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Evaluation of the Antibacterial Activity of Isatin against *Campylobacter jejuni* and *Campylobacter coli* Strains

Claudia B. Barroso [1,2], Liliane M. Seki [3,*], Wagner T. C. Esteves [3], Michele C. Nascimento [4] and Aurea Echevarria [2,5,*]

[1] Departamento de Ciências Biológicas, Escola Nacional de Saúde Pública Sérgio Arouca FIOCRUZ, Rio de Janeiro 21041-210, Brazil; cbastosbarroso@gmail.com
[2] Programa de Pós-Graduação em Ciência, Tecnologia e Inovação em Agropecuária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Seropédica 23890-000, Brazil
[3] Laboratório de Zoonoses Bacterianas-Setor de *Campylobacter*, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro 21040-361, Brazil; wthadeu@ioc.fiocruz.br
[4] Departamento de Controle da Qualidade, Instituto de Tecnologia em Imunobiológicos, FIOCRUZ, Rio de Janeiro 21040-090, Brazil; michelecn83@gmail.com
[5] Instituto de Química, Universidade Federal Rural do Rio de Janeiro, Seropédica 23890-000, Brazil
[*] Correspondence: miyuki@ioc.fiocruz.br (L.M.S.); echevarr@ufrrj.br (A.E.); Tel.: +55-(21)-26827829 (A.E.)

Abstract: Antibiotic resistance, particularly against fluoroquinolones and macrolides, has emerged globally among thermophilic Campylobacters (*Campylobacter jejuni* and *Campylobacter coli*), giving rise to concerns about the efficacy of antibiotic treatment of these bacteria. Thus, developing new antibacterials with excellent activity is important. Isatin (IST) and its derivatives have exhibited promising antibacterial activities in several pathogenic bacteria. However, its activity against *Campylobacter* is unknown. Therefore, this study was conducted to evaluate the antibacterial activity of isatin against 29-*Campylobacter* strains (*C. jejuni*-17 and *C. coli*-12) and investigate the effects at the cellular level. The minimal inhibitory concentrations (MICs) of isatin were between <1.0 and 16.0 µg/mL in *Campylobacter* strains. Most strains presented with MIC = 8.0 µg/mL (76%). The minimal bactericidal concentration (MBC) was determined to be 16.0 µg/mL for 72% of the *Campylobacter* strains tested. The 50% inhibitory concentration (IC_{50}) value for isatin was 125.63 µg/mL on the MRC-5 normal cell line, suggesting that isatin can be considered a safe substance in terms of cytotoxicity. In this study, we demonstrated the potential of isatin based on its low toxicity and effectiveness in vitro against antibiotic-resistant *Campylobacter* strains, which indicates that this compound could be an attractive candidate for future use in multidrug-resistant *Campylobacter* treatment.

Keywords: isatin; *Campylobacter*; antibacterial activity; low toxicity

1. Introduction

Campylobacteriosis is a type of gastroenteritis caused by pathogenic *Campylobacter* species and is an important public health concern. In Europe, the actual number of cases is close to nine million each year and the estimated cost of these infections is approximately EUR 2.4 billion per year [1]; in the US, the Centers for Disease Control (CDC) estimates that *Campylobacter* infections affect more than 1.5 million people each year [2].

Currently, the genus *Campylobacter* is composed of 61 species and 16 subspecies [3], and *C. jejuni* and *C. coli* are the most common causes of severe bacterial gastroenteritis, with the majority (over 90%) of these cases being caused by *C. jejuni* and to a lesser extent *C. coli* [4]. These thermophilic species, *C. jejuni* and *C. coli*, which are frequently isolated from poultry and pigs, respectively [5], are found in the microbiota of warm-blooded animals and, in most cases, are associated with asymptomatic infections [6]. Meat products, mainly poultry, cattle, pigs, sheep, and ostriches, have become the main vehicle of transmitting human campylobacteriosis through the consumption of undercooked meat [7].

Enteric *Campylobacter* infections are generally self-limiting and do not require antimicrobial drug treatment, but in patients with severe and prolonged symptomatology and in immunocompromised individuals, antimicrobial treatment may be necessary. In these cases, fluoroquinolones and macrolides are the first-line agents used to treat the disease, while tetracycline serves as a choice for treating clinical *Campylobacter* infection [8]. However, fluoroquinolones (e.g., ciprofloxacin) are often used in veterinary and human medicine, especially for enteric infections. Thus, a high prevalence of resistance to this class of antimicrobials has been reported in several studies [9,10]. Moreover, cross-resistance to fluoroquinolones and macrolides (e.g., erythromycin), other antimicrobials of choice for the treatment of *Campylobacter* infections, has recently been noted [11–13].

In Brazil, campylobacteriosis has been a neglected disease, and there are insufficient data to estimate the commonality of this pathogen in the country [14]. However, there are Brazilian studies that have also confirmed phenotypic resistance to ciprofloxacin and/or erythromycin and/or tetracycline in *Campylobacter* strains [15–17].

The phenomenon of antimicrobial resistance therefore represents a growing global health threat, which must be urgently addressed to protect public health, animal production, agriculture, and the environment, as resistance makes the effective treatment of various infections difficult and results in longer-lasting diseases and higher mortality [7]. Because of this concern, antibiotic-resistant *Campylobacter* has been designated as one of the high-priority pathogens in the WHO list for the development of new antibiotic therapies [18]. This situation has forced scientists to search for new antimicrobial substances from various sources, such as medicinal plants or synthetic compounds. We can highlight some research in the literature for *Campylobacter* that used essential oils and isolated terpenoid compounds [19], extracts plants of Chinese and Korean origin [20], agent Auranta 3001 (a mixture of citric and lactic acids) [21], synthetic compounds combined with ciprofloxacin (CIP) and erythromycin (ERY) [22], and peroxyacetic acid [23] for the purpose of reduced presence of *Campylobacter* after adding to animal feed [19] or to reduce the virulence of *C. jejuni* [20] or to increase antibacterial effects [21–23].

In recent years, isatin and its derivatives have been screened for their antibacterial activities, and some of these molecules have demonstrated promising in vitro and in vivo potency [24]. Furthermore, isatin and its derivatives have demonstrated diverse biological activities, such as anti-inflammatory, antioxidant, anticancer, anticonvulsant, and antimicrobial activities [25].

Isatin (1*H*-indole-2,3-dione) is a heterocyclic compound containing an indole group and was first synthesized by Erdman and Laurent in 1840 as a product of the oxidation of indigo dye by nitric acid and chromium [26]. For over approximately 100 years, isatin was thought to be an exclusively synthetic compound. However, isatin was extracted from plants of the species *Isatis tinctoria*, *Couroupita guianensis* Aubl, *Melochia tomentosa*, and *Boronela koniamboensis* and was found to be endogenously present in animals, such as in the secretions of the parotid gland of the Bufo frogs, in the egg masses of the Australian mollusk *Dicathais orbita*, and in humans, as it is a metabolic derivative of adrenaline [27,28]. The synthetic route to obtain isatin is the most promising, as several examples with good yields are described in the literature, while very low quantities are obtained from natural sources.

The antibacterial activity of isatin and its derivatives has been investigated in gram-positive and gram-negative bacteria, and the results have shown low MIC values against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [29]. Another study evaluated hybrid-containing acyl-hydrazones and isatin against *S. aureus* and *Bacillus cereus* and also the yeast-like fungus *Candida albicans*, and the results showed similar MIC values with bactericidal and bacteriostatic activity. In addition, the compounds did not show cytotoxicity in human lung and liver cells in the range of MICs [30]. Song and collaborators in a recent review focusing on isatin hybrids with antibacterial activity showed the important need to search for new agents to treat bacterial infections [31]. MDR and XDR *M. tuberculosis* strains have also been studied for isatin derivates [32]. Regarding the evaluation of the cytotoxicity of isatin and its derivatives, these have been shown

to have lower toxicity and be safer in several investigations [24,33]. However, although several bacterial strains have been evaluated, the genus *Campylobacter* and effects at the cellular level have not been investigated.

Thus, based on literature reports about the antibacterial effects of isatin and its hybrids, we investigated the anti-*Campylobacter* effects in 29 strains with or without resistance to tetracycline, ciprofloxacin, and erythromycin from animal, food, and human sources. Furthermore, the bacteriostatic or bactericidal effect and cytotoxicity in the MRC-5 human diploid cell line derived from normal lung tissue were investigated.

2. Material and Methods

2.1. Isatin and Solution Preparation

Isatin (IST) was purchased from Sigma-Aldrich® Co. and was solubilized at 1% (*m/v*) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich® Co., St. Louis, MO, USA), sterilized using a 0.22 μm pore size filter(Millex® Millipore Carrigtwohill, Co., Cork, Ireland), and stored at 2–8 °C. For experimental use, a fresh solution was prepared by diluting the stock solution according to the determined minimum inhibitory concentration (MIC) values.

2.2. Bacterial Strains

To determine the antibacterial activity of IST, the following strains from the collection of *Campylobacter* of the Bacterial Zoonoses Laboratory of the Oswaldo Cruz Institute, FIOCRUZ of Rio de Janeiro were studied; the collection included 17 strains of *Campylobacter jejuni*, 12 strains of *Campylobacter coli*, and *Campylobacter jejuni* (ATCC 33560) that have been maintained in a freezer (ColdLab model CL 374-86V, Piracicaba city, São Paulo state, Brazil) at a low temperature of −70 °C. These collected strains included those with or without resistance to antimicrobials (tetracycline, ciprofloxacin, and erythromycin) and were isolated from humans (4 strains), food (16 strains), and animals (9 strains) from Rio de Janeiro and Rio Grande do Sul States in the southeast and south regions of Brazil, respectively, between 1999 and 2016 (Table 1). Strains were cultured in BBL^{TM} Columbia Agar Base supplemented with charcoal, FBP (0.05% ferrous sulfate, 0.05% sodium pyruvate, and 0.05% sodium metabisulfite) and incubated under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 42 °C ± 2 °C for 48 h. The strains were maintained in BBL^{TM} Brucella semi-solid Agar for all experiments.

2.3. Determination of Minimal Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of isatin was determined by the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) with modifications [34]. Briefly, *Campylobacter* strains were incubated in BBL^{TM} Columbia Agar Base supplemented with charcoal (Neon, São Paulo, SP, Brazil), FBP (0.05% ferrous sulfate (Labsynth Ltd.a, Diadema, SP, Brazil)), 0.05% sodium pyruvate (Vetec Ltd.a, Duque de Caxias, RJ, Brazil), and 0.05% sodium metabisulfite (Labsynth Ltd.a, Diadema, SP, Brazil) diluted in sterile water under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 42 °C ± 2 °C for 48 h. The bacterial cells were diluted in sterile saline solution to a turbidity of 0.5 McFarland standard, or 1.5×10^8 CFU/mL. Subsequently, these samples were diluted in sterile saline 1:10 to yield a concentration of 10^7 UFC/mL. After that, 5 μL of bacterial suspension was dispensed into each well, giving a final concentration of approximately 10^4 CFU/mL for the inoculum in each well. Previously, using a multichannel pipette, 45 μL of MHB (Oxoid^{TM}, Basingstone, Hampshire, England) had been transferred to each well of a 96-well microtiter plate. Then, 50 μL of the antimicrobial agent (isatin) was dispensed into the first well of the plate, and serial twofold dilutions were made to obtain final concentrations of treatment ranging from 1 to 1024 μg/mL. The final volume in each well was 100 μL. The contents of each well were mixed for 1 min prior to incubation at 37 °C for 24 h in microaerobic conditions using BD Gaspak EZ^{TM} Campy. The MIC was determined as the lowest concentration at which bacterial growth was inhibited. To indicate respiratory activity, the color was determined after adding 10 μL/well

of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride, Sigma-Aldrich®) dissolved in water (INT 2 mg/mL) and incubating the plates under appropriate cultivation conditions for 30 min at 37 °C in the dark [35]. Cells that were not treated with isatin were diluted with 1% DMSO and MHB medium and were used as a negative control; ciprofloxacin (5 µg/L) from Sigma-Aldrich® was used as the positive control. Experiments were carried out with three replicates. The stability of the antimicrobial agent and the reproducibility of the method were evaluated simultaneously in all the analyses performed using inoculation of the strain ATCC 33560 of *Campylobacter jejuni*.

Table 1. Results of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for isatin against *Campylobacter jejuni* and *Campylobacter coli* strains.

Entry	CCAMP	Year of Isolation	State of Brazil *	Source	Specie	Antimicrobial Resistance **	MIC (µg/mL)	MBC (µg/mL)
1	499	1999	RJ	Human	*C. jejuni*	CIP-ERI-TT	8.0	16.0
2	1504	2007	RJ	Animal	*C. coli*	CIP-ERI-TT	<1.0	<1.0
3	1505	2007	RJ	Animal	*C. coli*	CIP-ERI-TT	<1.0	<1.0
4	1507	2007	RJ	Animal	*C. coli*	CIP-ERI-TT	8.0	16.0
5	1509	2007	RJ	Animal	*C. coli*	CIP-ERI-TT	8.0	8.0
6	1510	2007	RJ	Animal	*C. coli*	CIP-ERI-TT	8.0	8.0
7	1023	2008	RJ	Food	*C. jejuni*	CIP-TT	8.0	16.0
8	1051	2009	RJ	Food	*C. coli*	Sensitive	8.0	16.0
9	1052	2009	RJ	Food	*C. coli*	Sensitive	8.0	16.0
10	1057	2009	RJ	Food	*C. coli*	Sensitive	8.0	16.0
11	1477	2009	RJ	Human	*C. jejuni*	CIP-ERI-TT	8.0	16.0
12	1062	2010	RJ	Food	*C. coli*	CIP-ERI-TT	8.0	8.0
13	1064	2010	RJ	Food	*C. coli*	Sensitive	8.0	8.0
14	1067	2010	RJ	Food	*C. coli*	CIP	8.0	8.0
15	1490	2011	RJ	Human	*C. jejuni*	CIP-TT	16.0	16.0
16	1519	2014	RS	Food	*C. jejuni*	CIP- ERI-TT	8.0	8.0
17	1520	2014	RS	Food	*C. jejuni*	CIP- ERI-TT	8.0	16.0
18	1521	2014	RS	Food	*C. jejuni*	CIP- ERI-TT	8.0	16.0
19	1523	2014	RS	Food	*C. jejuni*	CIP- ERI-TT	8.0	16.0
20	1536	2014	RS	Food	*C. jejuni*	CIP-ERI-TT	8.0	16.0
21	1543	2014	RS	Food	*C. jejuni*	CIP-ERI	16.0	16.0
22	1547	2014	RS	Food	*C. jejuni*	CIP-ERI	16.0	16.0
23	1549	2014	RS	Food	*C. jejuni*	CIP-ERI	16.0	16.0
24	1552	2014	RS	Food	*C. jejuni*	CIP-ERI	16.0	16.0
25	1499	2015	RJ	Human	*C. jejuni*	CIP-ERI-TT	8.0	16.0
26	1538	2015	RS	Animal	*C. jejuni*	CIP	8.0	16.0
27	1554	2015	RJ	Animal	*C. coli*	CIP-ERI-TT	8.0	16.0
28	1555	2015	RJ	Animal	*C. jejuni*	CIP	8.0	16.0
29	1574	2016	RJ	Animal	*C. jejuni*	CIP	8.0	16.0

* RJ (Rio de Janeiro State); RS (Rio Grande do Sul State). ** CIP (ciprofloxacin); ERI (erithromycin); TT (tetracycline).

2.4. Determination of Minimal Bactericidal Concentration (MBC)

The minimal bactericidal concentration (MBC) was measured using a subculture of 10 µL derived from the wells, with no visible bacterial growth on Mueller Hinton Agar (Oxoid™, Basingstone, Hampshire, Englandlcontaining 5% (*v/v*) defibrinated horse blood plates. The MBC was the lowest concentration at which no *Campylobacter* growth could be observed on the plates after incubation at 37 °C for 24–48 h under microaerobic conditions using BD Gaspak EZ™ Campy packs in closed jars. Experiments were carried out with three replicates.

2.5. Cytotoxicity Test to Determine the IC$_{50}$ (50% Inhibitory Concentration)

The cytotoxicity of isatin was evaluated using the MRC-5 cell line derived from normal lung tissue by the neutral red uptake (NRU) method [36] according to OECD (Organization for Economic Co-operation and Development) Guide 129 (Guidance document on using

cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests), with modification [37]. For the experiments, normal MRC-5 cells were used, and the exposure times for the evaluation were 24, 48, and 72 h. Three experiments were carried out with six replicates per dilution. The isatin concentrations used ranged from 8 to 1024 μg/mL. The positive control was a solution of sodium dodecyl sulfate (SDS) dissolved in 0.5% phosphate-buffered saline (PBS). The SDS concentrations were 100 μg/mL, 68.02 μg/mL, 46.28 μg/mL, 31.48 μg/mL, 21.41 μg/mL, 14.57 μg/mL, 9.91 μg/mL, and 6.74 μg/mL. The experiments were carried out with six replicates per dilution. The negative control was the basal medium. Neutral red (25 μg/mL) was added to the plate and the plates were incubated at 37 °C for 3 h under 5% CO_2 conditions. For elution, 100 μL of eluent (50% ethanol, 1% acetic acid, and 49% water) was added per well and allowed to sit for 20–45 min in the dark. The absorbance reading was performed at 540 nm on a Versamax® microplate reader from Molecular Devices (San Jose, CA, USA). Graph Pad Prism 10 (GraphPad Software, La Jolla, CA, USA) was used for the calculations.

2.6. Statistical Analysis

All experiments were performed with a minimum of three replicates. The statistical analysis was executed using Graph Pad Prism. The results of the MIC and MBC assessments for isatin were the averages of three independent replicates and considered significant with a standard deviation <5%. Statistical analysis for IC_{50} values was performed using ANOVA. $p < 0.001$ was considered statistically significant.

3. Results and Discussion

The significance of *Campylobacter* as a major enteric pathogen with a high risk of antibiotic resistance has increased the need to develop new and alternative strategies to combat infections caused by antibiotic-resistant *Campylobacter* [38]. Concerned with the rising rates of resistance to fluoroquinolone (e.g., ciprofloxacin) and macrolide (e.g., erythromycin) in thermophilic *Campylobacters*, many recent studies have evaluated natural and synthetic products; however, the search for new alternatives must be continued.

We were interested in the effectiveness of the heterocyclic compound isatin against antimicrobial-resistant *C. jejuni* and *C coli* strains from different sources and years of isolation and from two states of Brazil. The species *C. jejuni* and *C. coli* were selected because they cause most cases of *Campylobacter* infection [4]. In this study, four *Campylobacter* strains that were sensitive to antimicrobials and one *C jejuni* ATCC strain were assessed and used for comparison with the results of antimicrobial-resistant strains.

According to data in the literature, the antimicrobial activity of isatin and its derivatives was previously tested in various pathogenic bacteria, such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhi*, and isatin showed moderate activity in these bacteria [25]. Other isatin derivatives have also been screened for their antibacterial activities against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus cereus*, and *Enterococcus faecalis*, and some of them have demonstrated promising potency in vitro and in vivo [24,39]. Despite advances in developing isatin derivatives as potential antibacterial agents, no antimicrobial activities have been previously reported for *Campylobacter* spp.

Initially, to determine the MIC values, culture was carried out in 96-well microplates without isatin, and satisfactory growth of *Campylobacter jejuni* ATCC 33560 was verified in all wells incubated at 37 °C under a microaerophilic atmosphere for 24 h and 48 h, and no significant difference in readings was observed. Therefore, the incubation time was set to 24 h. Thus, the antimicrobial activity of isatin against 29 *C. jejuni* and *C. coli* strains from foodborne, animal, and human samples was examined in the present study and their potency was assessed by MIC (minimum inhibitory concentration) and MBC (minimal bactericidal concentration); the results are shown in Table 1.

The MIC values of isatin were between <1.0 and 16.0 μg/mL. The highest MIC values were 8.0 μg/mL (76%), followed by 16.0 μg/mL (17%) and <1.0 μg/mL (7%). Inoculation

of the ATCC strain of *C. jejuni* (ATCC 33560) in all MIC studies allowed us to evaluate the antimicrobial agent's stability and the reproducibility of the method; identical results were observed in all tests. The MIC for the ATCC strain was 8.0 μg/mL.

When comparing the high MIC values of antibiotics using different *Campylobacter* isolates of several countries for ciprofloxacin resistance ($\geq$32 μg/L) and for levofloxacin resistance (in the range of 6 μg/L–32 μg/L) and also erythromycin and azithromycin resistance ($\geq$256 μg/L) [40] with the MIC values obtained in this work for isatin (8 μg/L and 16 μg/L) for ciprofloxacin-resistant, erythromycin-resistant, and tetracycline-resistant strains, and also a smaller percentage with MIC < 1 μg/L,′ we can observe very promising values for isatin.

In addition, several investigations using isatin and its derivatives have also shown similar results of low MIC values in gram-positive and gram-negative bacteria. Lian and collaborators investigated seven isatin derivatives, and two of these compounds showed MIC values of 0.03–0.05 μmol/mL against *S. aureus* and MICs of 0.672 μmol/mL and 0.830 μmol/mL against *Escherichia coli* and *Pseudomonas aeruginosa*, respectively [29]. A minireview reported the recent advances of four fluoroquinolone–isatin hybrids, evaluating their antibacterial activities against *Salmonella typhi*, *E. coli*, *Vibrio cholera*, *S. aureus*, *S. epidermidis*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Shigella flexnari*, and *Citrobacter freundi,* and their MIC values ranging from 0.10 to 2.50 mg/mL were also considered to be low concentrations against the nine strains tested [41].

Regarding the species *C. coli* (10 strains) and *C. jejuni* (12 strains), similar susceptibilities to isatin were observed, with MIC values of 8.0 μg/mL. However, two *C. coli* strains of animal origin from the state of Rio de Janeiro isolated in 2007 showed MICs < 1.0 μg/mL, and five strains of *C. jejuni*, one of human origin and four of foodborne origin from the states of Rio de Janeiro and Rio Grande do Sul isolated in 2011 and 2014, respectively, showed MICs of 16.0 μg/mL (Table 1). In addition, four strains that were not resistant to antibiotics (CCAMP 1051, CCAMP 1052, CCAMP 1057, and CCAMP 1064) presented similar susceptibilities, with MIC values of 8.0 μg/mL, in contrast to strains resistant to antibiotics (Table 1). Therefore, based on the MIC results, isatin was effective in both nonresistant strains and those with resistance to antibiotics (ciprofloxacin: CIP, tetracycline: TT, and erythromycin: ERI).

The minimal bactericidal concentration (MBC) was determined to be 16.0 μg/mL for 72%, 8 μg/mL for 20%, and 1 μg/mL for 8% of the *Campylobacter* strains tested. The MBC value for the ATCC strain was also 16.0 μg/mL (Table 1). The strains that presented with MBC values <1.0 μg/mL were the same ones that presented with MIC values <1.0 μg/mL. Six strains presented with MBC values of 8.0 μg/mL; five strains were of *C. coli*, two of animal origin and three of foodborne origin, all from the Rio de Janeiro state and isolated in 2007 and 2010, respectively; and one strain was *C jejuni* of foodborne origin isolated in 2014 from the state of Rio Grande do Sul (Table 1). A concentration of 16.0 μg/mL was sufficient to kill the tested bacteria.

Considering concerns about antibiotic resistance, our findings of MIC/MBC with isatin are very important, particularly with antibiotic-resistant strains. Similar investigations have shown the potential of isatin and its derivatives to take the lead in the treatment and drug development against drug-resistant pathogenic bacterial. Recently, a study with isatin–pyrimidine hybrids exhibited effective activity on MDR- and XDR-resistant *Mycobacterium tuberculosis* strains, and the experiments showed low MIC values of 0.48 and 3.9 μM, respectively [32]. Singh and collaborators investigated thymol–isatin hybrid activity against resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA). The results showed low concentrations of MIC = 1.9 μM and MBC = 3.9 μM, that they were also capable of inhibiting biofilm formation, and that they decreased virulence factor staphyloxanthin production [33].

Based on the promising MIC and MBC values of isatin against *Campylobacter*, the mean inhibitory concentration of isatin (IC_{50}) in MRC-5 cells was evaluated to determine the level of cytotoxicity. Assays were performed using the neutral red uptake (NRU) method

with modification. In this method, neutral red dye uptake activity by lysosomes of viable cells is measured.

Cell morphology was assessed, and the cell control remained intact at the end of the observation period (Figure 1A,B). There was a marked decrease in cell density and a change in cell morphology at the highest concentrations of isatin, which were 1024 μg/mL (Figure 1C,D) and 512 μg/mL; with a concentration of 256 μg/mL (Figure 1E,F), there was a gradual increase in cell density and a normalization of cell morphology, whereas at a concentration of 8 μg/mL (Figure 1G,H), no difference was noted compared to control cells.
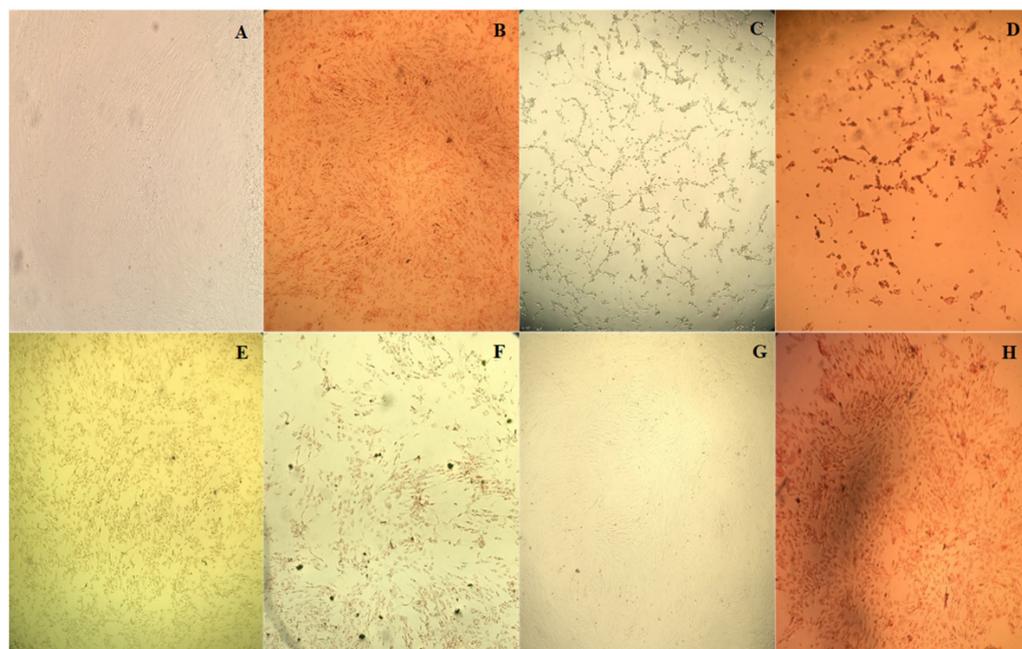
Figure 1. Cellular morphology after 48 h exposure. (**A**): Control of intact cells without staining; (**B**): control of intact cells stained with neutral red; (**C**): cells without staining and exposed to isatin 1024 μg/mL; (**D**): stained with neutral red and exposed to isatin 1024 μg/mL; (**E**): no staining and exposed to isatin 256 μg/mL; (**F**): stained with neutral red and exposed to isatin 256 μg/mL; (**G**): no coloring and exposed to isatin 8 μg/mL; (**H**): stained with neutral red and exposed to isatin 8 μg/mL. 40× magnification.

The IC_{50} values for isatin were calculated and the Grubbs method was applied to check for discrepant values; no IC_{50} result was considered an outlier. For an exposure time of 48 h, as is standardized in the OECD guide 129 [35], the IC_{50} value for isatin was 125.63 μg/mL. When considered in relation to the highest MIC value, 16 μg/mL, these data suggest that isatin can be considered a safe substance in terms of cytotoxicity. Isatin demonstrated low toxicity in normal cell cultures. IC_{50} values in normal human cells for isatin derivatives, despite different lineages, indicate values like those obtained in this work. Isatin derivatives with more complex structures, such as the hydrazide-hydrazone class, showed an IC_{50} value >100 μM in the HEK-293 cell line of normal human embryonic kidney cells [42]. Isatin–thiadiazoline hybrids assayed with WI-38 normal human lung cell line showed IC_{50} in the range of 64.62 to >100 μM [43], and a series of multi-substituted isatin was tested with normal human renal epithelial cells (2937) and normal human umbilical vein endothelial cells (UVEC), obtaining IC_{50} > 100 μM and 61.83 μM, respectively [44].

The cytotoxicity value of isatin (IC_{50} = 125.63 μg/mL for MRC-5 cell line derived from normal human lung tissue) was higher than that of ciprofloxacin; that is, with IC_{50} = 23.89 μg/mL (72.1 mM for RWPE-1 cell line from normal human epithelial prostate cells) [45], this indicates that isatin is less toxic despite the different cell lines assayed.

The initial assessment of pharmacokinetic properties (absorption, solubility, skin permeation, P-gp substrate, and CYP450 inhibition) of isatin was obtained by the free online program SwissADEME [46]. Lipinski set out a rule of five to determine the likeliness of the use of a compound, enabling a maximum of one violation [47]. Isatin did not show any violation of Lipinski's rule, as it was not found to have any rotatable bonds but had two hydrogen bond acceptors, one hydrogen bond donor, a topological polar surface area as recommended (46.17 Å^2), and $\log P_{o/w} = 0.72$ (consensus). In addition, isatin showed high gastrointestinal adsorption of $\log S = -1.38$ and was therefore of soluble class (<-2 to <0 very soluble); it also had $\log K_p = -6.61$ cm/s related to skin permeation. Furthermore, this study indicated that isatin showed no inhibition to CYP450 and no substrate of P-gp. Concerning synthetic accessibility, a value of 1.34 was obtained, being very easy (very easy is 1 and very difficult is 10) [46].

Success stories of natural product derivatives of medicinal plants or synthetic compounds have been investigated in several studies as multidrug-resistant modulators in bacteria by inhibiting or otherwise stopping their growth, according to a review by Zhai and collaborators [48]; for *Campylobacter*, we can highlight a review by Salimikia and collaborators that described active compounds of medicinal plants that are nature-based antibiotic agents that could be effective against *Campylobacter* [49]. However, there is an urgent need to coordinate efforts for meaningful research. Comparison of the antimicrobial activity of isatin in *Campylobacter* is difficult, especially without any related reports. However, considering the low MIC and MBC concentrations of isatin against *Campylobacter* cells, we can see its great potential.

Our study may be the first report on the anti-campylobacter activity of isatin against MDR *Campylobacter*. We hope that our results will provide a starting point for further investigations of this compound to exploit new antimicrobial agents that are effective and safe for *Campylobacter* treatment.

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