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Article

Synthesis and Anticancer Activity of Homodimeric Morita-Baylis-Hillman Adducts Based on 3-Hydroxyindolin-2-one Core

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Cancer treatment represents one of the main scientific study targets today, mainly due to the pronounced side effects arising from chemotherapy. This study reports the synthesis, characterization, and anticancer activity of ten compounds from the Morita-Baylis-Hillman reaction. Ethylene glycol diacrylate was used as a double Michael acceptor in reactions with isatin derivatives to give homodimers of 3-hydroxyindolin-2-one core, recognized in the literature for its extensive pharmacological profile. The use of 1,4-diazabicyclo[2,2,2]octane (DABCO) as a catalyst and room temperature were the optimal conditions for the study reaction. The isolated yields were up to 63%, with most reaction times inferior to 24 h, some as fast as 15 min. The anticancer potential of the synthesized dimers was evaluated *in vitro* against three cancer strains, resulting in average inhibitory concentrations up to 0.72 μM. It was also found that the best performing homodimers are more active than their monomeric counterparts. Considering the promising selectivity indices observed, the preliminary results obtained here act as a basis for broader tests regarding the effectiveness of homodimeric adducts against cancer cells.

Keywords: MBHR, C–C bond formation, 3-hydroxyindolin-2-one, molecular symmetry, isatin, homodimer

Introduction

The development of methodologies aimed at the treatment and consequent cure of cancer is a huge scientific challenge because of the similarities between cancerous and normal cells in the body, making the selectivity of drugs quite difficult.[1] The complexity of this pathology requires combining therapies among the alternatives available today including chemotherapy, that is, the administration of drugs called chemotherapeutics in order to minimize the rampant multiplication of cancer cells or even destroy them.[2] As with other drug-based interventions, the main obstacles to cancer chemotherapy are pronounced side effects and the development of molecular resistance to commercially available drugs.[3] Such inconveniences imply a constant search for new candidates for anticancer agents.

Isatin is one of the main indole derivatives that has been widely explored in Medicinal Chemistry, given its low production cost and synthetic versatility with regard to the presence of different reactivity sites.[4] From a biological perspective, the importance of isatin reflects the pharmacological profile exhibited by some of its derivatives, including antibacterial, antiviral, antiprotozoal, anti-inflammatory, antioxidant, and anticonvulsant activities.[5,6] The discovery of the antineoplastic potential of several natural products containing a 2-oxindole core, such as maremycin B and paratunamide D (Figures 1a and 1b), has been an important starting point for the molecular design of new drugs based on derived analogs of isatin aiming antitumor evaluation.[7] Sunitinib is a multi-targeted receptor tyrosine kinase inhibitor based on the 2-oxindole moiety

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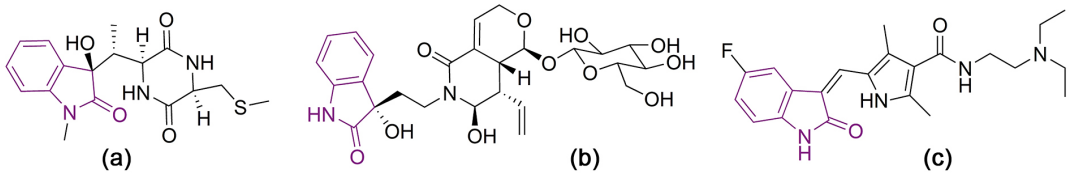


Figure 1. Anticancer compounds endowed with the 2-oxindole moiety: (a) maremycin B; (b) paratunamide D and (c) sunitinib.

(Figure 1c), approved for use in the treatment of several types of cancer, as it exhibits potent anti-angiogenesis and anti-tumor growth activities.[8]

Morita-Baylis-Hillman reaction (MBHR) of substituted isatins yielded oxindole-based compounds with cytotoxic activity in appreciable levels of selectivity against leukemia cells (HL-60)[9] and lung cancer cells (NCI-H292).[10] MBHR is an important synthetic methodology in the formation of C–C bonds between the α position of activated alkenes and the electrophilic carbon of aldehydes, ketones or imines. The reaction occurs in the presence of organocatalysts, such as phosphines or tertiary amines, leading to the corresponding Morita-Baylis-Hillman adducts (MBHA).[11-13] Among the advantages of MBHR over other C–C bond-forming reactions are the atom economy, mild temperature conditions, and metal-free catalysts. In addition, many of these reactions may be conducted in an aqueous medium[14] or even in solvent-free conditions.[15,16]

Homodimerization of MBHA as well as other bioactive molecules is one of the most relevant techniques among various strategies to potentialize pharmacological activity. The homodimerization of molecules has as its main objective the production of more selective and/or potent drugs than their monomeric units, exhibiting the same or even different selectivity profiles and pharmacokinetic properties.[17] The dimers may show a higher affinity for the same receptor as the monomer or act on a different receptor.[17,18]

da Silva *et al.*,[19] for example, describe the synthesis of MBHA homodimers as well as their evaluation against *Leishmania donovani*. These dimers proved to be more potent compared to the respective monomeric adducts, reaching an activity level 400 times higher than that presented by the base monomer, with no toxicity towards human red blood cells compared to amphotericin B. Promising results in terms of anticancer activity have also been reported for isatin-based dimer compounds tethered via different positions.[20] Based on the antineoplastic potential of homodimeric isatin derivatives previously reported, Attia *et al.*[21] evaluated the activity of bis-isatins condensed as hydrazones against colon (HT-29), breast (ZR-75) and lung (A-549). The authors found a pronounced effect against the cells tested, with average percentages of inhibition higher than those obtained by the reference drug (Sunitinib). Following the same trend, Chen *et al.*[22] also described the design and biological activity evaluation of bis-isatin derivatives as potent dimeric DJ-1 inhibitors, a target of pivotal role in tumorigenesis and cancer progression.

Inspired by literature reports, which demonstrate the feasibility of using twin drugs in the development of new anticancer agents, this paper presents the synthesis and exploration of the anticancer potential of new dimers of isatin derivatives based on MBHR, using an ethylene diacrylate compound as a double Michael acceptor and, consequently, as a homodimeric spacer. Some of the molecules shown here exhibit excellent inhibitory effects against cancer cells, being relatively more active than their respective monomers.

Results and Discussion

Synthesis of homodimeric MBHA

The intermediates *N*-alkyl isatin derivatives (**1a-1j**) for MBHRs were prepared from alkylation reactions of isatin or substituted isatins (5-chloroisatin, 5,7-dichloroisatin and 5-nitroisatin)[23,24] with the respective alkyl halides, according to a literature procedure (see Supplementary Information section).[25] Ethylene glycol diacrylate was obtained by esterification reaction between ethylene glycol and acrylic acid, using *p*-toluenesulfonic acid as catalyst.[19]

Preliminary optimization assays for the double MBHR involving *N*-methyl isatin and the dimeric Michael acceptor ethylene glycol diacrylate to give a homodimeric MBHA (**2a**) were carried out (Scheme 1 and Table 1). The reaction progress was followed by thin layer chromatography (TLC). In all cases, conversion to the homodimeric product was not complete, and starting materials as well as the presence of mono MBHA from this reaction were detected, being separated from the desired product by flash column chromatography. The best conditions we found involved the use of the molar equivalent of 1,4-diazabicyclo [2,2,2]octane (DABCO) in *N,N*-dimethyl-formamide (DMF) and room temperature.

DABCO is a base catalyst well-established for MBHR involving isatin.[26-28] At first, the proportion of DABCO was fixed at 50 mol% in relation to the Michael acceptor, and the

Table 1. Optimization of the reaction conditions for obtaining the homodimeric MBHA **2a**

entry	Catalyst	Amount of catalyst / mol%	Solvent	Temperature / ºC	time	Yield[a] / %
1	DABCO	50	DMF	r.t.	5 days	34
2	DABCO	50	DMF	80	1 h	trace
3	DABCO	50	DMF	100	1 h	trace
4	DABCO	50	DMF	120	1 h	trace[b]
5	DABCO Cu/Mn-IDA	50	DMF	r.t.	3 days	20
6	DABCO	50	[Bmim]BF_4	r.t.	5 days	24
7	DABCO	100	DMF	r.t.	11 days	43
8	DBU	100	DMF	r.t.	3 days	28
9	DABCO	50	ChCl/EG	r.t.	1 day	trace
10	DABCO	100	ChCl/EG	r.t.	1 day	trace

[a]Isolated yield; [b]thermal decomposition of reagents observed by TLC; DABCO: 1,4-diazabicyclo[2,2,2]octane; Cu/Mn-IDA: Cu/Mn iminodiacetate; [Bmim]BF_4: 1-butyl-3-methylimidazolium tetrafluoroborate; DBU: 1,8-diazabicyclo[5,4,0]undec-7-ene; ChCl/EG: DES choline chloride/ethylene glycol; r.t: room temperature; DMF: *N,N*-dimethyl-formamide.

reaction temperature was varied. The reaction yielded 34% of the isolated product after 5 days of reaction when carried out at room temperature in DMF (Table 1, entry 1). The rise in the reaction temperature to 80, 100, and 120 ºC (Table 1, entries 2, 3, and 4, respectively), by microwave irradiation as a heating source, was ineffective in improving the yield. On the contrary, the reactions with high temperatures gave only traces of the product, detected by TLC, and some thermal decomposition of the reaction components was also observed after irradiation time of 1 h at 120 ºC.

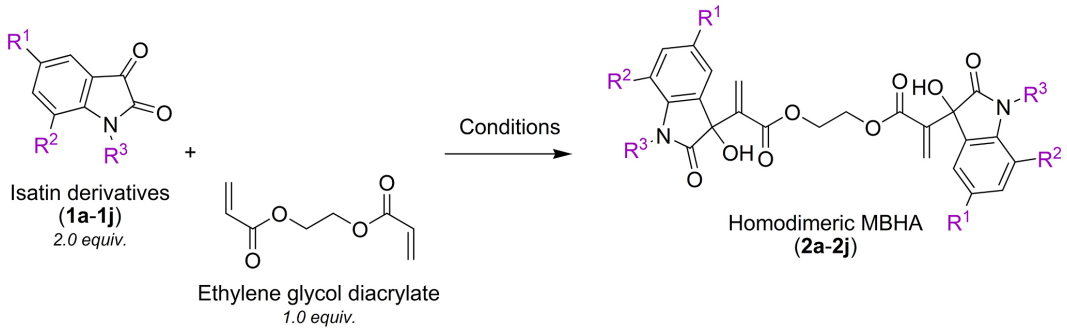
Based on recently published work with satisfactory results for MBHR between aromatic aldehydes and methyl acrylate,[29] a coordination polymer Cu/Mn-IDA (Cu/Mn iminodiacetate) was explored as a reaction co-catalyst, conducting the reaction at room temperature in DMF. Unfortunately, after 3 days the reaction yielded only 20% of the isolated product (Table 1, entry 5). Replacement of the DMF solvent with 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF_4) was also tested, considering the positive impact of imidazolic and tetrafluoroborate ionic liquids on some reported MBHRs.[30-32] The use of an ionic liquid did not show any improvement in yield or reaction time compared to the reaction in DMF (Table 1, entry 6).

In another try, the mass of DABCO in DMF was increased to an equimolar amount related to the Michael acceptor. This change raised the reaction yield to 43%, however, with an increase in reaction time to 11 days (Table 1, entry 7). In addition, the replacement of DABCO by 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) did not result in significant improvements for the reaction (Table 1, entry 8). A deep eutectic solvent (DES) composed of choline chloride and ethylene glycol (ChCl/EG 1:2) was also evaluated in the studied reaction, based on literature reports regarding the co-catalytic potential of DES in MBHR involving acrylonitrile and acrylic ester as Michel acceptors.[33,34] In the tests presented here, only traces of the desired product were formed (entries 9 and 10) using different ratios of DABCO.

The homodimeric MBHA **2a-2j** were synthesized according to the Scheme 1. Table 2 shows the results for the reaction times and isolated yields. The MBHAs were obtained in yields ranging from 24 to 63%, due to the formation of a co-product of the reaction. In all reactions, the dimeric adduct showed greater polarity than this co-product, as verified by TLC. For some MBHRs the reaction time was about hours or even minutes. In those reactions, there was a total conversion of the reagents. On the other hand, for reactions with a longer time than 1 day, conversion to products was not complete and the reactions were finished when no significant changes in TLC were detected.

MBHRs involving unsubstituted *N*-alkyl isatins (**1a-1c**) were significantly slower compared to the reactions of chlorinated or nitrated ones. The electron-withdrawing nature of these substituents on the aromatic ring increases the power of the carbon electrophile at keto carbonyl of isatin. The synthesis of adduct **2f** from 5,7-dichloro-1-methylindoline-2,3-dione was remarkably short, about 15 min, and gave the best yield (63%). The positive impact of electron-withdrawing substituents on reaction time was also observed for MBHA from aromatic aldehydes.[19] Authors[19] described that MBHRs using nitro-substituted benzaldehydes were about 20 times faster than non-substituted ones.

From our results, the influence of the alkyl group bonded to the nitrogen heterocycle was more evident in reactions from 5-nitro and 5,7-dichloro substituted isatins



Scheme 1. General reaction for obtaining the homodimeric MBHAs. For optimization of the reaction conditions see Table 1; for **2a**-**2j**, DABCO (1 eq.), DMF, rt.

Table 2. Obtaining homodimeric MBHA from ethylene glycol diacrylate and isatin derivatives[a]

MBHA	Substituent			time	Yield[a] / %
	R^1	R^2	R^3		
2a	H	H	methyl	11 days	43
2b	H	H	allyl	3 days	30
2c	H	H	benzyl	4 days	40
2d	Cl	H	methyl	3.5 h	52
2e	Cl	H	allyl	3 h	37
2f	Cl	Cl	methyl	15 min	63
2g	Cl	Cl	allyl	1 h	25
2h	Cl	Cl	benzyl	30 min	24
2i	NO_2	H	methyl	23 h	59
2j	NO_2	H	allyl	1 h	56

[a]Isolated yield. MBHA: Morita-Baylis-Hillman adducts.

rather than from the 5-choro monosubstituted isatin. For **2f**, **2g** and **2h** the increasing size of the alkyl group gave poor reaction yields. On the other hand, nitro substituted MBHA **2i** and **2j** were obtained in similar yields (the best yields among all synthesized adducts, 59 and 56% respectively), but with very different reaction times. The MHBR with methyl substituent was about 20 times slower than the reaction with allyl substituent. Based only on our experiments, it is not possible to explain entirely the reason for those differences, and it is feasible that electronic effects as well as the size of the substituent may be involved.

An experiment using commercial isatin as a substrate (without an *N*-substituent) were also performed, but the reaction did not lead to the desired product. In a basic medium (DABCO) acid amidic hydrogen abstraction can generate a good nucleophile for Michael addition, resulting in side-reactions.

The chemical structures of all synthesized MBHAs were fully characterized by Fourier transform infrared (FTIR) spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy spectra and high-resolution mass spectrometry (HRMS) (Figures S1-S46, see Supplementary Information section). The spectroscopic data obtained from FTIR demonstrate the conversion of the isatin derivatives to the expected products from the appearance of a band at approximately 3300 cm^{-1} referring to the stretching vibrations of the hydroxyl group formed from the MBHR. In addition, ^{1}H NMR spectra show two doublets close to 6.50 ppm due to vinylic protons, and a resonance at around 4.00 ppm due to methylene hydrogens of the spacer chain. ^{13}C NMR spectra of the homodimers exhibited shifts corresponding to the carbinolic carbons (δ ca. 75 ppm), and the methylene portion of the spacer (δ ca. 62 ppm). The carbon atoms of the C=C vinyl bond are also preserved at 140.9 and 128.5 ppm. All the products were obtained as a mixture of meso and stereoisomer, due to the two asymmetrical centers in the symmetrical molecule. This is evidenced by expanding the ^{13}C NMR spectra, as shown in the Supplementary Information section (Figures S7, S16, S21, S39, S44).

In silico analysis

In silico screening tools in the exploitation of the properties of a drug candidate may provide relevant information on parameters related to pharmacokinetic and pharmacodynamic behavior and toxicological effects (absorption, distribution, metabolization, excretion and toxicity (ADMET)). Thus, it is possible to select compounds with suitable properties and avoid compounds with undesirable ones.[35] Lipinski's rule is one of the main approaches to evaluate the properties obtained via *in silico* testing.[36] It considers some physicochemical criteria that the compounds must obey for better absorption and distribution in the human organism. This analysis may also predict whether drugs are metabolically stable.

Based on this, the prediction of the ADME parameters and some physicochemical descriptors of the homodimeric compounds **2a-2j** was performed, as part of a preliminary study of their pharmacokinetics and pharmacodynamics (Table 3). Lipinski's parameters of molecular weight (MW), number of acceptors (HBA) and hydrogen donors

Table 3. Lipinski's parameters, molar refractivity and TPSA of each homodimeric compound predicted via SwissADME

Compound	Lipinski's parameter					Molar refractivity	TPSA
	MW / (g mol^{-1})	HBA	HBD	Log P	nViol		
2a	492.48	8	2	1.78	0	133.33	133.68
2b	544.55	8	2	2.73	1	151.61	133.68
2c	644.67	8	2	4.06	1	182.30	133.68
2d	561.37	8	2	2.80	1	143.35	133.68
2e	613.44	8	2	3.78	1	161.63	133.68
2f	630.26	8	2	3.86	1	153.37	133.68
2g	682.33	8	2	4.79	1	171.65	133.68
2h	682.33	8	2	6.16	2	171.65	133.68
2i	582.47	12	2	0.29	2	150.97	225.32
2j	634.55	12	2	1.31	2	169.25	225.32

MW: molecular weight; HBA: hydrogen bond acceptor; HBD: hydrogen bond donor; Log P: octanol/water partition coefficient; nViol: number of violations; TPSA: topological polar surface area.

(HBD), lipophilicity (Log P) were calculated using the SwissADME software.[37,38] The molar refractivity and topological polar surface area (TPSA) were also evaluated using this same software.

The results given in Table 3 show that most homodimers violate at least one of the parameters of Lipinski's rule. The molecular weight is relevant for predicting solubility and consequently the drug's ability to cross cell membranes. For this parameter, all MBHAs are above 500 g mol^{-1}, except for **2a** (molecular weight of 492.48 g mol^{-1}).

The polarity of drug candidates may be related to their ability to form hydrogen bonds; therefore, it is explored in terms of the presence of HBA and HBD in the structure. These bonds are broken when the molecule goes from a hydrophilic to a hydrophobic medium. Lipinski's Rule limits the numbers of HBA and HBD to 10 and 5 atoms, respectively, since too many hydrogen bonds hinder the penetration of the drug through the lipid bilayer of cell membranes.[36] None of the evaluated compounds violated the HBD number. On the other hand, **2i** and **2j** exceeded the HBA limit established by the rule, due to the poor solubility nature known for nitroaromatic compounds.

Lipophilicity (Log P) is the result of a theoretical octanol/water partition coefficient. Compounds with Log P > 5 exhibit difficulties to access the membrane surface, due to low solubility in aqueous solutions.[39] Log P value between 0 and 3 indicates an effective gastrointestinal absorption after oral drug administration.[40] MBHAs **2a**, **2b**, **2d**, **2i**, and **2j** showed Log P in this range of 0 to 3, and indeed most of the compounds showed lipophilicity below the value established by Lipinski (Log P < 5). **2h** was the only compound presenting a Log P larger than 5. It may be due to the presence of the benzyl group, which significantly reduces its polarity.

As well as lipophilicity, molar refraction (MR) is a factor of great influence in the binding of a drug to the receptor site, being a fragmental constant that quantitatively represents the dispersive interactions involved in this arrangement.[41] All compounds showed values above the reference standard established in the literature (40 ≤ MR ≤ 130).[41] The topological polar surface area (TPSA) expresses the molecular surface area resulting from the summation of the surface contributions of the 2D polar fragments (O or N atoms and H atoms attached to them).[42,43] TPSA values ≤ 140 Å^2 indicate oral bioavailability.[44] All compounds showed TPSA within this range of oral bioavailability values (TPSA of 133.68 Å^2). The exceptions were the nitro-substituted **2i** and **2j** (TPSA of 225.32 Å^2).

In vitro anticancer assays

MBHAs **2a-2j** were tested for anticancer activity against three specific strains: A-549 (epithelial-like lung carcinoma), HL-60 (promyelocytic leukemia), and K-562 (chronic myeloid leukemia). Preliminary screening for the susceptibility of tumor cells to the final compounds was performed at a single concentration of 25 μg L^{-1}. The results are shown in Table 4. Except for **2a**, all other compounds exhibited good activity with percentages of inhibition predominantly higher than 80%.

The average inhibitory concentrations (IC_{50}) for selected compounds **2b-2j** against the same cell lines are summarized in Table 5. IC_{50} assays provided results ranging between 0.72 and 11.35 μM, which were classified according to the following criteria: $IC_{50} \leq 3$ μM (high activity); $3 < IC_{50} \leq 6$ μM (good activity); $6 < IC_{50} \leq 12$ μM (moderate activity); $12 < IC_{50} \leq 24$ μM (low activity); and $IC_{50} > 24$ μM (inactive compound).[45] The studied MBHAs

Table 4. Tumor cell growth inhibition of compounds at a single concentration (25 μg L^{-1})

Compound	Inhibition (± SD) / %		
	A-549	HL-60	K-562
2a	31.5 ± 12.5	22.1 ± 11.9	59.5 ± 17.1
2b	90.1 ± 5.2	87.9 ± 3.0	89.5 ± 5.7
2c	89.6 ± 5.4	90.2 ± 4.9	91.4 ± 4.5
2d	87.1 ± 7.3	88.8 ± 3.2	86.8 ± 6.6
2e	90.9 ± 4.9	81.1 ± 8.2	87.5 ± 6.3
2f	92.5 ± 4.1	85.7 ± 7.2	89.8 ± 5.4
2g	90.9 ± 4.8	94.9 ± 2.4	91.4 ± 4.6
2h	92.2 ± 4.1	88.3 ± 7.9	92.9 ± 3.8
2i	46.7 ± 14.4	61.3 ± 13.8	87.9 ± 6.2
2j	89.5 ± 5.5	75.7 ± 4.1	87.2 ± 6.4
Doxorubicin	46.5 ± 15.0[a]	90.1 ± 8.9[b]	90.8 ± 8.4[a]

[a]Doxorubicin 5 μg mL^{-1}; [b]doxorubicin 10 μg mL^{-1}. SD: standard deviation.

showed predominantly good and high activities, especially against the HL-60 strain. Using MRC-5 (fibroblasts, originally developed from the lung tissue) as a control strain for the selectivity of the evaluated compounds, satisfactory results were observed against A-549 by the compounds **2c** and **2f**, which combine lower IC_{50} with selectivity indexes (SI) greater than 1. From this same perspective, compounds **2f**, **2h** and **2j** presented the best results against HL-60, especially compound **2j**, which reached a selectivity index of 7.29. Compound **2f** also showed promising activity against the K-562 strain, approaching in SI the reference compound, doxorubicin.

Comparing compounds by substituent groups in the aromatic moiety (Figure 2) it is verified that, in general, 5,7-dichlorinated MBHAs (**2f**-**2h**) presented the lowest IC_{50} values, except for **2g** against the cell line A-549. The benzylated compound **2h** stood out with the lowest ones, but the methylated compound **2f** obtained the best selectivity index. Grouping the molecules by *N*-alkyl substituent, the benzylated ones (**2c** and **2h**) are highlighted in all cell lines, with the lowest IC_{50} values. In addition, the allylated compounds (**2b**, **2e**, **2g** and **2j**) showed greater selectivity, especially against the HL-60 cell line.

A direct comparison of biological activities between monomers and homodimers against HL-60 was performed. For this, three homodimeric MBHAs from this work and three monomeric analogues of MBHA previously synthesized in our research group were used.[10] The chemical structures and results are given in Table 6. Structurally, **3b**, **3f** and **3j** correspond to the monomeric equivalents of the dimers **2b**, **2f** and **2j**. The comparative analysis of these IC_{50} values showed that, despite being active against HL-60, the monomers were less potent than their respective dimers, which, on the other hand, showed an average activity

Table 5. Inhibitory concentration (IC_{50}) with confidence intervals, and selectivity index against tumor cells (SI) of synthesized compounds[a]

Compound	MRC-5[b] IC_{50} / μM	A-549		HL-60		K-562	
		IC_{50} / μM	SI	IC_{50} / μM	SI	IC_{50} / μM	SI
2b	21.11 (12.77-34.71)	8.54 (7.21-10.07)	2.47	4.52 (3.57-5.69)	4.67	11.35 (8.48-15.12)	1.86
2c	8.80 (4.99-15.47)	3.75 (2.85-4.89)	2.35	3.95 (2.52-6.17)	2.23	5.27 (3.51-7.87)	1.67
2d	9.34 (5.03-17.26)	9.64 (7.60-11.99)	0.97	5.31 (3.04-9.25)	1.76	7.87 (6.35-9.70)	1.19
2e	4.48 (3.28-6.07)	8.83 (6.51-11.90)	0.51	4.39 (2.97-6.48)	1.02	10.35 (8.66-12.30)	0.43
2f	6.91 (4.74-10.03)	3.20 (2.55-4.01)	2.16	1.18 (0.96-1.46)	5.84	3.83 (3.04-4.80)	1.80
2g	3.49 (3.09-3.94)	10.42 (8.85-12.21)	0.33	2.47 (1.70-3.57)	1.41	4.89 (3.64-6.53)	0.71
2h	1.35 (0.61-2.90)	2.24 (1.72-2.90)	0.60	0.72 (0.44-1.18)	1.87	1.65 (1.37-1.97)	0.82
2i	36.13 (28.88-45.21)	–[c]	–	7.94 (5.27-11.92)	4.55	6.08 (3.45-10-63)	5.95
2j	16.31 (7.60-34.79)	7.39 (5.90-9.21)	2.21	2.24 (1.83-2.72)	7.29	7.93 (5.11-12.25)	2.06
Doxorubicin	6.09 (3.49-10.45)	1.24 (0.74-2.07)	4.91	0.43 (0.32-0.58)	14.16	2.71 (1.95- 3.77)	2.25

[a]IC_{50} values obtained by non-linear regression; data represent the means of three independent experiments, with each concentration tested in triplicate; [b]non tumorigenic cell line; [c]IC_{50} > 25 μg L^{-1}. NT: not tested. MRC-5: fibroblasts; A-549: epithelial-like lung carcinoma; HL-60: promyelocytic leukemia; K-562: chronic myeloid leukemia.

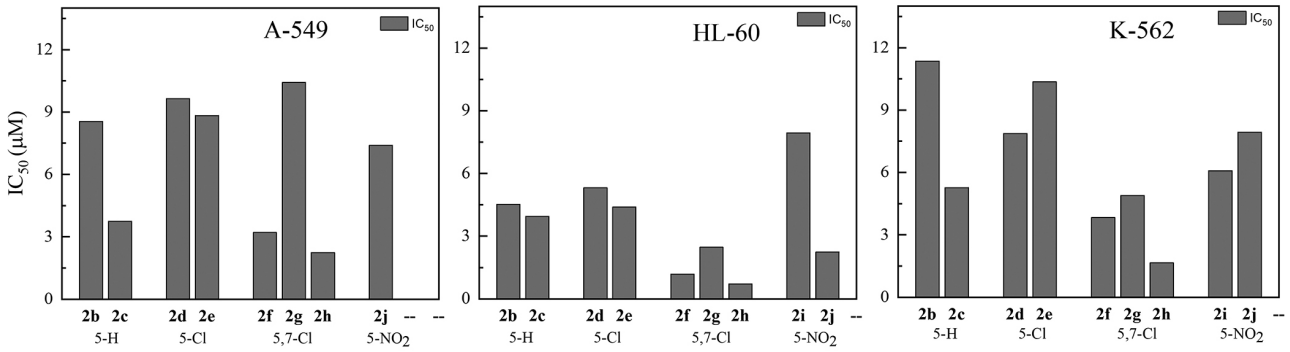
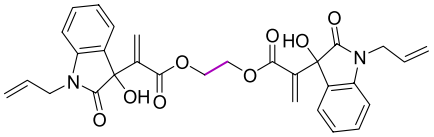
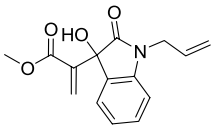
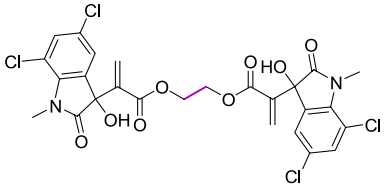
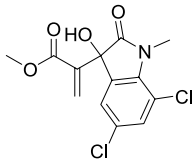
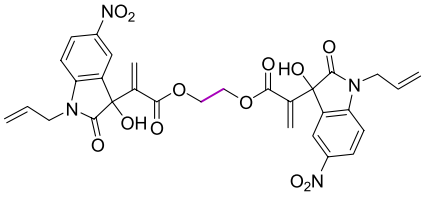
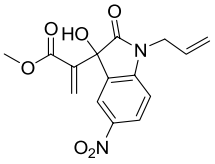


Figure 2. Inhibitory concentration (IC_{50}) of homodimeric MBHAs against A-549, HL-60 and K-562 tumor cells.

Table 6. Comparison between the IC_{50} of homodimers and their respective monomers against the HL-60 cell line

Homodimer (IC_{50} / μM)	Monomeric equivalent (IC_{50} / μM)
 **2b** (4.52)	 **3b** (16.08)
 **2f** (1.18)	 **3f** (4.20)
 **2j** (2.24)	 **3j** (5.42)

IC_{50}: average inhibitory concentration.

three times more expressive. These findings indicate the homodimerization technique's effectiveness as a strategy to obtain more potent bioactive compounds.

Conclusions

Considering the results presented here, it was possible to verify the possibility of using ethylene glycol diacrylate, already described to obtain a simpler compound, to obtain dimeric MBHAs derived from isatin. Although other catalysts and solvents were evaluated to optimize the MBHR, the best results in terms of yield were obtained with the use of DABCO (100 mol%) and DMF, at room temperature. These conditions made it possible to obtain ten novel homodimers, reaching yields of 63%. The reaction times ranged from 15 min to 4 days, and it was observed that the reactions involving 5-substituted and 5,7-disubstituted isatin derivatives proceeded faster than the others. Biological assays against cancer strains showed moderate to excellent inhibitory effect, with attention to compounds **2b**, **2f** and **2j**, which also showed an attractive selectivity index against some tested strains. In addition, the dimers proved to be more potent compared to their respective monomers, indicating the efficiency of homodimerization to potentiate the therapeutic effects of MBHAs. Our results point to the feasibility of *in vivo* studies involving these adducts in order to broaden the understanding of their anticancer activity, contributing to the development of alternative chemotherapeutics in the pharmaceutical market.

Experimental

General

All commercially available reagents were used without further purification. Reactions were monitored by TLC using silica gel 60 UV254 pre-coated plates (Macherey-Nagel, Düren, Germany). Flash column chromatography was performed on 300-400 mesh silica gel (Silicycle Inc., Quebec, Canada) using an ethyl acetate (Neon, Suzano, Brazil) and hexane (Synth, Diadema, Brazil) mixture as eluent. FTIR spectra were recorded on an IR-Prestige-21 spectrophotometer (Shimadzu, Tokyo, Japan) using KBr pellets and frequencies are expressed in cm^{-1}. ^1H and ^{13}C NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ (Cambridge Isotope Laboratories, Tewksbury, USA) with a Bruker Avance II spectrometer (Bruker Daltonik GmbH, Bremen, Germany) (500 or 400 MHz for ^1H; 126 or 100 MHz for ^{13}C). ^1H NMR chemical shift (δ) values are expressed as parts *per* million (ppm) downfield from tetramethylsilane (TMS; δ 0.00 ppm) with *J* values in hertz. ^{13}C NMR chemical shifts are reported in scale relative to $CDCl_3$ and DMSO-d_6. In HRMS analysis, the electrospray source was operated in positive mode. High-purity nitrogen (> 98%) was used as desolvation (200 °C; 4 L min^{-1}) nebulizer and collision gas. Nebulizer pressure was kept at 4 bar, the dry gas 9 L min^{-1}, and the capillary voltage set at 4500 V. The Q-TOF conditions were as follows: end plate offset 500 V; funnel 1 200 Vpp; funnel 2 200 Vpp; hexapole RF 100 Vpp; collision RF 180 Vpp; transfer time 72 µs; pre pulse storage 5 µs; ion energy quadrupole 5 eV; rolling average: 2 × 1 Hz. The mass spectra were acquired and processed using Bruker Compass DataAnalysis Software (Bruker Daltonik GmbH, Bremen, Germany).

Synthesis of ethylene glycol diacrylate

Ethylene glycol diacrylate was synthesized according to a previous protocol in the literature,[19] by reacting 15 mmol of ethylene glycol (Synth, Diadema, Brazil) with 30 mmol of acrylic acid (Sigma-Aldrich, Barueri, Brazil) (2.0 equiv.) in 10 mL of cyclohexane (Neon, Suzano, Brazil). Then, *p*-toluenesulfonic acid (Sigma-Aldrich, Barueri, Brazil) (3 mmol) and hydroquinone (Sigma-Aldrich, Barueri, Brazil) (9 mmol) were added to the system. The flask was connected to a Dean-Stark apparatus and a condenser, keeping the system under heating (110 ºC) and magnetic stirring for 6 h. At the end of the reaction, the pH was adjusted to 7 with an aqueous solution of 1 mol L^{-1} NaOH (Neon, Suzano, Brazil) and a saturated solution of NaCl (Synth, Diadema, Brazil). The product was extracted with dichloromethane (Vetec, Rio de Janeiro, Brazil) and purified by flash column chromatography using ethyl acetate/hexane as eluent (1:9) as eluent.

Synthesis of dimeric MBHA from isatin derivatives

In a 25 mL flask containing 1 mL of DMF (Synth, Diadema, Brazil), the appropriate isatin derivative **1a**-**1j** (1.0 mmol), ethylene glycol diacrylate (0.5 mmol), and DABCO (Sigma-Aldrich, Barueri, Brazil) (0.5 mmol) were solubilized. The flask was kept under magnetic stirring and at room temperature until consumption of the limiting reagent or until the reaction stabilized, monitored by TLC. At the end of the process, liquid-liquid extraction was performed with ethyl acetate (Neon, Suzano, Brazil) and distilled water. The organic phase was dried with anhydrous calcium chloride (Neon, Suzano, Brazil) and concentrated under reduced pressure on a rotary evaporator. Purification was carried out by flash column chromatography.

Ethane-1,2-diyl bis(2-(3-hydroxy-1-methyl-2-oxoindolin-3-yl) acrylate) (compound **2a**)

Pale yellow solid (43% yield); ^1H NMR (400 MHz, DMSO-d_6) δ 7.32-7.22 (m, 2H, Ar–H), 7.04-6.90 (m, 6H, Ar–H), 6.60 (d, *J* 1.7 Hz, 2H, $=CH_2$), 6.44 (s, 2H, OH), 6.36 (d, *J* 1.3 Hz, 2H, $=CH_2$), 3.90 (s, 4H, CH_2), 3.09 (s, 6H, CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.1, 163.4, 144.4, 139.3, 130.8, 129.3, 128.0, 127.9, 123.0, 122.1, 108.4, 74.7, 62.1, 26.0; HRMS *m/z*, calcd. for $C_{26}H_{24}N_2O_8$ $[M + H]^+$: 493.1605, found: 493.1635.

Ethane-1,2-diyl bis(2-(1-allyl-3-hydroxy-2-oxoindolin-3-yl) acrylate) (compound **2b**)

Yellow oil (30% yield); ^1H NMR (500 MHz, DMSO-d_6) δ 7.34-7.21 (m, 2H, Ar–H), 7.03 (d, 2H, Ar–H), 6.98-6.93 (m, 2H, Ar–H), 6.89 (d, 2H, Ar–H), 6.67 (d, 2H, $=CH_2$), 6.47 (d, 2H, CH_2), 6.39 (s, 2H, OH), 5.90-5.78 (m, 2H, =CH), 5.37 (ddt, 2H, *J* 17.3, 3.8, 1.7 Hz, $=CH_2$), 5.19 (ddt, 2H, *J* 10.4, 3.8, 1.7 Hz, $=CH_2$), 4.26 (t, 4H, CH_2), 4.07-3.88 (m, 4H, CH_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.2, 163.8, 143.7, 139.2, 132.0, 130.8, 129.5, 128.3, 123.1, 122.2, 117.1, 109.2, 74.9, 62.2, 41.7; HRMS *m/z*, calcd. for $C_{30}H_{28}N_2O_8$ $[M + H]^+$: 545.1918, found: 545.1956.

Ethane-1,2-diyl bis(2-(1-benzyl-3-hydroxy-2-oxoindolin-3-yl) acrylate) (compound **2c**)

Pearly whit solid (40% yield); ^1H NMR (500 MHz, DMSO-d_6) δ 7.43 (d, *J* 9.5 Hz, 4H, Ar–H), 7.33 (t, *J* 7.4 Hz, 4H, Ar–H), 7.27 (d, *J* 6.1 Hz, 2H, Ar–H), 7.17 (t, *J* 7.7 Hz, 2H, Ar–H), 7.01 (d, *J* 7.3 Hz, 2H, Ar–H), 6.93 (t, *J* 7.6 Hz,

2H, Ar–H), 6.80 (d, *J* 7.8 Hz, 2H, Ar–H), 6.74 (s, 2H, OH), 6.49 (s, 2H, =CH_2), 6.41 (s, 2H, =CH_2), 4.91 (d, *J* 16.0 Hz, 2H, CH_2), 4.80 (d, *J* 15.9 Hz, 2H, CH_2), 3.93 (d, *J* 8.9 Hz, 2H, CH_2), 3.84 (d, *J* 8.9 Hz, 2H, CH_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.5, 163.8, 143.5, 139.2, 136.3, 130.8, 129.4, 128.5, 128.5, 127.4, 127.3, 123.1, 122.2, 109.1, 74.9, 62.1, 42.9; HRMS *m/z*, calcd. for $C_{38}H_{32}N_2O_8$ $[M + H]^+$: 645.2231, found: 645.2284.

Ethane-1,2-diyl bis(2-(5-chloro-3-hydroxy-1-methyl-2-oxoindolin-3-yl)acrylate) (compound **2d**)

Pearly white solid (52% yield); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.36 (dd, 2H, *J* 8.3, 2.2 Hz, Ar–H), 7.06-6.98 (m, 4H, Ar–H), 6.80 (s, 2H, OH), 6.47 (dd, 2H, *J* 7.2, 1.2 Hz, =CH_2), 6.39 (dd, 2H, *J* 13.9, 1.2 Hz, =CH_2), 3.99 (s, 4H, CH_2), 3.10 (d, 6H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.0, 163.6, 143.4, 138.6, 132.9, 129.4, 128.7, 126.1, 123.1, 110.2, 74.8, 62.4, 26.2; HRMS *m/z*, calcd. for $C_{26}H_{22}Cl_2N_2O_8$ $[M + H]^+$: 561.0826, found: 561.0918.

Ethane-1,2-diyl bis(2-(1-allyl-5-chloro-3-hydroxy-2-oxoindolin-3-yl)acrylate) (compound **2e**)

White solid (37% yield); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.33 (d, *J* 8.2 Hz, 2H, Ar–H), 7.05 (d, *J* 4.4 Hz, 2H, Ar–H), 6.94 (s, 2H, Ar–H), 6.83 (s, 2H, OH), 6.49 (d, *J* 10.4 Hz, 2H, =CH_2), 6.41 (d, *J* 15.0 Hz, 2H, =CH_2), 5.86-5.77 (m, 2H, =CH), 5.39-5.31 (m, 2H, =CH_2), 5.19 (dd, *J* 6.7, 5.2 Hz, 2H, =CH_2), 4.27 (s, 4H, CH_2), 4.04 (s, 4H, CH_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.7, 163.7, 142.5, 138.5, 132.8, 131.5, 129.4, 128.8, 126.1, 123.1, 117.2, 110.8, 74.7, 62.3, 41.7; HRMS *m/z*, calcd. for $C_{30}H_{26}Cl_2N_2O_8$ $[M + H]^+$: 613.1139, found: 613.1190.

Ethane-1,2-diyl bis(2-(5,7-dichloro-3-hydroxy-1-methyl-2-oxoindolin-3-yl)acrylate) (compound **2f**)

White oil (63% yield); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49-7.46 (m, 2H, Ar–H), 7.04 (dd, *J* 10.5, 2.1 Hz, 2H, Ar–H), 6.98 (d, *J* 2.1 Hz, 2H, =CH_2), 6.50 (s, 2H, OH), 6.45-6.41 (m, 2H =CH_2), 4.05 (d, *J* 8.9 Hz, 4H, CH_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.0, 139.3, 138.2, 133.3, 132.0, 129.6, 129.5, 128.7, 123.2, 116.6, 62.5, 33.2, 30.0; HRMS *m/z*, calcd. for $C_{26}H_{20}Cl_4N_2O_8$ $[M + H]^+$: 629.0047, found: 629.0260.

Ethane-1,2-diyl bis(2-(1-allyl-5,7-dichloro-3-hydroxy-2-oxoindolin-3-yl)acrylate) (compound **2g**)

Yellow oil (25% yield); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.46 (s, 2H, Ar–H), 7.06 (d, *J* 5.6 Hz, 2H, Ar–H), 7.03 (s, 2H, OH), 6.52 (d, *J* 4.6 Hz, 2H, =CH_2), 6.44 (s, 2H, =CH_2), 5.99-5.88 (m, 2H, =CH), 5.27 (ddq, *J* 17.0, 2.9, 1.6 Hz, 2H, =CH_2), 5.16 (ddq, *J* 10.3, 4.5, 1.5 Hz, 2H, =CH_2), 4.55 (s, 4H, CH_2), 4.09 (s, 4H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.5, 163.8, 138.8, 138.1, 135.7, 133.6, 130.9, 129.7, 127.0, 122.6, 116.1, 114.9, 74.3, 62.7, 43.3; HRMS *m/z*, calcd. for $C_{30}H_{24}Cl_4N_2O_8$ $[M + H]^+$: 681.0360, found: 681.0415.

Ethane-1,2-diyl bis(2-(1-benzyl-5,7-dichloro-3-hydroxy-2-oxoindolin-3-yl)acrylate) (compound **2h**)

White solid (24% yield); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.41 (t, 2H, *J* 2.3 Hz, Ar–H), 7.35-7.31 (m, 8H, Ar–H), 7.29-7.22 (m, 2H, Ar–H), 7.15 (d, 2H, *J* 1.4 Hz, Ar–H), 7.11 (dd, 2H, *J* 11.0, 2.1 Hz, =CH_2), 6.56 (d, 2H, *J* 7.3 Hz, =CH_2), 6.50 (s, 2H, OH), 5.22-5.17 (m, 4H, CH_2), 4.12 (s, 4H, CH_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.9, 163.7, 138.6, 137.9, 137.6, 135.6, 135.5, 130.8, 130.7, 129.6, 128.4, 127.1, 127.0, 127.0, 126.1, 122.6, 122.5, 114.8, 74.2, 62.5, 44.4; HRMS *m/z*, calcd. for $C_{38}H_{28}Cl_4N_2O_8$ $[M + H]^+$: 781.0673, found: 781.0666.

Ethane-1,2-diyl bis(2-(3-hydroxy-1-methyl-5-nitro-2-oxoindolin-3-yl)acrylate) (compound **2i**)

Yellow solid (59% yield); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (dd, 2H, *J* 8.7, 2.3 Hz, Ar–H), 7.81 (dd, 2H, *J* 4.1, 2.4 Hz, Ar–H), 7.23 (d, 2H, Ar–H), 7.05 (s, 2H, OH), 6.51 (d, 2H, =CH_2), 6.43 (d, 2H, =CH_2), 3.97 (s, 4H, CH_2), 3.19 (d, 6H, CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.8, 163.5, 150.6, 142.3, 138.0, 131.7, 129.3, 126.7, 118.1, 108.7, 74.2, 62.3, 26.4; HRMS *m/z*, calcd. for $C_{26}H_{22}N_4O_{12}$ $[M + H]^+$: 583.1307, found: 583.1336.

Ethane-1,2-diyl bis(2-(1-allyl-3-hydroxy-5-nitro-2-oxoindolin-3-yl)acrylate) (compound **2j**)

Yellow oil (56% yield); ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.35-8.23 (m, 2H, Ar–H), 7.85 (t, 2H, *J* 2.6 Hz, Ar–H), 7.17 (dd, 2H, *J* 8.7, 2.8 Hz, Ar–H), 7.11 (s, 2H, OH), 6.54 (d, 2H, *J* 4.2 Hz, =CH_2), 6.46 (d, 2H, *J* 8.1 Hz, =CH_2), 5.96-5.74 (m, 2H, =CH_2), 5.44-5.30 (m, 2H, =CH_2), 5.28-5.15 (m, 2H, =CH), 4.38 (s, 4H, CH_2), 4.04 (s, 4H, CH_2); ^{13}C NMR (126 MHz, DMSO-d_6) δ 175.4, 170.4, 149.7, 142.5, 138.0, 131.9, 131.1, 129.5, 126.9, 118.4, 117.5, 109.6, 74.2, 62.5, 42.0; HRMS *m/z*, calcd. for $C_{30}H_{26}N_4O_{12}$ $[M + H]^+$: 635.1625, found: 635.1663.

In vitro cytotoxicity for cancer cells

The cell lines HL-60 (promyelocytic leukemia), K-562 (chronic myeloid leukemia) and A-549 (epithelial-like lung carcinoma) were obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). They were cultured in RPMI 1640 media (Sigma-Aldrich, São Paulo, Brazil)

supplemented plus 10% of fetal bovine serum (Gigco Life Technologies, Gaithersburg, USA) and 1% of antibiotics (Gigco Life Technologies, Gaithersburg, USA), and kept in incubator at 37 ºC with 5% CO_2. The samples were diluted in pure sterile dimethyl sulfoxide (DMSO) (Vetec, Rio de Janeiro, Brazil).

The cells HL-60 and K-562 were plated at a concentration of 3×10^7 cells mL^{-1} and the cell line A-549 was plated at a concentration of de 10^5 cells mL^{-1}. The substances were dissolved in DMSO and serial diluted in RPMI media to obtain the final concentrations. They were added in 96-well plates (100 µL *per* well). The plates were incubated for 72 h in an incubator at 5% CO_2 at 37 ºC. After that, 25 µL of 3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide solution (MTT) (Sigma-Aldrich, St. Louis, USA) was added and the plates were incubated for 3 h. The absorbance was read at 570 nm in a plate spectrophotometer after dissolution of the precipitate with pure DMSO. Doxorubicin (5 or 10 µg mL^{-1}) (Sigma-Aldrich, St. Louis, USA) was used as the positive control. IC_{50} was calculated by a non-linear regression in the program GraphPad Prism.[46] Each sample was tested in triplicate.

Supplementary Information

Supplementary information (additional experimental details, ^1H and ^{13}C NMR spectra, and HRMS) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

M. C. Coelho was responsible for conceptualization, investigation, methodology, writing original draft preparation; A. Castro for writing review and editing, formal analysis; T. R. Olegário for conceptualization, investigation; R. Cristiano for conceptualization, writing review and editing; B. G. Vaz for formal analysis; G. F. dos Santos for formal analysis; L. S. Machado for formal analysis; G. C. G. Militão for formal analysis; P. B. N. da Silva for formal analysis; M. L. A. A. Vasconcellos for conceptualization, resources, funding acquisition, supervision; C. G. Lima-Junior for conceptualization, resources, funding acquisition, project administration, writing review and editing.

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