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Streptomyces hygroscopicus UFPEDA 3370: A valuable source of the potent cytotoxic agent nigericin and its evaluation against human colorectal cancer cells

Iza Mirela Rodini Garcia-Princival [a], Jefferson Luiz Princival [b], Emmanuel Dias da Silva [b], Sandrine Maria de Arruda Lima [a], Jhonattas Carvalho Carregosa [c], Alberto Wisniewski Jr [c], Caio Cézar Oliveira de Lucena [a], Fernando Halwass [b], José Adonias Alves Franca [b], Luiz Felipe Gomes Rebello Ferreira [d], Marcelo Zaldini Hernandes [d], Karina Lidianne Alcântara Saraiva [e], Christina Alves Peixoto [e,f], Blandine Baratte [g,h], Thomas Robert [g,h], Stéphane Bach [g,h], Dayene Correia Gomes [i], Patricia Maria Guedes Paiva [i], Pascal Marchand [j], Maria do Desterro Rodrigues [a], Teresinha Gonçalves da Silva [a,*]

[a] *Departamento de Antibióticos, Rua Prof. Moraes Rego, 1235, Universidade Federal de Pernambuco, Recife, Pernambuco, 50670-901, Brazil*
[b] *Departamento de Química Fundamental, Av. Jornalista Anibal Fernandes, s/n, Universidade Federal de Pernambuco, Recife, Pernambuco, 50740-560, Brazil*
[c] *Departamento de Química, Av. Marechal Rondon, s/n, Universidade Federal de Sergipe, Aracaju, Sergipe, 49100-000, Brazil*
[d] *Laboratório de Química Teórica Medicinal (LQTM), Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco, Av. Prof. Artur de Sá - Cidade Universitária, Recife, PE, 50740-521, Brazil*
[e] *Laboratório de Ultraestrutura. Instituto Aggeu Magalhães (IAM), Fundação Oswaldo Cruz (FIOCRUZ), Recife, PE, Brazil*
[f] *Instituto de Ciências e Tecnologia em Neuroimunomodulação (INCT-NIM), Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, RJ, Brazil*
[g] *Sorbonne Université, CNRS, UMR8227, Integrative Biology of Marine Models Laboratory (LBI2M), Station Biologique de Roscoff, F-29680, Roscoff, France*
[h] *Sorbonne Université, CNRS, FR2424, Kinase Inhibitor Specialized Screening Facility - KISSf, Station Biologique, F-29688, Roscoff, France*
[i] *Departamento de Bioquímica, Rua Prof. Moraes Rego, SN, Universidade Federal de Pernambuco, Recife, Pernambuco, 50670-420, Brazil*
[j] *Universite de Nantes, Cibles et médicaments des infections et du cancer, IICiMed, EA 1155, Nantes, F-44000, France*

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ABSTRACT

Streptomyces hygroscopicus UFPEDA 3370 was fermented in submerged cultivation and the biomass extract was partitioned, obtaining a fraction purified named EB1. After purification of EB1 fraction, nigericin free acid was obtained and identified. Nigericin presented cytotoxic activity against several cancer cell lines, being most active against HL-60 (human leukemia) and HCT-116 (human colon carcinoma) cell lines, presenting IC_{50} and (IS) values: 0.0014 μM, (30.0) and 0.0138 μM (3.0), respectively. On HCT-116, nigericin caused apoptosis and autophagy. In this study, nigericin was also screened both *in vitro* and *in silico* against a panel of cancer-related kinases. Nigericin was able to inhibit both JAK3 and GSK-3β kinases *in vitro* and its binding affinities were mapped through the intermolecular interactions with each target *in silico*.

1. Introduction

According to estimates from the World Health Organization (WHO) in 2015, cancer is the first or second leading cause of death before age 70 years in 91 of 172 countries, and it ranks third or fourth in an additional 22 countries [1]. It was noticed that age-standard cancer mortality rates have decreased for all types of malignancies, in spite of newer drugs only contribute to a small percentage of this improvement [2]. Moreover, the fact that tumor cells usually display chemotherapy resistance in patients is becoming increasingly worrying [3]. Thus, there is an urgent need for alternative therapeutic agents around on the world.

The use of cytotoxicity screening models keeps providing preliminary data of great relevance in the search for bioactive extracts with potential antineoplastic application for future works [4]. Currently, about 60% of the small approved molecules used in clinic are related to natural products, and for antimicrobial drugs this percentage reaches

* Corresponding author.
E-mail addresses: baratte@sb-roscoff.fr (B. Baratte), bach@sb-roscoff.fr (S. Bach), teresinha.goncalves@pq.cnpq.br (T. Gonçalves da Silva).

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69% [5,6]. Natural products have played a very important role as established cancer chemotherapeutic agents, either in their naturally occurring or through semi-synthesis [7], so derived from microorganisms ranks as one of the most usual alternatives for the discovery of potential anticancer agents. Although several natural products have been identified for this purpose, the achievement of substantial amounts of isolated substances from crude extracts containing cytotoxic activity is urgently needed.

Streptomyces sp., an actinomycetes Gram-positive filamentous bacterium with fungal morphology and widely found in soil, are known as a valuable source of many biologically active compounds for medicine purposes [8]. It has been especially used as source of antitumor drugs mostly used to treat different types of cancer. As examples, we can mention doxorubicin, produced from *Streptomyces peucetius* [9]; dactinomycin also known as actinomycin D, produced from *Streptomyces parvulus* [10]; bleomycin produced from *Streptomyces verticillus* [11]; and immunosuppressive drugs rapamycin and ascomycin, produced by *Streptomyces hygroscopicus* [12]. Nigericin is another natural product of great importance produced from *Streptomyces hygroscopicus* [13]. This substance, also known as polyetherin A, azalomycin M, helixin C, antibiotic K178 and antibiotic X-464, is an ionophore commercially obtained as a byproduct, or contaminant, at the fermentation of geldanamycin. Structurally, ionophores consist of a mono carboxylic acid containing polyethers that conformationally forms an oxygen rich hydrophilic interior capable to efficiently transport monovalent ions across the membrane (K^+, Rb^+, Na^+, Cs^+, Li^+, H^+). There are over 120 naturally occurring ionophores known so far [14,15] giving these natural products, released by *Streptomyces* sp., an economically importance that represents for about $150 million in sales annually [16]. In addition to nigericin free acid (**1**), on Fig. 1 are depicted some important ionophores containing mono carboxylic acid in a polyether skeleton, like grisorixin (**2**), lonomycin (**3**) and mutalomycin (**4**).

Cancer cells have a reversed internal pH if compared to normal cells (slightly alkaline internal pH favors cancer cell proliferation). Thus, ionophores are able to acidify the intracellular medium of the cytoplasm [17,18]. This pH disturbance has been used as a tumor cell sensitizer in combination with other drugs [19,20]. For instance, we can mention the potentiation of the clinically used anticancer drug paclitaxel [21] when associated with the ionophore inostamycin. In this case, after cancer cells have had their internal pH diminished, the DNA replication becomes not properly. The cytotoxicity of the drugs towards cancer cells was improved by this approach.

Colorectal cancer is the third most diagnosed cancer in males and the second in females, being one of the most common type of cancers. This disease has becoming a predominant cancer leading to accounts for approximately 10% of cancer-related mortality in western countries [22,23]. New treatments for primary and metastatic colorectal cancer have emerged, providing additional options for patients. These treatments include laparoscopic surgery, radiotherapy for rectal cancer and neoadjuvant or palliative chemotherapies. However, these new treatment options have had limited impact on cure rates and long-term survival [24]. In recent years, targeted therapies have made some progress in the treatment of colorectal cancer and kinase proteins have been identified as important molecular targets for the discovery of new drugs.

To target proteins kinases have emerged as one of the most intensely objectives pursued in current pharmacological research, especially for cancer, due to its critical roles in signaling pathways [25]. Kinase proteins regulate a variety of signaling pathways, which are particularly vital for cellular growth and proliferation [26]. In colorectal carcinoma, the dysregulation of JAK-3 (Janus kinase-3) leads to increased invasion and progressive growth [27]. Ruxolitinib was the first JAK inhibitor to reach the US market and it was approved in 2012 for the treatment of myelofibrosis and polycythemia vera. In the last decade, new JAK inhibitors have been investigated as potential treatment for other types of cancer, including colorectal cancer [28,29].

Another protein kinase involved in cancer is glycogen synthase kinase 3 (GSK-3), a serine/threonine kinase existing as two isoforms named GSK-3α (51 KDa) and GSK-3β (47 KDa), expressed in most tissues and encoded by two different genes [30]. The mechanism by which GSK-3 affects neoplastic transformation and tumorigenesis is largely investigated but remains controversial. It has been known to have tumor suppressor and promotor activity [31]. GSK-3 role in cancer is largely investigated, so in some cases, its activity has been associated with tumor progression, while in other cases the suppression of GSK-3 activity has been associated with cancer progression, through stabilization of components of the β-catenin complex [32]. GSK-3 is overexpressed in various cancer such as colon, liver, and pancreatic tumors. Interestingly, GSK-3β downregulation prevented pancreatic cancer growth, angiogenesis, and vascular endothelial growth factor expression [33]. Therefore, the chemomodulation of GSK-3 activity is considered as a strategy for the development of new treatment modalities [34,35].

Therefore, this work aimed to carry out the purification and structural characterization of nigericin achieved by fermentation of *Streptomyces hygroscopicus* UFPEDA 3370, and to evaluate the inhibitory activity against protein kinases *in vitro* and *in silico,* in addition to anticancer activity. This strategy was encouraged after that preliminary testing of a fraction, containing an until then unidentified compound, showed a very marked cytotoxic activity against various tumor cell lines. Thus, the present work relies in our efforts to pursue the investigations on the biological interest of actinomycetes soil-derived metabolites from

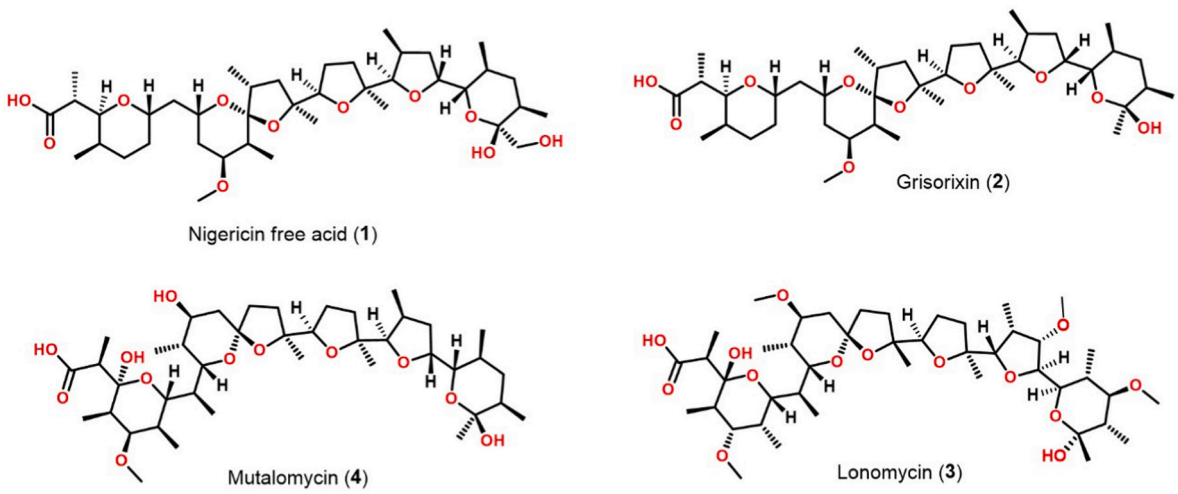

Fig. 1. Structures of ionophores with polyether skeleton.

Amazon (Brazil) and to consolidate a study which began with the identification of a *Streptomyces hygroscopicus* UFPEDA 3370 that enabled to isolate the bioactive macrolide elaiophylin [36].

2. Materials and methods

2.1. General

Chemicals and nigericin identification parameters are depicted on SI (supplementary information). MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). The absorbance in cytotoxicity assay was measured in a well plate micro analyzer 3550 BIO-RAD, Inc. and fluorescence measured on a BD accuri C6 Flow Cytometer System.

The *Streptomyces hygroscopicus* UFPEDA 3370 strain was provided by the Collection of Microorganisms of Antibiotics Department, from Federal University of Pernambuco (UFPEDA, Recife, Brazil). The following cancer cell lines used in this study were purchased from Rio de Janeiro Cell Bank (Brazil): HCT-116 (human colorectal carcinoma), MCF-7 (human breast adenocarcinoma), NCI–H292 (human mucoepidermoid lung), HL-60 (human acute promyelocytic leukemia), P815 (mouse mastocytoma) and L929 (mouse fibroblasts).

2.2. Obtention of the fraction EB1 from Streptomyces hygroscopicus UFPEDA 3370

Morphological and biochemical identification of the actinomycetes was previously reported [36]. The obtention of the extract, denominated EB1, from the fermentation of the *Streptomyces hygroscopicus* ACTSM-9H strain was performed according to procedure previously described [36].

During fermentation in submerged cultivation, *S. hygroscopicus* UFPEDA 3370 grew well in soya flour medium. The biomass separated from submerged cultivation was extracted with methanol to produce methanol extract from biomass (EMeOH 9H–B). Afterwards, approximately 1.3 g of the methanolic biomass extract (EMeOH 9H–B) from *S. hygroscopicus* UFPEDA 3370 was subjected to liquid-liquid partition with n-hexane, ethyl acetate, and 2-butanol. The butanolic phase (FBuOH) showed promising antimicrobial and cytotoxic activities in previous study, being selected for fractionation in flash column chromatography. The fractions F1–F11 were grouped and concentrated in a rotary evaporator under vacuum at 50 °C to produce the EB1 fraction as a very slimy red brown oil.

2.3. Purification of nigericin by column chromatography

To a round bottomed flask with capacity of 10 mL containing the fraction EB1 (1.0 g) was sequentially added MeOH (3 mL) and silica (1.0 g). The methanol was then removed in a rotary evaporator under vacuum leading to the formation of a powder extract. The silica powder solid was placed under a glass column (25 cm x 2,5 cm) packaged with silica (10.0 g) and the components were purified using a mix of n-hexane/ethyl acetate (1:1) as eluent. The fractions (R.F. = 0.75) was concentrated furnishing the nigericin as a pale yellow solid (0.3 g) 30% w/w yield. Spectral data for the isolated (+)- nigericin free acid from *Streptomyces hygroscopicus* UFPEDA 3370 are shown on Supporting information and were compared with the literature [37].

2.4. Cytotoxic activity

The cytotoxic activity of nigericin was tested at different concentrations against a panel of cancer cell lines: HCT-116 (human colon carcinoma), HL-60 (human acute promyelocytic leukemia), MCF-7 (human breast adenocarcinoma), NCI–H292 (human mucoepidermoid lung), P815 (mouse mastocytoma) and L929 (mouse fibroblasts). The MTT assay was performed according to the literature [38]. The cancer cell lines HCT-116, HL-60 and MCF-7 were grown in RPMI-1640 medium and NCI–H292, P815 and L929 were grown in Dulbecco's Modified Eagle's Medium (DMEM). Both media were supplemented with fetal bovine serum (FBS) (10% of final concentration) followed by treatment with penicillin and streptomycin as antibiotics (1% of final concentration).

The cells (3×10^5 cells/mL) were plated in 96-well plates and incubated in the presence of CO_2 atmosphere (5%) for 24 h at 37 °C. Then, nigericin free acid in concentration ranging from 0.0007 μg/mL to 25 μg/mL (final concentration) was added and incubated at 37 °C for 72 h. Then, 25 μL of MTT (5 mg/mL in PBS) was added to each well and the plates were left for another 3 h in incubator at 37 °C and at the end of that period the supernatant was aspirated. To perform the reading, 100 μL of dimethylsulfoxide (DMSO) was added in each well for the dissolution of the formazan crystals. The amount of formazan was measured by reading the absorbance at 560 nm. Concentration leading to 50% inhibition of viability (IC_{50}) was calculated by regression analysis using GraphPad Prism 5.0. Each sample was tested in triplicate in two independent experiments. Doxorubicin was used as positive control.

2.5. Isolation of human peripheral blood mononuclear cells (PBMCs) assay

The project was submitted and approved by the Human Research Ethics Committee of the Federal University of Pernambuco, in accordance with Resolution 466/12 of the National Health Council and approved with CAAE 23236618.1.0000.5208. In this assay, human peripheral blood mononucleated cells (PBMCs), including lymphocytes and monocytes, were collected from healthy middle-aged volunteers. The result of this assay enables to calculate the selectivity index that measure the selective toxicity of a tested compound toward cancer cells. The peripheral blood assay was performed according to the literature [39]. Peripheral blood sample was transferred aseptically into 50 mL polystyrene heparin tubes and isolated by centrifugation (30 min at 400 g) through Ficoll-Hypaque® gradients (Sigma- Aldrich, UK). After centrifugation, the cells were harvested, washed with phosphate buffer (PBS) and resuspended in RPMI 1640 medium. Using a Neubauer chamber, cells were counted and plated in a 96-well plate at a concentration of 1×10^6 cells/mL and incubated in the presence of CO_2 atmosphere (5%) at 37 °C for 24 h.

2.5.1. Cytotoxicity assessment on peripheral blood mononuclear cells (PMBC)

After 24 h of incubation, nigericin was dissolved in DMSO and further diluted in RPMI 1640 medium until concentration ranging from 0.0007 μg/mL to 25 μg/mL (final concentration) and added to each well. The plates were incubated at 37 °C for 72 h, followed by addition of 25 μL of MTT (5 mg/mL solution). The plate was incubated for 4 h and after was centrifuged at 1500 rpm for 10 min. The supernatant was aspirated in order to remove the excess of MTT. To perform the reading, 100 μL of DMSO was added for dissolving formazan crystals and the cell viability was quantitatively measured at 560 nm in a plate spectrophotometer. The 50% inhibitory concentration (IC_{50}) was calculated by regression analysis using GraphPad Prism 5.0. Each sample was tested in triplicate in two independent experiments. Doxorubicin was employed as positive control. The products were tested in μg/mL because the tests were performed before the structural elucidation of nigericin, so on the end we transformed the values to molar concentrations.

2.6. Annexin V/propidium iodide (PI) cell death assay

HCT-116 cells (0.3×10^6 cells/mL) were incubated at 37 °C with 5% CO_2 for 48 h and exposed to different concentrations of nigericin free acid. After then, the cells were stained with Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions. The rate of apoptotic cells was analyzed using a dual laser flow cytometer (BD Accuri C6 Plus personal flow cytometer) and estimated using the C6

BD software (BD Biosciences).

2.7. Transmission electron microscopy

HCT-116 cells were treated for 48 h with nigericin free acid (0.0138 μM). After, cells were fixed for 2 h in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer solution. The samples were washed twice in the same buffer and post-fixed in a solution containing 1% osmium tetroxide, 2 mM calcium chloride and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone as previously reported [40] and were then embedded using a Fluka Epoxy Embedding kit (Fluka Chemie AG, Buchs, Switzerland). Polymerization was performed at 60 °C for 24 h. Ultrathin sections (70 nm) were placed on 300-mesh nickel grids and then counterstained with 5% uranyl acetate and lead citrate, followed by examination using a transmission electron microscope (Tecnai Spirit Biotwin, FEI Company, Hillsboro, OR, USA).

2.8. Kinase inhibition assays

Kinase activities were assayed in appropriate kinase buffer, with either protein or peptide as substrate in the presence of 10 μM ATP in a final volume of 6 μL using the ADP-Glo™ assay kit (Promega, Madison, WI, described in Zegzouti et al. [41]). Controls were performed with appropriate dilutions of DMSO (solvent of the tested compounds). Peptide substrates were obtained from Proteogenix (Schiltigheim, France). The buffer A was used for kinase assays: 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris-HCl pH 7.5, 50 μg/mL heparin.

A panel of five recombinant human protein kinases was used during this study: *Hs*CDK9/CyclinT (human, recombinant, expressed by baculovirus in Sf9 insect cells) was assayed in buffer A with 0.27 μg/μL of the following peptide: YSPTSPSYSPTSPSYSPTSPSKKKK, as substrate; *Hs*GSK-3β (human, recombinant, expressed by baculovirus in Sf9 insect cells) were assayed in buffer A with 0.010 μg/μL of GS-1 peptide, a GSK-3-selective substrate (YRRAAVPPSPSLSRHSSPHQSpEDEEE, "Sp" stands for phosphorylated serine); *Hs*CK1ε (human, recombinant, expressed by baculovirus in Sf9 insect cells) was assayed in buffer A with 0.022 μg/μL of the following peptide: RRKHAAIGSpAYSITA as CK1-specific substrate; *Hs*JAK3 (human, recombinant, expressed by baculovirus in Sf9 insect cells) was assayed in buffer A with 0.17 μg/μL of the following peptide: GGEEEEYFELVKKKK as substrate; *Hs*ABL1 (human, recombinant, expressed by baculovirus in Sf9 insect cells) was assayed in buffer A with 0.17 μg/μL of the following peptide: EAIYAAPFAKKK as substrate.

2.9. Molecular modeling

The structures of both JAK3 and GSK-3β have been obtained through the Protein Data Bank repository (rcsb.org), under the codes 6AAK [42] and 1Q41 [43], respectively. The structure of the nigericin has been obtained from DrugBank Database (drugbank.ca, code: DB14056). For comparison purposes of *in silico* approaches, molecules peficitinib (which is the reference as co-crystallized ligand at structure 6AAK), indirubin and a monoxime derivative (which are the reference as co-crystallized ligand at structure 1Q41) were also used. The addition of missing hydrogen atoms and the generation of the 3D conformation for nigericin have been made using Marvin Sketch 20.10.0 software [44]. The molecular docking calculations has been performed using the AutoDock 4.2 software [45], using increased values for two important parameters of the genetic algorithm search: i) ga_run: increased from 10 to 50; and ii) ga_num_evals: increased from 2,500,000 to 25, 000, 000. The central coordinates of the search space grid box have been defined on the respective co-crystalized ligands for each target, and the default value (0.375 Å) for the spacing between the grid points has been used. For JAK3: center coordinates determined by the peficitinib ligand (Center X: 39.439, Center Y: 6.199 and Center Z: 36.168) and the search space size was defined as a cube of 40 points on each dimension (40 × 40 x 40). For GSK-3β: center coordinates determined by the indirubin-3′-monoxime ligand (Center X: 11.811 Center Y: −38.319 and Center Z: 49.192) and the search space size was defined as a rectangle (40 × 54 x 62). The addition of polar hydrogens, gasteiger charges and torsions branches, for all ligands, were achieved by using the software AutoDock Tools [45]. In order to take into account some important receptor's degrees of freedom and, thus, better simulate the induced fit phenomena at pharmacodynamics, the following amino acid residues were treated as flexible during the calculations: i) for JAK3, the residues LEU828, VAL884, TYR904, ARG953, ASN954, LEU956 and ASP967; and ii) for GSK-3β, the residues LYS85, LEU132, THR138, ARG141, LEU188, CYS199 and ASP200. Then, the BINANA [46] software has been used to analyze the intermolecular interactions present on the docking solutions. Finally, the PyMol software [47] has been used to generate the images of molecular interactions. All hydrogen bonds are presented in donor-acceptor distances. All distances are in Angstroms (Å).

3. Results

3.1. Isolation and structural elucidation of the nigericin free acid from Streptomyces hygroscopicus UFPEDA 3370

While detailed biochemical assays related to nigericin free acid isolated from *Streptomyces hygroscopicus* UFPEDA 3370 were performed, a fully structure characterization was recognized on the basis of spectral data analysis concomitantly (supplementary material). The (+)-nigericin free acid was characterized in accordance with the literature data.

3.2. Cytotoxic activity of nigericin

As shown in Table 1, nigericin showed cytotoxicity against various tested cancer cell lines with IC_{50} values in the nanomolar range, with more potent activity against HL-60, HCT-116, P815 and NCIH-292. Cytotoxicity of nigericin was also evaluated upon peripheral blood mononuclear cell (PBMCs) in order to determine if there is pronounced effect when compared to tumor cell lines. Therefore, selectivity index (SI) was calculated in order to demonstrate the ratio of IC_{50} between normal cells and tumor cells to estimate the compound safety profile (Table 2). As reported here, nigericin free acid is nearly 30 times more potent towards HL-60 leukemic cells than the non-cancerous PBMCs. This compound also showed favorable selectivity toward HCT-116 and

Table 1
IC_{50} values (μM) of nigericin free acid against tumor cell lines or Peripheral Blood Mononuclear Cells (PBMCs) performed by the MTT test after 72 h of incubation. SI = Selectivity Index. *The data are presented as IC_{50} values (95% confidence interval) of four independent experiments; **IC_{50} was calculated using nonlinear regression in GraphPad Prism 5.0 after 72 h; ***SI = IC_{50} (PBMC)/IC_{50} (cancer cell).

		IC_{50} (μM)/95% confidence interval		selectivity index (SI)***	
	Lines Cells	Nigericin	Doxorubicin	Nigericin	Doxorubicin
1	HCT-116	0.0138 (0.0096–0.019)	0.4231 (0.1655–0.5931)	3.0	4.4
2	MCF-7	0.1104 (0.0041–0.0317)	0.8277 (0.2320–0.8552)	0.4	2.3
3	NCIH-292	0.0414 (0.0276–0.0689)	0.2391 (0.0965–0.3310)	1.0	7.8
4	HL-60	0.0014 (0.0012–0.0027)	0.6806 (0.3448–0.7449)	30.0	2.7
5	P815	0.0124 (0.0083–0.0138)	0.1471 (0.0552–0.2345)	3.3	12.7
6	L929	0.3036 (0.1305–0.3862)	0.9793 (0.7448–1.3655)	0.1	1.9
7	PBMC	0.0414 (0.0138–0.1241)	1.8759 1.082–1.9832	–	–

Table 2
Kinase inhibitory activity of nigericin.

Compound	Kinases/IC_{50} (μM)				
	*Hs*CDK9/CyclinT	*Hs*JAK3	*Hs*CK1ε	*Hs*GSK3β	*Hs*ABL1
Nigericin	36.12	8.82	23.03	5.38	14.73

Data expressed as IC_{50} of at least three assays.

P815 cell lines with SI values of 3.0 and 3.3, respectively.

3.3. Detection of apoptosis using flow cytometry

In order to study the cell death process induced in HCT-116 cells after 48 h treatment with nigericin free acid, a flow cytometric test was performed after double staining with annexin V and PI. Annexin V and PI values were set as the horizontal and vertical axes, respectively, for the plot construction. Mechanically damaged or necrotic cells, late apoptotic cells, viable cells, and early apoptotic cells were located in the

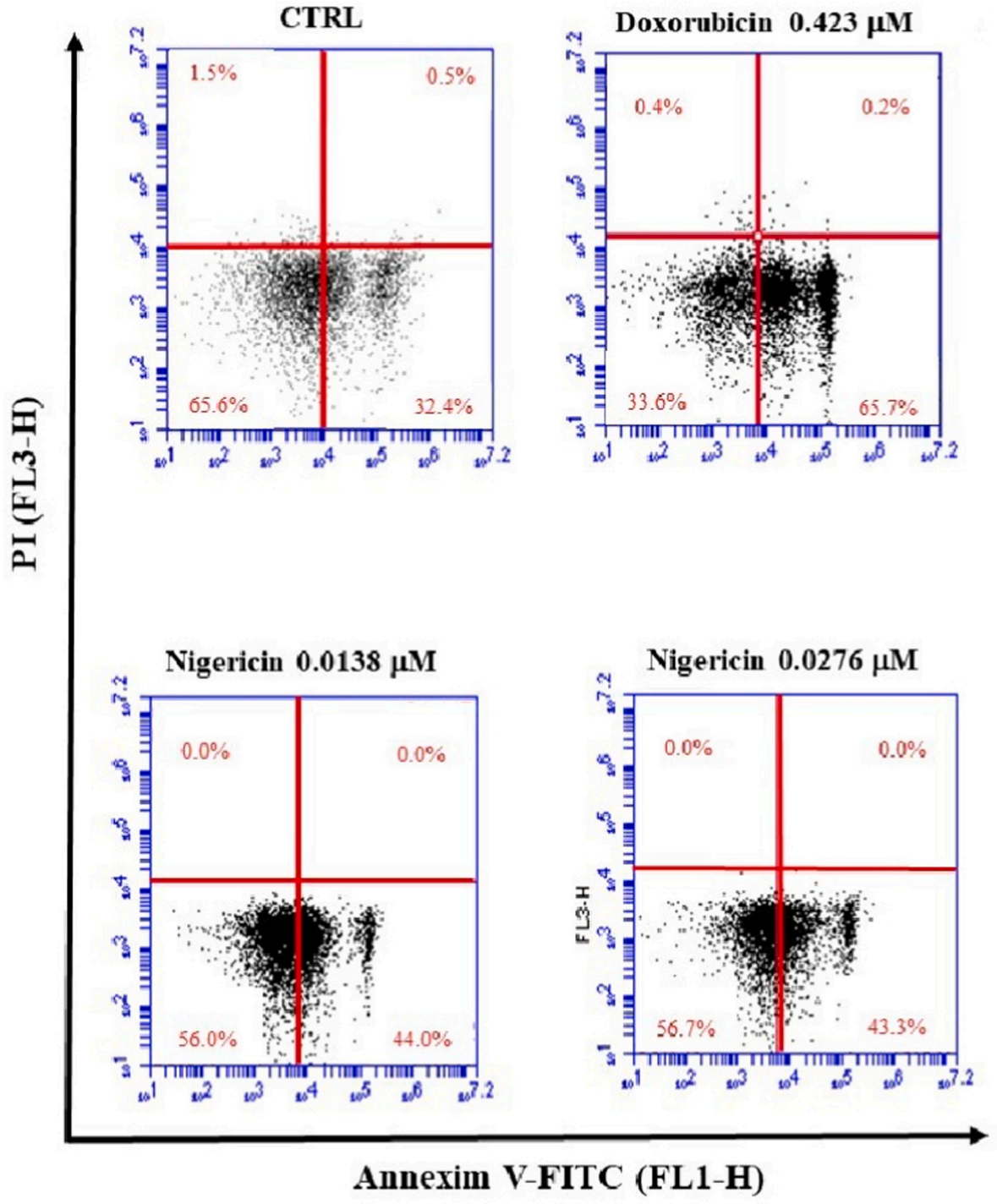

Fig. 2. Measurement of cell death by Annexin V binding and PI uptake. HCT-116 cells were treated with nigericin (0.0138 and 0.0276 μM) for 48 h. After the incubation time, the samples were analyzed by flow cytometry. Doxorubicin was used as positive control. Lower left: live cells; Lower right: apoptotic cells.

upper left, upper right, lower left and lower right quadrants of the flow cytometric dot plot, respectively. The results are shown in Fig. 2. Apoptosis was observed in 44.0%, 43.3% and 65.7% of the cell treated with nigericin (0.0138 or 0.0276 μM) or doxorubicin (0.423 μM), respectively.

3.4. Ultrastructural analyses

HCT-116 control cells showed characteristic morphology such as round nucleus, numerous mitochondriae, saccules of endoplasmic reticulum and Complex of Golgi. In contrast, HCT-116 cells treated with 0.0138 or 0.0276 μM (corresponding to 1 x IC_{50} and 2 x IC_{50} values, respectively) of nigericin free acid exhibited several morphopathological alterations, such as mitochondriae with osmiophilic deposit-like inclusions and loss of cristae, nucleus containing round osmiophilic inclusion and double-membrane vesicles, as well as nuclear tubular invaginations. Moreover, these cells showed inclusion of osmiophilic content inside the smooth endoplasmic reticulum (SER), fragmentation

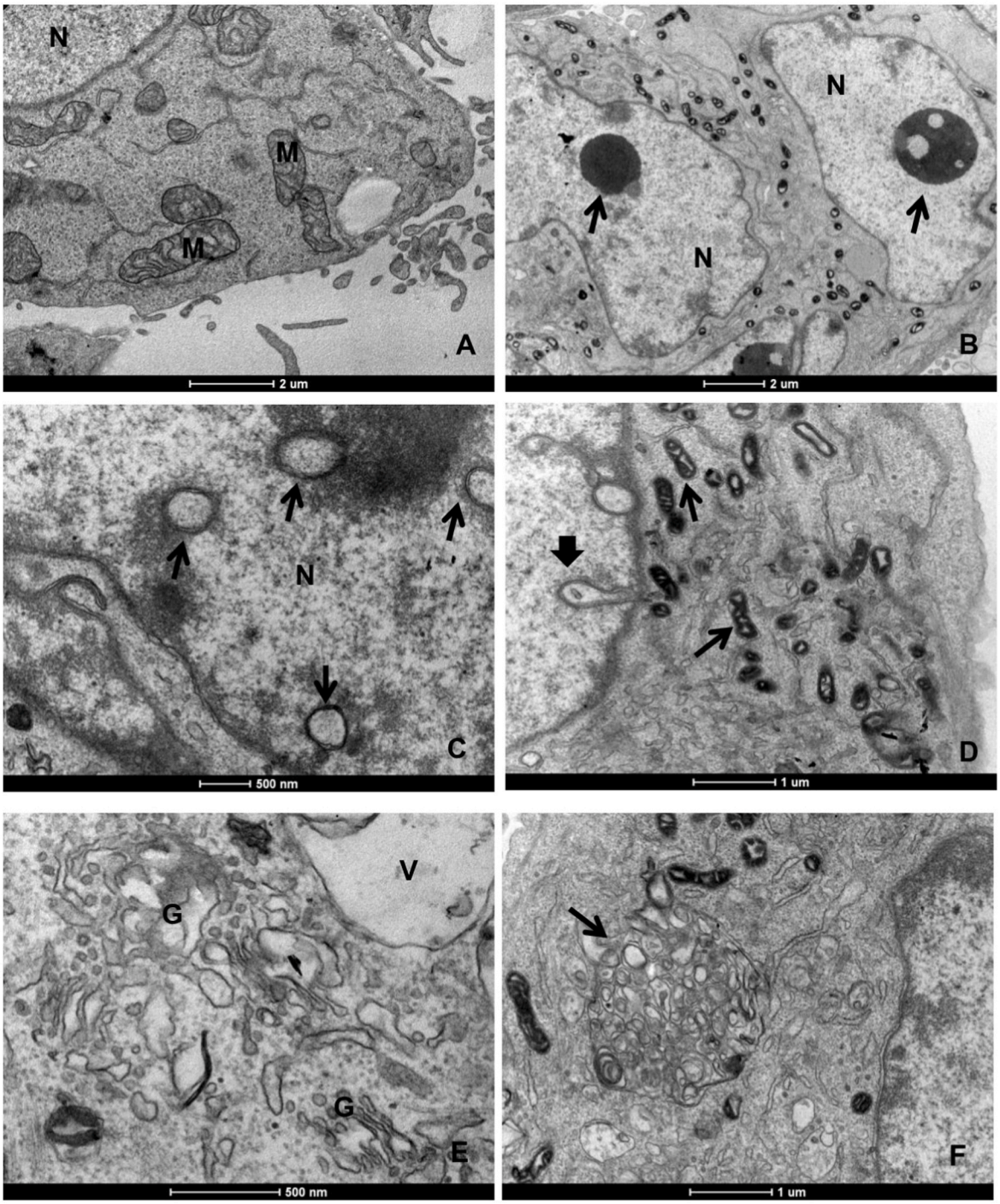

Fig. 3. Ultrastructural analysis of HCT-116. (A) Control cell; (B, C, D, E and F) HCT-116 cells treated with 0.0138 μM of Nigericin free acid. (B) Observe osmiophilic nuclear inclusion (arrows), (C) double-membrane nuclear vesicles (arrows), (D) tubular invaginations of the nuclear membrane (large arrow) and osmiophilic mitochondriae (thin arrows), (E) swollen and fragmented Golgi Complex, (F) autophagosome-like structure (arrow). N, nucleus; G, Golgi, M, mitochondria, V, vacuole.

of Golgi complex and presence of autophagosome-like structures encircled by ER-like membrane saccules, containing cytoplasmic osmiophilic organelles together with part of the cytoplasm. There was no difference between the two doses tested on the morphology of the treated cells. The results are shown in Fig. 3.

3.5. Kinase inhibition assays

Nigericin was tested against five different human protein kinases that are involved in various human disorders, including cancer and inflammatory diseases [48,49]. Nigericin displayed kinase inhibitory activity on *Hs*JAK3 and *Hs*GSK-3β kinases with IC_{50} values of 8.82 and 5.38 μM, respectively. In addition, nigericin proved to be less active towards CDK9, CK1ε and ABL1. The results are shown in Table 2.

3.6. Molecular modeling

The results highlighted on JAK3 and GSK-3β kinases *in vitro* prompted us to study the binding poses of nigericin. For the JAK3 target, one can see that nigericin (Fig. 4a) performed three hydrogen bonds, with distances of 2.8 Å (GLN827), 2.9 Å (LYS830) and 2.7 Å (ARG953), and hydrophobic contacts with the residues LEU828, GLY829, VAL836, ALA853, GLY908, CYS909, ASN954, ILE955, LEU956, ALA966 and ASP967. On the other hand, the peficitinib (Fig. 4b) performed three hydrogen bonds, with distances of 3.2 Å (ASP967), 2.9 Å and 2.9 Å (LEU905), one pi-pi stacking parallel displaced with the residue TYR904, and hydrophobic contacts with the residues ALA853, VAL884, MET902, CYS909 and LEU956. By looking into their intermolecular interactions, it is possible to conclude that although both compounds have performed three hydrogen bonds, the peficitinib was the only compound to perform a pi-pi stacking parallel displaced. Additionally, while the nigericin performed hydrophobic contacts with eleven residues, the peficitinib performed with only five residues.

Continuing the analysis of the intermolecular interactions between these compounds and the JAK3's binding site, the pi-pi stacking interaction formed by peficitinib, and absent on nigericin, may have contributed to stabilize this compound in the JAK3's binding site. Another key difference can be observed on the formed hydrogen bonds. While the peficitinib formed hydrogen bonds with the residues GLN827, LYS830 and ARG953, nigericin formed two hydrogen bonds with the residue LEU905 and one with the residue ASP967, with slightly longer distances when compared to peficitinib. Therefore, these seem to be the reasons, at the intermolecular level, why peficitinib presented a higher predicted binding affinity ($\Delta G = -11.24$ kcal/mol) for the JAK3 target than nigericin ($\Delta G = -10.22$ kcal/mol). This finding may indicate that in order to increase the potency of a drug candidate targeting JAK3, the molecule may need to be able to form a pi-pi stacking along with the hydrogen bonds in order to stay stabilized in this binding site.

For the GSK-3β target, the nigericin (Fig. 5a) formed three hydrogen bonds, with distances of 3.3 Å (VAL61), 3.2 Å and 3.4 Å (both with ASP200), one salt bridge (ARG141) and hydrophobic contacts with residues ILE62, GLYS63, ASN64, VAL70, LYS85, TYR134, GLU137,

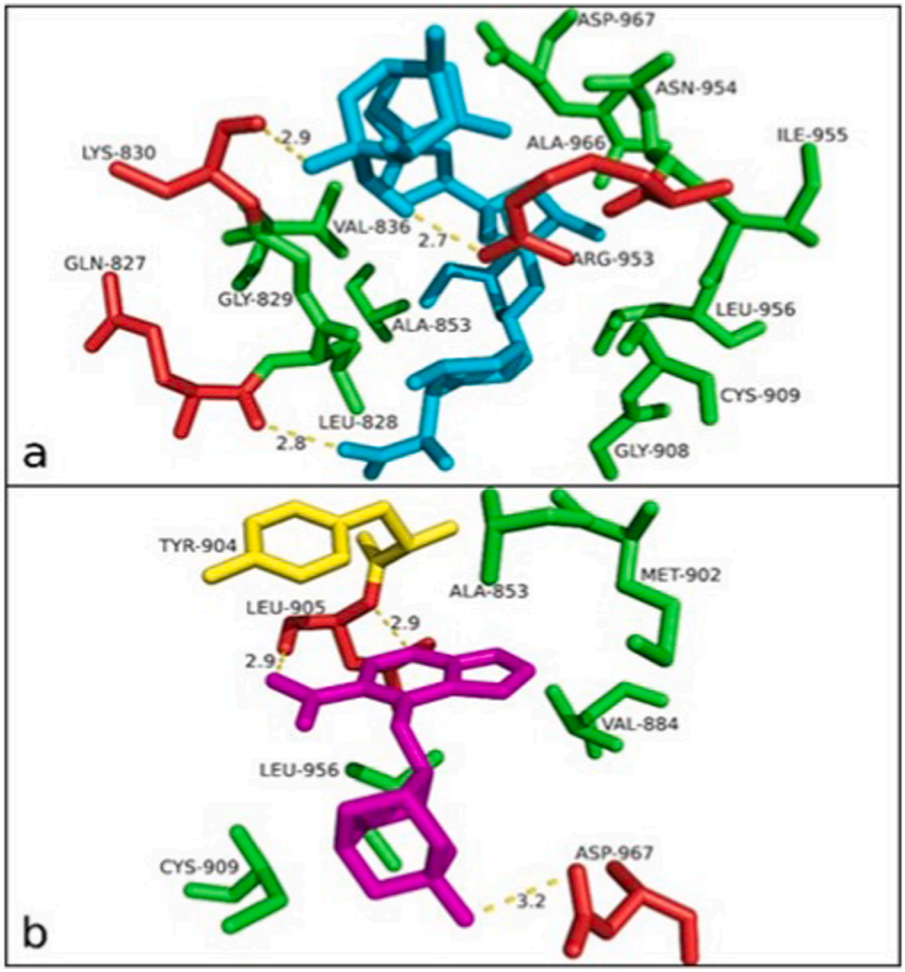

Fig. 4. The intermolecular interactions between the JAK3's binding site residues and the (a) Nigericin (cyan) and (b) Peficitinib (magenta) compounds. The residues participating in hydrophobic contacts are represented in green, hydrogen bonds in red and pi-pi stacking in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

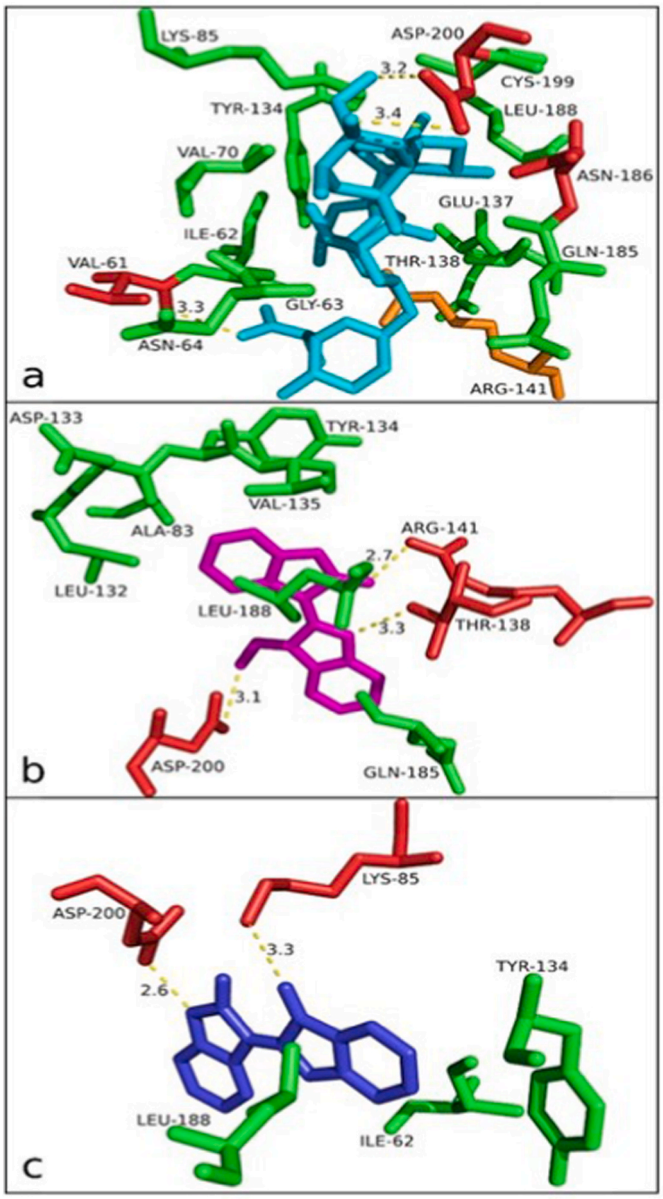

Fig. 5. The intermolecular interactions formed between the GSK3-β's binding site residues and (a) Nigericin (cyan), (b) Indirubin-3′-monoxime (magenta) and (c) Indirubin (blue) compounds. The hydrophobic contacts are in green, hydrogen bonds in red and salt bridge in orange. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

THR138, GLN185, LEU188 and CYS199. On the other hand, the indirubin-3′-monoxime (Fig. 5b) has formed three hydrogen bonds, with distances of 3.3 Å (THR138), 2.7 Å (ARG141) and 3.1 Å (ASP200), and hydrophobic contacts with the residues ALA83, LEU132, ASP133, TYR134, VAL135, GLN185 and LEU188. Finally, the indirubin (Fig. 5c) formed two hydrogen bonds, with distances of 3.3 Å (LYS85) and 2.6 Å (ASP200), and hydrophobic contacts with residues ILE62, TYR134 and LEU188.

By looking into their intermolecular interactions with this target (GSK-3β), it is possible to conclude that all these three compounds (Indirubin-3′-monoxime/ΔG = −10.56 kcal/mol, Indirubin/ΔG = −6.12 kcal/mol and nigericin/ΔG = −8.38 kcal/mol) performed hydrogen bonds with the residue ASP200. Another key interaction seems to occur with the residue ARG141, although it is absent for the indirubin, but performed a salt bridge with the Nigericin and one short (more stable) hydrogen bond with the Indirubin-3′-monoxime, the top ranked compound in this comparison. The intermolecular interactions seem to explain again the predicted affinities of these molecules with this target. In this way, it seems that a good drug candidate targeting GSK-3β could benefit by performing intermolecular interactions with these two residues.

4. Discussion

A column chromatography purification was made in order to identify the most biologically active metabolite from the 2-butanolic fraction that proved to be the most cytotoxic extract within the *S. hygroscopicus* UFPEDA 3370 strain fermentation. Thus, it was possible to isolate, in high concentration (30% w/w), the substance responsible for the high cytotoxic activity in the EB1 fraction.

As nigericin free acid showed cytotoxicity against all tested cell lines, especially against HCT 116 (IC_{50} = 0.0138 μM), HL-60 (IC_{50} = 0.0014 μM) and P815 cells (IC_{50} = 0.0124 μM) (Table 1), the selectivity index of nigericin free acid was determined to evaluate its safety profile.

In this context, it is important to highlight the ratio of the obtained IC_{50} values for nigericin free acid toxicity on tumor cell lines and peripheral blood mononuclear cell (PBMCs). From these values, it is possible to calculate the selectivity index (SI) where the higher the value of this index is (above 1), the greater the selectivity of the compound is, in relation to the tumor cell. Values above 2 is desirable, so the compound can be applied at twice its concentration without showing toxicity in the healthy cell [50]. Since colorectal cancer is one of the most incident in human, and due to the fact that nigericin have shown a selectivity index above 2 for the HCT-116 strains, this cell was chosen for further studies.

Despite nigericin has been reported in the literature and have been described to be potentially used in cytotoxic assays, its use is only restricted to the Sigma-Aldrich commercially sodium salt to date. In addition, it has been reported that nigericin treatment induces autophagy, however the mechanism of action is still poorly understood. To our knowledge, this is the first study showing of nigericin acid free action on protein kinases.

Through flow cytometry, it was observed that nigericin free acid caused apoptosis in about 40% of the treated cells, with no difference between the tested doses. On the other hand, in the ultrastructural analysis it was possible to observe characteristics of cell death by autophagy on HCT-116 treated with nigericin free acid, such as: mitochondriae with osmiophilic deposit-like inclusions and loss of cristae, nucleus containing round osmiophilic inclusion and double-membrane vesicles, as well as nuclear tubular invaginations.

Autophagy and apoptosis are well-regulated biological processes that have important roles in tumor development and progression. However, the crosstalk between autophagy and apoptosis are unclear [51,52]. Studies have demonstrated that autophagy has an anti-apoptotic role in numerous types of cancer [53,54], whereas other studies reported its essential pro-apoptotic role in certain cancer, including colorectal cancer cells [55,56].

Previous study reported that nigericin, like salinomycin, selectively inhibits Wnt signaling cascade in HEK293 cells at nanomolar concentrations [57], and could suppress colorectal cancer metastasis by inhibition of epithelial–mesenchymal transition. [58]. Wang et al. [56] reported for the first time that nigericin regulates the expression of GSK-3β, a Wnt/βcatenin signaling protein that activates the canonical Wnt pathway. However, the specific mechanism of this process remains unclear. However, it is known that GSK-3 kinase inhibits autophagy through the mammalian target of rapamycin (mTOR) complex 1 (mTORC1). In fact, overexpression of either GSK-3α or GSK-3β activates mTORC1 and suppresses autophagy in MCF-7 breast cancer cells. The opposite way, the treatment of cells with GSK-3 inhibitors inhibits mTORC1 activity and increases autophagic flux [59]. These studies corroborate, at least in part, with our results *in vitro* and *in silico* on

GSK-3β target. In this study, nigericin caused autophagy probably by inhibition of mTORC1.

Targeting PI3K/AKT/mTOR signaling can not only induce apoptosis to inhibit the proliferation of tumor cells, but also induce autophagy, however, this crosstalk between these two pathways was unclear [60, 61]. In our study, nigericin caused both apoptosis and autophagy.

Nigericin free acid also presented kinase inhibitory activity on JAK-3 (IC_{50} = 8.82 μ/M). This result was confirmed *in silico*. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction pathway is an evolutionarily conserved pathway present in *Drosophila melanogaster* through *Homo sapiens* [62]. JAK family of non-receptor protein-tyrosine kinases consists of JAK1, JAK2, JAK3, and TYK2 (tyrosine kinase-2), all of which play a role in different cytokine and growth factor receptor mediated signaling pathways [63]. JAK3 belongs to a family of cytoplasmic non-receptor tyrosine kinases. Although most JAK family members are ubiquitously expressed, JAK3 is mainly restricted to the hematopoietic lineage, playing a vital role in lymphoid cell development and homeostasis [64].

JAK3 activating mutations that result in persistent activation of the JAK-STAT signaling have been described in various leukemias and lymphomas. JAK-STAT dysregulation results in autoimmune disorders such as rheumatoid arthritis, ulcerative colitis, and also plays a role in the pathogenesis of myelofibrosis, polycythemia vera, and other myeloproliferative illnesses [65]. Tofacitinib is a small-molecule inhibitor of JAK tyrosine kinases (JAK1/3, dubbed as pan-JAK inhibitor) and has been approved for the treatment of arthritis, psoriatic arthritis and ulcerative colitis; while ruxolitinib have been approved to treat myeloproliferative neoplasms [66]. Although the effective JAK blockade is well established for inflammatory diseases, the involvement of JAK/-STAT3 pathway in the most common forms of sporadic colorectal cancer has been documented. These tumors arise from the aberrant activation of the WNT/β-catenin pathway in more than 80% of cases, which results mainly as a consequence of homozygous loss or inactivation of the tumor's APC (Adenomatous polyposis coli) gene suppressor [67].

Activation of JAK also triggers the activation of other signaling pathways such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3′-kinase (PI3K) and AKT/rapamycin target in mammals (mTOR) [68]. mTOR, the best known regulator of autophagy, is a serine/threonine kinase conserved throughout eukaryotes and exists in two functionally distinct complexes termed mTORC1 and mTORC2; mTORC1 integrates multiple signals from growth factors, oxygen, energy levels and nutrients to promote cell growth and proliferation, and inhibition of catabolic process such as autophagy. On the other hand, mTORC2 only responds to growth factors and regulates actin/cytoskeleton organization and cell survival through the pathways as shown above. The activity of mTORC1 is inversely correlated with autophagy induction. The mTORC1 inhibitor rapamycin potently induces autophagy, even in the presence of abundant nutrients [69]. Despite being a preliminary study, evidence points that nigericin causes autophagy through inhibition of the mTOR pathway, the likeness of rapamycin, since both are ionophores isolated from *S. hygroscopicus*. The interaction of nigericin with JAK-3 and GSK-3β may converge to mTOR inhibition, however, other experiments need to be carried out to confirm this hypothesis. Taken together these data can be used to establish a preliminary hypothetical scheme of the mechanism of action of nigericin related to kinase inhibition (illustrated in Fig. 6).

5. Conclusion

A novel source of appreciable amount of nigericin was identified in *Streptomyces hygroscopicus* UFPEDA 3370 isolated from rhizosphere of *Paullinia cupana*, from Amazon (Brazil). Our data confirmed that nigericin free acid, itself, showed to possess a broad antiproliferative spectrum against an array of cell lines in a nanomolar IC_{50} values, being especially efficient and selective in inhibiting HCT-116 (human colon carcinoma) and HL-60 (human acute promyelocytic leukemia).

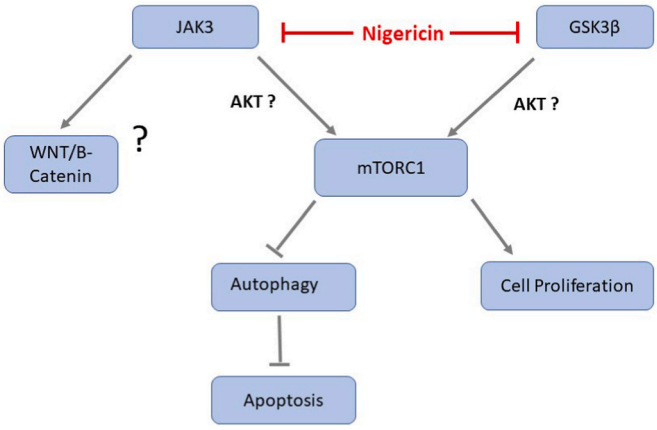

Fig. 6. Scheme of the hypothetical mechanism of action of nigericin via inhibition of JAK3 and GSK3-β kinases. Arrows Activation of the signaling pathway. Arrows inhibition of the signaling pathway.

Nigericin free acid caused cell death by both apoptosis and autophagy, these effects are related to inhibition of the JAK3 and GSK-3β kinases, which in turn, would probably inhibit the activation of mTORC1, resulting in increased autophagy and apoptosis. These findings provide a basis for further investigation and development of nigericin as a potential drug for colorectal cancer. However, further research would be required to verify the antitumor activity of nigericin *in vivo* and proteins involved in cell signaling.

Sample credit author statement

Iza Mirela R.G. Princival, Teresinha G. Silva, Maria D. Rodrigues, Sandrine M.A. Lima: Conceptualization, Methodology; Marcelo Z. Hernandes, Luiz F.G.R. Ferreira: Molecular docking: Emmanuel D. Silva, Jhonattas C. Carregosa, Alberto Wisniewski-Jr., Fernando Halwass, José A.A Franca: Data curation. Christina A. Peixoto, Karina L.A. Saraiva: Electron microscopy analysis; Blandine Baratte, Thomas Robert, Stéphane Bach: Tests of protein kinases; Patricia M.G. Paiva, Dayene C. Gomes, Caio C·O Lucena: Visualization, Investigation. Teresinha G. Silva: Supervision; Pascal Marchand, Jefferson L. Princival, Teresinha G. Silva: Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cbi.2020.109316.

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