WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.632
Volume 9, Issue 1, 266-280 Research Article ISSN 2278 – 4357

CYTOTOXIC ACTIVITY OF COUMARIN DERIVATIVE ON VERO AND HELA CELL LINES

Erwelly B. Oliveira[1,2]*, João S.B. Luz[1,2], Gleyka D.M. Santos[1,2], José Maria Barbosa-Filho[5], Rodrigo S.A. Araújo[5], Cláudio G. Rodrigues[2,4], Dijanah C. Machado[4], Eliete C. Silva[1,2,3] and Paloma L. Medeiros[1,2,3]

[1]Laboratório de Cultura de Tecidos (LCT) do Departamento de Histologia e Embriologia, Universidade Federal de Pernambuco, 50.670-901, Cidade Universitária, Recife-PE, Brasil.

[2]Programa de Pós-graduação em Inovação Terapêutica, Universidade Federal de Pernambuco, 50.670-901, Cidade Universitária, Recife-PE, Brasil.

[3]Programa de Pós-graduação em Morfotecnologia, Universidade Federal de Pernambuco, 50.670-901, Cidade Universitária, Recife-PE, Brasil.

[4]Departamento de Biofísica e Radiobiologia, Universidade Federal de Pernambuco, 50.670-901, Cidade Universitária, Recife-PE, Brasil.

[5]Instituto de Pesquisa em Fármacos e Medicamentos (IPeFarM), Universidade Federal da Paraíba, Cidade Universitária, 58051900, João Pessoa-PB, Brasil.

Article Received on
04 November 2019,

Revised on 25 Nov. 2019,
Accepted on 15 Dec. 2019

DOI: 10.20959/wjpps20201-15310

***Corresponding Author**
Erwelly B. Oliveira
Laboratório de Cultura de Tecidos (LCT) do Departamento de Histologia e Embriologia, Universidade Federal de Pernambuco, 50.670-901, Cidade Universitária, Recife-PE, Brasil.

ABSTRACT

Natural products have been considered good tools for prospecting of new active drugs or models for new therapeutic drugs. Coumarins are a group of natural phenolic compounds that shows several pharmacological activities and the 3-hydroxycoumarin represents as target valuable molecules against several diseases. The aim of present work was to investigate the cytotoxic activity of 3-hydroxycoumarin on Vero and HeLa cells and its action on the morphology of these cells line. Cytotoxicity was measured using MTT assay and the morphological features were evaluated by phase-contrast microscopy. Significant toxicity was determined for both cell lines and morphological changes were observed from 100 µg/mL. Further studies are needed to investigate other activities of this coumarin and determine potential applications.

KEYWORDS: 3-hydroxycoumarin, cytotoxic activity, HeLa cells, Vero cells, morphological analysis.

INTRODUCTION

The use of plants based in folk medicine have been a major target of the pharmaceutical industry which has been trying to find new prototypes useful for the drugs directed to the treatment of various diseases. This has led to a resurgence of interest in secondary metabolites produced as phenolic compounds, among which highlight the coumarin.[1] These purified substances exhibit potent and relevant biological activities, in addition to its low mammalian toxicity. This set of benefits keeps the coumarins as target on current research and promotes pharmaceutical interest worldwide.[2]

Coumarins (2H-1-benzopyran-2-one) owe their class name to "Coumarou", the vernacular name of the tonka bean, *Dipteryx odorata* (Aubl.) Willd. (Fabaceae), from which coumarin itself was isolated in 1820.[3] Coumarins are distributed in nature and are a class of natural phenolic substances found in plants,[4] bacteria[2] and fungi,[5] widely used as additives in food, perfumes, cosmetics, pharmaceuticals.[6] Nearby, 1.300 coumarins were identified from natural resources and reported in about 150 species distributed in 30 different families in higher plants, richest sources being Rutaceae, Umbelliferone and Clusiaceae.[7] This secondary metabolite is distributed over all parts of the plant, but occurs in large quantities in fruits, followed by the roots, stalksand leaves.[8]

Coumarin and its derivatives have a large number of properties and applications that justify the interest in these compounds. Synthetic and natural coumarins are valuable structures in drug design and development, due to broad spectrum of biological properties including anti-HIV,[9] antimicrobial,[10,11] anticancer,[12] anti- inflammatory,[13,14] anti-Alzheimer,[15] antifungal,[16,17] anticonvulsant,[18] and antidepressant,[19] activities.

Hydroxycoumarins represent a class of coumarin derivatives that have diverse pharmacological and biochemical properties and play important roles in the prospect of pharmacologically active compounds, some of which may be of potential pharmaceutical interest.[20] 4-hydroxycoumarin is used as intermediate in the synthesis of important pharmaceuticals, such as warfarin and acenocoumarol, which are used in medical practice as an anticoagulant.[21,22] Some derivatives of 7-hydroxycoumarin (umbelliferone) show significant antioxidant activity and anti-inflammatory.[23,24]

Although some studies have showed several biological activities of natural and synthetic coumarin derivatives, additionally the activity of certain hydroxycoumarins such as 3-hydroxycoumarin has not been investigated. In this context, the present study aimed to evaluate the cytotoxic activity of 3-hydroxycoumarin on Vero and HeLa cells and its action on the morphology of these cells line.

MATERIALS AND METHODS

Chemicals

3-hydroxycoumarin was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) and stored at 5°C. MTT powder [3- 4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide], cell culture medium (DMEM), fetal calf serum (FCS), phosphate-buffered saline (PBS), trypsin-EDTA, penicillin-streptomycin mixture and L-glutamine were from Gibco BRL (Life Technologies, Paisly, UK).

Cell culture

HeLa (Human cervical carcinoma) and Vero cells (*Cercopithecus aethiops* Green monkey kidney epithelial cell line) were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Gibco BRL, Life Technologies, Paisly, UK). The culture was maintained at 37 ºC in an atmosphere of 5% CO_2 and 95% of relative humidity.

Cell viability assay

The cytotoxic activity *in vitro* was evaluated using the MTT assay.[25] Briefly, Vero cell (1×10^5 cells/mL) and HeLa cell (2×10^5 cells/mL) were seeded in 96-wells plates and incubated for 24 h. 3-hydroxycoumarin was dissolved in dimethyl sulfoxide and added in different concentrations (0.78 - 400 µg/mL). Negative control was DMSO. The absorbance was measured by using a multi-well scanning spectrophotometer (SkanIt Software 2.4.5 RE for Varioskan Flash, Thermo Scientific, Massachusetts, USA) at 595 nm wavelength. The results were expressed as percentage of relative viability of cell compared to control group. Three independent experiments were performed in triplicate.

Morphologic analysis

Vero and HeLa cell lines were cultured in 96-well plates for 72 h, in the presence of different concentrations of 3-hydroxycoumarin. The morphological aspects of these cells were evaluated by an inverted phase-contrast microscope (Leica DMIL, microsystems, Wetzlar,

Germany), equipped with digital camera (MOTICAM BA 2.000, Campinas, Brazil) and the digital photographs were taken using the Motic Images Plus 2.0 software.

Statistical Analysis

All results obtained in this study were presented as mean values ± standard deviation (SD) of three independent experiments, performed in triplicate. The data were analyzed using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). ANOVA (Analysis of Variance) was performed and followed by Tukey's post-test ($p < 0.05$). The 50 % cytotoxicity concentrations (CC_{50}) values were determined by GraphPad Prism (version 5.0) and were calculated by non-linear regression.

RESULTS AND DISCUSSION

Cytotoxic activity of 3-hydroxycoumarin on Vero cell

In vitro cytotoxic activity of 3-hydroxycoumarin on Vero cells was first evaluated at different concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) and CC_{50} value was determined in 143 ± 34.0 µg/mL. Low concentrations of 3-hydroxycoumarin (0.78 to 50 µg/mL) exhibited greater viability when compared to negative control (DMSO). 3-hydroxycoumarin exhibit concentration- dependent cytotoxicity from 100 µg/mL (**Figure 1**).

According to some authors, the coumarins should bear at least one hydroxyl group to show antioxidant activity, since it is proven that hydroxyl groups of hydroxycoumarins are potent electron/hydrogen donors to free radical.[26]. Coumarins have shown antioxidant or pro-oxidant properties depending on their intracellular concentration and some derivatives may present an antioxidant activity at low concentrations and pro- oxidant activity at higher concentrations, which induces an intracellular overproduction of Reactive Oxygen Species (ROS) leading to cell death.[27]

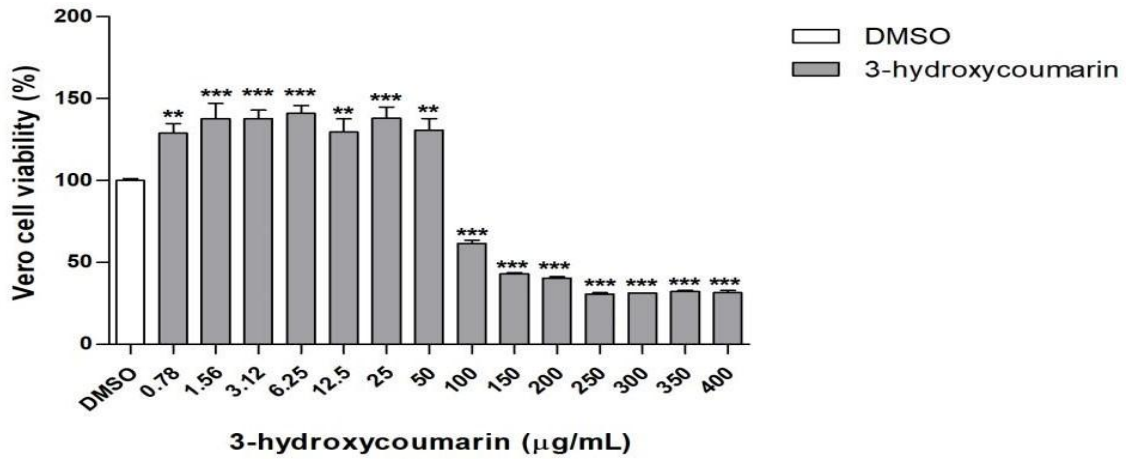

Figure 1: Cytotoxic activity of 3-hydroxycoumarin on Vero cell line. 50 % cytotoxicity concentrations (CC50) values were determined at concentrations ranging from 0.78 to 400 µg/mL. Statistical analysis of growth differences between treated and control (DMSO) cultures were performed using the ANOVA followed by Tukey's test ($p < 0.05$). The results were expressed as mean values ± standard deviation (SD) of three independent experiments tested by the MTT assay.

Despite having a potential antioxidant activity due to oxygen atoms in the ortho- position, 3-hydroxycoumarin is an understudied compound.

In recent study, has been shown that hydroxyl or methoxy groups were able to increase the affinity of coumarin with its molecular target, enhancing its cytotoxic effect.[28]

Morphological evaluation of Vero cells

Vero cells were cultured under effect of different concentrations of 3-hydroxycoumarin for 72 h, as illustrated in **Figure 2**. The cell morphology was clearly altered with the increase of concentration of 3-hydroxycoumarin. At low concentrations (0.78 to 50 µg/mL) cells grew as an adherent monolayer similar to the control and significant changes in cell morphology were observed at the highest concentrations (100 to 400 µg/mL).

Marked cytoplasmic retractions were found from 100 µg/mL and at highest concentrations (250 to 400 µg/mL), dramatic changes in cell morphology were observed such as decrease in cell density, as well cell rounding and shrinking. These results corroborate with the cytotoxic activity determined for of 3-hydroxycoumarin. In order, the cells demonstrated failure to reestablish intercellular associations and the growth pattern as adherent monolayer.

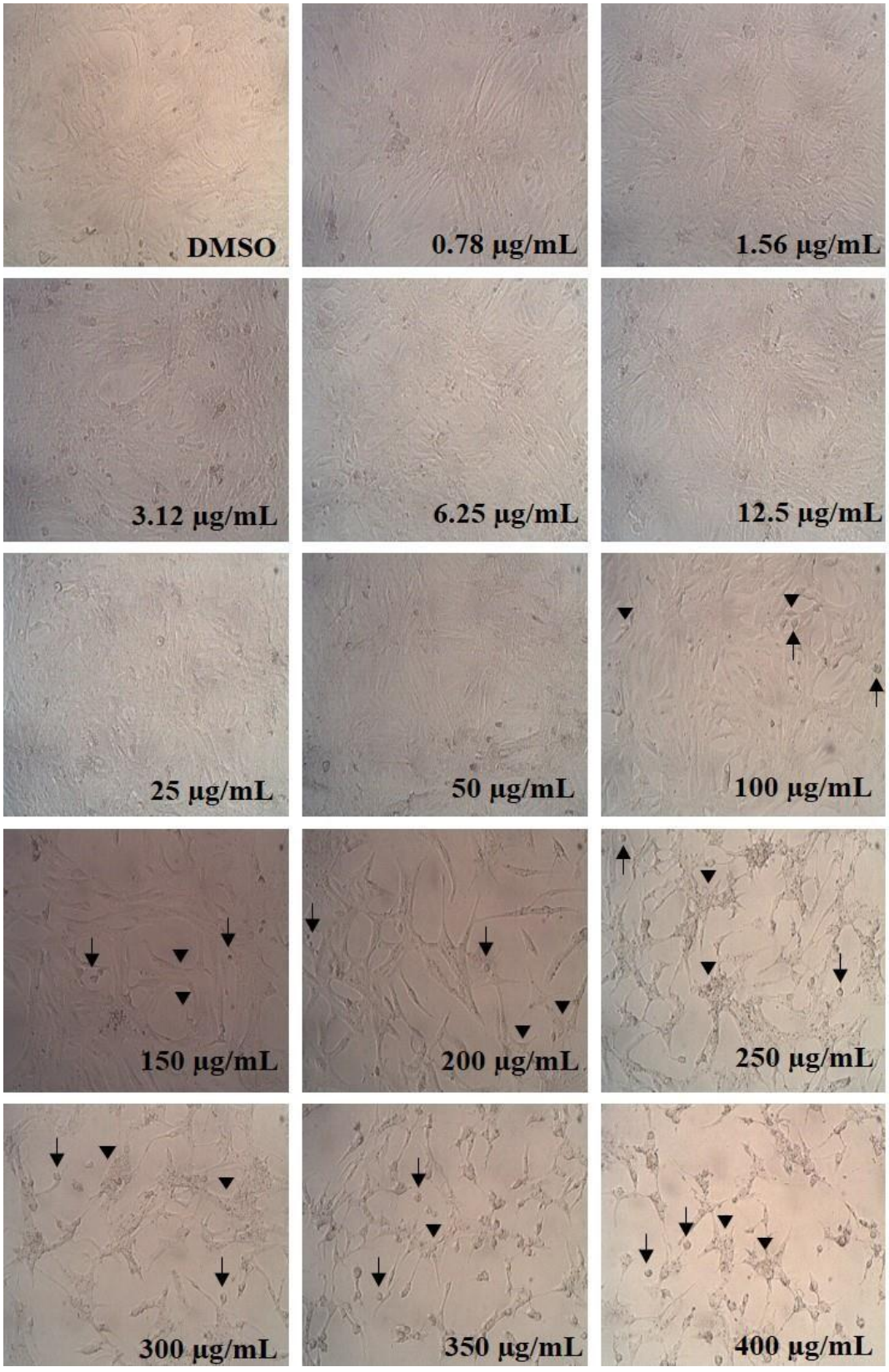

Figure 2: Phase-contrast photomicrographs of Vero cell line treated with 3-hydroxycoumarin. Different concentrations of this derived coumarin (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) and negative control (DMSO) were used. Note cells cultivated as adherent monolayer at 0.78 to 50 µg/mL.

Morphological alterations revealed a decrease in cell density, as well cell rounding (arrow) and shrinking (arrowhead) at the highest concentrations (100 to 400 µg/mL). Magnitude of all photos: 100 ×.

Very few systematic studies have been reported on structure-antioxidant activity correlations in coumarins, but their activity is probably due to their structural analogy with flavonoids and benzophenones.[29] Therefore, the coumarins possess a great structural diversity, since the replacements can occur at any of the six available sites of their basic molecular moiety (1,2-benzopyrone).[30]

Phenolic compounds are bioactive substances that have one or more aromatic rings in their structure, bearing one or more hydroxyl groups. This family of compounds acts as antioxidants and thereby protect from degenerative diseases in which reactive oxygen species (ROS) are involved.[26] In fact, overproduction of free radicals can cause oxidative damage to biomolecules (lipids, proteins, DNA), eventually leading to many chronic diseases.[31] It has been reported that deleterious effects of ROS on human cells may end in oxidative injury leading to programmed cell death.[32]

The properties of phenolic compounds are related to their chemical structure, which confers stability to the secondary free radical formed from the antioxidant reaction product with a free radical.[33] In the context, the hydroxycoumarins are phenolic compounds which act as potent metal chelators and free radical scavengers.[34-36]

Moreover, 7-HC enhances the inhibition of cell proliferation by interfering with mitotic spindle microtubule function,[19] by inhibiting the MAP kinase pathways,[20] and by blocking the cell cycle in the S, G1 or G2/M phases.[21,22]

Cytotoxic activity of 3-hydroxycoumarin on HeLa cell

The results showed the first report to cytotoxic activity of 3-hydroxycoumarin on HeLa cell line (**Figure 3**). Significant effect was evident of from concentration of 100 µg/mL. CC50 value was determined in 173.8 ± 28.2 µg/mL.

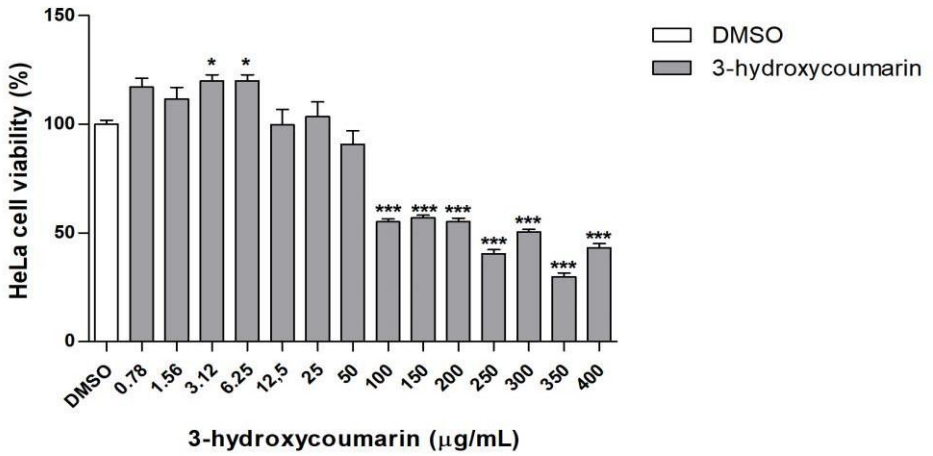

Figure 3: Effect of 3-hydroxycoumarin on *in vitro* viability of human cervical carcinoma cells (HeLa cells). The data are presented as percentage of increase (% viability) for each concentration of 3- hydroxycoumarin used in the control (DMSO). *, represent ($p < 0.05$) significant *versus* control, an analysis of variance (ANOVA) test followed by Tukey's.

Coumarins and their derivatives have been reported due to their cytostatic and cytotoxic effects against human carcinomas, *in vitro* and *in vivo*. The biological effects were correlated with chemical structure, since the change from methoxy group to hydroxyl increased the antiproliferative activity exerted by these substances in HT-29, A549, MCF-7 and OVCAR. This process of cell death occurred by necrosis due to an induction of intracellular ROS overproduction.[37]

The hydroxyl radical can damage all types of macromolecules and there are the ability of the cells to eliminate these reactive species through the use of endogenous antioxidants to protect their cytoplasmic organelles.[38] The results indicate that 3- hydroxycoumarin acted as an antioxidant by significant attenuated oxidative damage, increasing cell viability at lower concentrations. However, the protective effect decreased from 100 µg / mL, indicating that the cytotoxicity of this substance depends on the dose tested. This biphasic behavior with Reactive oxygen species (ROS) modulators has been already observed with resveratrol and can be attributed the basal level of ROS in each cell type, cell-specific properties and to the compound that can have antioxidant properties at low concentration.[39]

The coumarins and their derivatives may exert anticancer activity through several mechanisms: inhibition of telomerase enzyme, down regulation of oncogene expression.[40] and among the hydroxycoumarins, the 7,8-hydroxycoumarin may demonstrate this action by

generating oxidative stress due to production of free radical species in cancer cells, which leads to a pro-apoptotic effect in U-937 and HL-60 cells.[41]

The 7-hydroxycoumarin inhibits the growth of all lung cancer cell lines, and exhibited a cytostatic effect at concentration of 100 µg/mL and induces blocking the cell cycle in the G1 phase or apoptosis.[42] It has been reported in literature that esculetin (6,7-di-hydroxycoumarin) inhibits cell growth and cell cycle progression by inducing arrest in G1 phase in leukaemia HL-60, and CCRF-HSB-2 cell lines.[43,44]

Morphological evaluation of HeLa cells

The morphological aspects of HeLa cells under effect of 3-hydroxycoumarin at different concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) were evaluated with a phase-contrast microscope. HeLa cells were cultured as adherent monolayers at low-dose effects of 3-hydroxycoumarin (0.78 to 50 µg/mL) and morphological alterations were observed at the highest concentrations (100 to 400 µg/mL) revealing a decreased overall cell density as well as cell clusters with condensed chromatin, nuclear segmentation, shrinking and cellular debris (Figure 4).

Morphology changes of HeLa and MCF-10A cells were also induced by betaine treatment (0.0 -100 µg/mL, for 24-96h) and at 100 µg/mL the cells presented changes associated with apoptosis such as nuclear condensation and fragmentation and apoptotic bodies.[45] Betaine and coumarin are components derived from *Lycium chinense* an *Angelicae decursiva* (respectively) and these plants have been used for treatment of respiratory diseases in oriental medicine due to have various bioactivities effects.[46]

It was interesting, to see our work in agreement with others researches, which refers to 7-Hydroxycoumarin and 6,7-dihydroxycoumarin (esculetin) like coumarin derivatives that have been reported to exhibit antitumor activity, but the action mechanism underlying this activity remains unknown.[47]

Molecular docking studies have revealed that specific action in cancer cells might possibly be due to a dual inhibition of both binding sites (colchicine and GTP) on the α and β-tubulin.[48]

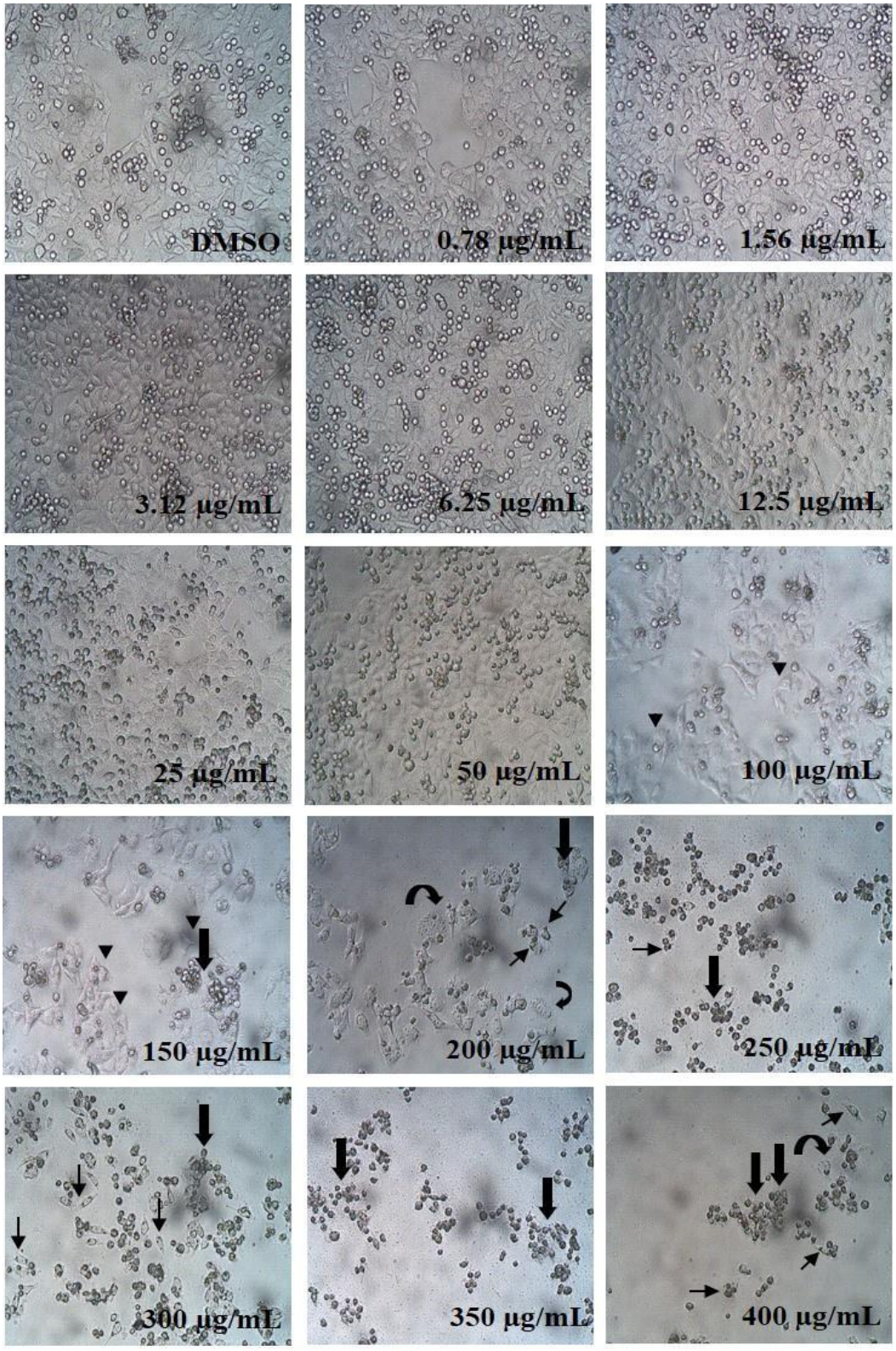

Figure 4: Phase-contrast photomicrographs of 3-hydroxycoumarin-treated HeLa cell lines. Different concentrations of this derived coumarin (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL) and negative control (DMSO) were used. HeLa cells showed as adherent monolayers at 0.78 to 50 µg/mL. Morphological alterations were observed at the highest concentrations (100 to 400 µg/mL) revealing a

decreased overall cell density as well as cell clusters with condensed chromatin (arrows full), nuclear segmentation (short arrows) shrinking (arrowheads) and cellular debris (curved arrows) Magnitude of all photos: 100 ×.

CONCLUSION

We report the cytotoxic effect of 3-hydroxycoumarin on Vero and HeLa cell lines for the first time in the present study. The 3-hydroxycoumarin-induced toxicity and remarkable morphological changes for both cell lines were evident from 100 µg/mL. Further studies to elucidate the detailed mechanism of these effects are underway.

Conflict of Interests

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

The authors like to express their sincere thanks to Professor José Maria Barbosa Filho, who provided the substance for conducting this research and to Dijanah Cota Machado for valuable technical help. The authors wish to express their sincere thanks to FACEPE (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco) and to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support.

REFERENCES

1. World Health Organization, WHO Traditional Medicine Strategy: 2014-2023, World Health Organization, Geneva, Switzerland, 2003.
2. Hoult JRS and Payá M. Pharmacological and Biochemical Actions of Simple Coumarins: Natural Products with Therapeutic Potential. Gen Pharmacol, 1996; 27(4): 713-722.
3. Murray RDH. Naturally Occurring Plant Coumarins. New York: Springer-Verlag, 1978; 1: 200-209.
4. Kaneko T, Baba N and Matsuo M. Protection of coumarins against linoleic acid hydroperoxide-induced cytotoxicity. Chem-Biol Interact, 2003; 142: 39–254.
5. Kawase M, Varu B, Shah A et al. Antimicrobial activity of new coumarin derivatives. Arzneimittelforschung, 2001; 51: 67-71.
6. Narayanasy VK, Gleiser RM, Kasumbwe K et al. Evalution of halogenated coumarins for antimosquito properties. Sci World J, 2014; 2014(189824): 6.
7. Stefanachi A, Leonetti F, Pisani L, Catto M, Carotti A. Coumarin: A natural, privileged and versatile scaffold for bioactive compounds. Molecules, 2018; 23(2): 250- 283.

8. Jain PK and Joshi W. Coumarin: Chemical and Pharmacological Profile. J Appl Pharm Sci, 2012; 2(6): 236-240.
9. Hamdy AM, Khaddour Z, Al-Masoudi NA, Rahman Q, HeringJunghans C, Villinger A, Langer P. Synthesis of arylated coumarins by Suzuki–Miyaura cross-coupling. Reactions and anti-HIV activity. Biorg Med Chem, 2016; 24(1): 5115–5126.
10. Mangasuli SN, Hosamani KM, Devarajegowda HC, Kurjogi MM, Joshi SD. Synthesis of coumarin-theophylline hybrids as a new class of anti-tubercular and anti- microbial agents. Eur J Med Chem, 2018; 146: 747–756.
11. Zavrsnik D, Halilovic SS and Softic D. Synthesis, structure and antibacterial activity of 3-substituted derivates of 4-hydroxycoumarin. Period Biol, 2011; 113(1): 93-97.
12. Thakur A, Singla R, Jaitak V. Coumarins as anticancer agents: A review on synthetic strategies, mechanism of action and SAR studies. Eur J Med Chem, 2015; 101: 476–495.
13. Lv HN, Wang S, Zeng KW, Li J, Guo XY, Ferreira D, Zjawiony JK, Tu P-F, Jiang Y. Anti-inflammatory Coumarin and Benzocoumarin Derivatives from Murraya alata. J Nat Prod, 2015; 78: 279–285.
14. Huang GJ, Deng JS, Liao JC et al. Inducible nitric oxide synthase of cyclooxygenase-2 participe in anti-inflammatory activity of imperatorin from *Glehnia littoralis.* J Agric Food Chem, 2012; 60(7): 1673-1681.
15. Singla S, Piplani P. Coumarin derivatives as potential inhibitors of acetylcholinesterase: Synthesis, molecular docking and biological studies. Biorg Med Chem, 2016; 24: 4587–4599.
16. Wang CM, Zhou W, Li CX, Chen H, Shi ZQ and Fran YJ. Efficacy of osthol, a potente coumarin compound, in controlling powdery mildew caused by *Sphaerotheca fuliginea*. J Asian Nat Prod Res, 2009; 11(9): 783-791.
17. Chiang CC, Cheng MJ, Peng CF, Huang HY and Chen IS. A novel dimeric coumarin analog and antimycobacterial constituents from *Fatoua Pilosa.* Chem Biodivers, 2010; 7(7): 1728-1736.
18. Luszczki JJ, Wojda E, Andres-Mach M et al. Anticonvulsant and acute neurotoxic effects of imperation, osthole and valproate in the maximal eletroshock seizure and chimney tests in mice: a comparative study. Epilepsy Res, 2009; 85(2-3): 293-299.
19. Sashidhara KV, Modukuri RK, Singh S, Rao KB, Teja GA, Gupta S, Shukla S. Design and synthesis of new series of Coumarin-aminopyran derivatives possessing potential anti-depresent-like activity. Bioorg Med Chem Lett, 2015; 25: 337-341.
20. Yasarawan N, Thipyapong K and Ruangpornvisuti V. Exploring molecular structures,

orbital interactions, intramolecular próton-transfer reaction kinetics, electronic transitions and complexation of 3-hydroxycoumarin species using DFT methods. J Mol Graph Model, 2014; 51: 13-26.

21. Hirsh J, Dalen J, Anderson DR, Poller L, Bussey H, Ansel J, Deykin D. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest, 2001; 119(1): 8S-21S.
22. Cesar JM, Garcia-Avello A, Navarro JL, Herraez MV. Aging and oral anticoagulante therapy using acenocoumarol. Blood Coagul Fibrinolysis, 2004; 15: 673- 676.
23. Vasconcelos JF, Teixeira MM, Barbosa-Filho JM et al. Effects of umbelliferone in a murine model of allergic airway inflammation. Eur J Pharmacol, 2009; 609(1-3): 126- 131.
24. Stanchev S, Hadjimitova V, Traykov T, Boyanov T and Manolova I. Investigation of the antioxidant properties of some new 4-hydroxycoumarin derivatives. Eur J Pharm Sci, 2009; 44: 3077-3082.
25. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983; 65: 55-63.
26. Yordi EG, Molina EP, Matos MJ, Villares EU. Antioxidant and pro-oxidant effects of polyphenolic compounds and structure-activity relationship evidence," In Nutrition, Well-Being and Health; J. Bouayed, T. Bohn, Eds.; InTech: Rijeka, Croatia, Chapter, 2012; 2: 23–48.
27. Jamier V, Marut W, Valente S et al. Chalcone-Coumarin Derivatives as Potential Anti-Cancer Drugs: An *in vitro* and *in vivo* Investigation. Anticancer Agents Med Chem, 2014; 14: 963-974.
28. Leite JCA, Castro TMX, Barbosa-Filho JM, Siqueira-Junior JP, Marques-Santos LF. Photoprotective effect of coumarin and 3-hydroxycoumarin in sea urchin gametes and embryonic cells. Journal of Photochemistry and Photobiology B: Biology, 2015; 146: 44-51.
29. 29.. Farombi EO and Nwaokeafor IA. Antioxidant mechanisms of kolaviron: studies on serum lipoprotein oxidation, metal chelation and oxidative membrane damage in rats. Clin Exp Pharmacol Physiol, 2005; 32: 667-674.
30. Beillerot A, Domínguez JCR, Kirsch G and Bagrel D. Synthesis and protective effects of coumarin derivatives against oxidative stress induced by doxorubicin. Bioorg. Med. Chem. Lett, 2008; 18: 1102-1105.
31. Tyagi YK, Kumar A, Raj HG et al. Synthesis of novel amino and acetyl amino-4-

methylcoumarins and evaluation of their antioxidant activity. Eur J Med Chem, 2005; 40: 413-420.

32. Salganik RI. The benefits and hazards of antioxidants: controlling apoptosis and other protective mechanisms in cancer patients and the human population. J Am Coll Nutr, 2001; 20(5): 464S-472S.
33. Hamdi N, Puerta C, Valerga P. Synthesis, structure, antimicrobial and antioxidant investigations of dicoumarol and related compounds. Eur J Med Chem, 2008; 43: 2541–2548.
34. Kostova I. Synthetic and natural coumarins as antioxidants. *Mini Review Medicine Chemistry*, 2006; 6: 365–374.
35. Panteleon V, Kostakis IK, Marakos P, Pouli N, Andreado I. Synthesis and free radical scavenging activity of some new spiropyranocoumarins. Bioorg Med Chem Lett, 2008; 18: 5781–5784.
36. Symeonidis T, Chamilos M, Hadjipavlou-Litina DJ, Kallitsakis M, Litinas KE. Synthesis of hydroxycoumarins and hydroxybenzo[f]- or [h]coumarins as lipid peroxidation inhibitors, Bioorg Med Chem Lett, 2009; 19: 1139-1142.
37. Jamier V, Wioleta M, Valente S, Chéreau C, Chouzenoux S, Nicco C, Lemaréchal H, Weill B, Kirsch G, Jacob C, Battex F. Chalcone-Coumarin Derivatives as Potential Anti-Cancer Drugs: An in vitro and in vivo Investigation. Anticancer Agents Med Chem, 2014; 14: 963-974.
38. Marnett LJ. Oxyradicals and DNA damage. Carcinogenesis, 2000; 21: 361–370.
39. Su D, Cheng Y, Liu M, Liu D, Cui H, Zhang B, Zhou S, Yang T, Mei Q. Comparision of Piceid and Resveratrol in Antioxidation and Antiproliferation Activities In Vitro. Plos One, 2013; 8(1): e54505.
40. Nasr T, Bondock S and Youns M. Anticancer activity of new coumarin substituted hydrazide-hydrazone derivatives. Eur J Med Chem, 2014; 76: 539-548.
41. Vázquez R, Riveiro ME, Vermeulen M et al. Structure-anti-leukemic activity relationship study of *ortho*-dihydroxycoumarins in U-937 cells: Key role of the -lactone ring in determining differentiation-inducing potency and selective pro-apoptotic action. Bioorg Med Chem, 2012; 20: 5537-5549.
42. Lopez-Gonzalez JS, Prado-Garcia H, Aguilar-Cazares D et al. Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines. Lung Cancer, 2004; 43: 275-283.
43. Kawaii S, Tomono Y, Ogawa K et al. The anti-proliferative effect of coumarins on

several cancer cell lines. Anticancer Res, 2001; 21: 917-23.

44. Wang CJ, Hsieh YJ, Chu CY, Lin YL, Tseng TH. Inhibition of cell cycle progression in human leukemia HL-60 cells by esculetin. Cancer Lett, 2002; 183: 163- 8.
45. Guo Y, Xu L-S, Zhang D et al. Betaine effects on morphology, proliferation, and p53-induced apoptosis of HeLa Cervical Carcinoma cells *in vitro.* Asian Pac J Cancer Prev, 2015; 16(8): 3195-3201.
46. Lee CJ, Lee JH, Seok JH et al. Effects of betaine, coumarin and flavonoids on mucin release from cultured hamster tracheal surface epithelial cell. Phytother Res, 2004; 18: 301-305.
47. Melendez FJ, Durand-Niconoff JS, Domínguez-Ortiz MA, García-Barradas O, Caballero NA and González E. Theoretical study of global and local reactivities of coumarin and its hydroxylated derivatives. Int J Quantum Chem, 2016; 116(9): 1-7.
48. Pingaew R, Saekee A, Mandi P et al. Synthesis, biological evaluation and molecular docking of novel chalcone-coumarin hybrids as anticancer and antimalarial agentes. *Eur* J Med Chem, 2014; 85: 65-76.