitomycin, mitoxantrones, and saintopin, which are clinically used in therapy of solid cancers (Verma, 2006).

## 5. Conclusions

The data here presented indicate that biflorin showed an important cytotoxic effect against tumor cell lines. In addition, substantial morphological changes and activation of late apoptotic stages were observed after biflorin treatment with increased concentrations of the *Capraria biflora* L. isolated biflorin. However, more studies are needed to better understand the bioactivity of this natural product both in tumor and normal cells. Elucidate the mechanism of action of this isolated product and better characterize the pathways involved in programmed cell death is also required.

## 6. Acknowledgment

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# 4 DISCUSSÃO GERAL

O desenvolvimento de princípios ativos derivados de plantas e seus análogos sintéticos e semi-sintéticos serviram para descoberta de novos produtos farmacêuticos da medicina moderna (Lee, 1999; Newman & Cragg, 2007). Várias drogas anti-neoplásicas ainda possuem limitação quanto ao uso por fatores como baixo potencial, baixa solubilidade em água, toxicidade e resistência tumoral. Sendo assim, faz-se necessários o desenvolvimento de agentes antitumorais alternativos, mais efetivos e seguros (Srivastava *et al.*, 2005).

Produtos naturais têm desempenhado um papel importante no mundo para o tratamento e prevenção de doenças humanas, uma vez que mais de 60% dos medicamentos utilizados atualmente têm a sua origem relacionada com fontes naturais (plantas, algas, entre outros) (Kirkpatrick *et al.*, 2002; Silva, 2006; Costa-Lotufo *et al.*, 2010). Quinonas são metabólitos secundários encontrados em plantas e apresentam uma variedade de efeitos biológicos, como anticancerígeno, antibacteriano, entre outros. Em particular, as naftoquinonas, em que a biflorina está inclusa, é um grupo promissor de compostos com propriedades anti-tumorais (Asche, 2005). Radicais quinona estão presentes em muitas drogas, tais como antraciclinas, daunorubicina, doxorubicina, mitomicina, mitoxantrones e saintopin, os quais são utilizados clinicamente no tratamento de câncer sólido (Verma, 2006).

No presente estudo, foi possível observar que a biflorina (6,9-dimetil-3-(4-metil-3-pentenil)nafta[1,8-bc]-piran-7,8-diona), uma ɵ-naftoquinona obtida a partir das raízes da planta *Capraria biflora L.*(Schrophulariaceae), apresentou efeito citotóxico seletivo dose-resposta em cultura de células tumorais de laringe (Hep-2), câncer cervical (HeLa), câncer de cólon (HT-29), melanoma (A-375), câncer epitelial alveolar (A-549) e com citotoxicidade seletiva para a linhagem não tumoral de rim (HEK-293). A mesma concentração de biflorina foi capaz de inibir a proliferação celular de forma mais eficaz em células tumorais do que em células não tumorais. Estudos anteriores do mesmo grupo testaram outras linhagens celulares usando diferentes concentrações de biflorina semelhantes às testadas neste trabalho que também apresentaram atividade antitumoral (Vasconcellos *et al.*, 2005; Vasconcellos *et al.*, 2007, Vasconcellos *et al.*, 2011). Recentemente, Montenegro *et al.* (2013b) demonstraram efeitos citotóxicos sobre a linhagem de câncer de mama (SK-BE3) tratadas com biflorina. O estudo revelou que as células apresentaram citotoxicidade seletiva contra células cancerígenas, em relação a

células normais, como observado no presente estudo. Os autores também demonstraram que a inibição da proliferação de células por biflorina poderia ser mediado pela regulação negativa da via de sinalização de EGFR, uma vez que a biflorina diminui os níveis de expressão de EGFR totais.

Os nossos resultados são pioneiros ao demonstrar que a biflorina participa na indução a apoptose tardia em células tumorais de laringe, câncer cervical, câncer de cólon, melanoma, câncer epitelial alveolar. Atualmente tem sido enfatizada a relação entre apoptose e câncer, sugerindo que a progressão da neoplasia envolve alteração dos estágios de apoptose em células normais. Apoptose é uma morte celular programada responsável por remover células disfuncionais, velhas ou danificadas (Yang *et al.*, 2006). Portanto, um importante processo fisiológico para manter o equilíbrio homeostático da regeneração de células teciduais ou remoção de células mortas. Uma vez iniciada a apoptose, ocorrem inúmeras variações morfológicas, como, por exemplo, perda de volume celular, condensação da cromatina, fragmentação nuclear, formação de vesículas citoplasmáticas que formam os chamados corpos apoptóticos (Mester & Redeuilh, 2008; Yang *et al*., 2006; Wu *et al*., 2001). O crescimento do tumor é determinado não somente pela progressão das células malignas, mas também pela velocidade com a qual estas células são mortas e eliminadas. Estudos que utilizam modelos animais e cultura de células têm demonstrado que a maioria dos cânceres apresenta alguma falha em programar a morte da célula neoplásica. Sendo assim, inibir o crescimento do tumor e induzir a morte nestas células tumorais acabam sendo estratégias terapêuticas alternativas (Yang *et al*., 2006).

Evidências sugerem que as células tumorais com alterações na função apoptótica, podem divergir para outro caminho de morte celular, como a necrose, apresentando perda da integridade física e promovendo portanto uma alternativa de abordagem terapêutica (Zong & Thompson, 2006; Degenhardt *et al.,* 2006; Zong *et al.*, 2004).

Dessa forma, é recomendável o desenvolvimento de novas estratégias de tratamento para o câncer, sendo necessário aprofundar o entendimento da integração dos múltiplos caminhos de morte celular. Na avaliação de apoptose induzida pela biflorina em todas as linhagens aqui estudadas pelo método *in situ* da anexina V, foi observado um maior efeito dose resposta de apoptose tardia/necrose nas concentrações testadas. Esses resultados sugerem que a indução de apoptose tardia/necrose podem interferir no processo terapêutico, possivelmente as células manteriam o processo de morte celular

(apoptose ou necrose) em etapas posteriores ao tratamento. Sendo assim, muitas células mortas por apoptose apresentam características fisiológicas semelhantes a uma célula morta por necrose após certo período de tratamento (Sun *et al.,* 2005). Estudo recente sugere um mecanismo de ação para biflorina em células de melanoma (MDA-MB-435) por provável inibição de proteínas da família da caderina, que também induzem apoptose em alguma etapa do metabolismo celular (Montenegro *et al.,* 2013a).

Muitos agentes terapêuticos para o tratamento do câncer, tais como a cisplatina, paclitaxel, isotiocianato e adriamicina foram criados para eliminar as células tumorais através da indução da morte celular por apoptose (Hanahan e Weinberg, 2011;. Tan *et al.,* 2009). Sabe-se que o crescimento tumoral é determinado não só pela progressão das células malignas, mas também pela velocidade com que estas células são eliminadas (Mester & Redeuilh, 2008). Portanto, a inibição do crescimento do tumor e indução a morte de células tumorais são estratégias terapêuticas alternativas para controlar a progressão do tumor (Mester & Redeuilh, 2008; Yang *et al.,* 2006). A ativação da morte programada de células deve ser focada em células tumorais, minimizando os efeitos colaterais por indução de apoptose em células normais. Assim, torna-se necessário o desenvolvimento de novas estratégias de tratamento de câncer, com foco em seletividade celular, e estudos moleculares que levem a melhor compreensão das múltiplas vias de morte celular e os mecanismos envolvidos no processo da carcinogênese.

# 5 CONCLUSÕES

Os dados obtidos neste estudo permitem concluir que:

- ✓ A biflorina é capaz de inibir o crescimento (dose-dependente) das seguintes células tumorais testadas neste trabalho: células tumorais de laringe (Hep-2), câncer cervical (HeLa), câncer de cólon (HT-29), melanoma (A-375), câncer epitelial alveolar (A-549);

- ✓ A biflorina apresentou seletividade para células não tumorais de rim (HEK-293);

- ✓ A biflorina induziu majoritariamente um mecanismo de apoptose tardia através de ensaios *in situ* utilizando coloração Anexina-V em todas as linhagens de células tratadas com diferentes concentrações de biflorina.

# 6 PERSPECTIVAS

Como continuidade deste trabalho seria importante:

- ✓ Testar estabilidade química da biflorina;
- ✓ Avaliar a citotoxicidade da biflorina em estudos *in vivo;*
- ✓ Isolar, avaliar e validar a expressão de proteínas diferencialmente expressas através de imunofluorescência indireta e *Western blot;*
- ✓ Avaliar dano de DNA utilizando ensaio cometa.

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