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Friends or Foes? Cytotoxicity, HPTLC and NMR Analyses of Some Important Naturally Occurring Hydroxyanthraquinones

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Abstract: Hydroxyanthraquinones from plants have been used as both medicinal active ingredients and adulterants in slimming food supplements. Although sensible doses of certain natural hydroxyanthraquinones for laxative effects are generally safe in the short term, chronic intake has been related to tumorigenic, carcinogenic, and genotoxic effects. However, an increasing number of researchers are reporting the antiproliferative properties of the same ingredients in cancer cells, pointing towards a potential nutraceutical value for cancer prevention. Previous studies have evaluated anthraquinones' anti-proliferative activity against various tumour cell lines and bioavailability in Caco-2 cells. However, there are scarce data about both their cytotoxicity in the later cell line and long-term stability. Therefore, this study will check the purity of several 'aged' samples using mutually complementary analytical techniques such as HPTLC and NMR assays as well as evaluate the anti-proliferative activity of the purest of these samples using the Caco-2 cell line. The chromatographic and spectroscopic analyses confirmed the long-term stability of those compounds, and their cytotoxic activity resulted in chrysazin (15 μg/mL) > catenarin (27.29 μg/mL) > rhein (49.55 μg/mL) > helminthosporin (52.91 μg/mL) > aloe-emodin (55.34 μg/mL). Our succinct review of the cytotoxicity of these compounds afforded two results: that this is the first clear report for catenarin being active in colon cancer cells and that this class of compounds needs to be better studied to clearly evaluate their benefit/risk profile in regard to both new chemo preventative nutraceuticals and anticancer therapies.

Keywords: anthraquinones; cancer; Caco-2; NMR; cytotoxicity

Citation: Al Kazman, B.S.M.; Prieto, J.M. Friends or Foes? Cytotoxicity, HPTLC and NMR Analyses of Some Important Naturally Occurring Hydroxyanthraquinones. *Nutraceuticals* **2021**, *1*, 13–30. https://doi.org/10.3390/nutraceuticals1010004

Academic Editor: Anna Iwaniak

Received: 15 September 2021
Accepted: 9 October 2021
Published: 18 October 2021

1. Introduction

Anthraquinones form the basis of the medicinal and economic uses of many plants since ancient times. Two of the most exploited aspects are their laxative [1] and colouring effects [2]. During the last half of the 20th century, an increasing number of reports discouraged their human use due to the genotoxic properties of some of the most commonly found anthraquinones [3]. Recently, it has been acknowledged that these very same toxicological properties are fundamental to unique anticancer activities mainly through DNA damage, cycle arrest, and apoptosis [4].

As conspicuously present natural products, they form part of many dietary ingredients in various quantities. Anthraquinone-based laxatives are safe when adequately used [5], but both their voluntary abuse and involuntary intake as adulterants in many slimming drinks [6] is widespread at a global scale. However, current dietary recommendations drastically warn against daily intakes above 10–50 ppm [7]. They are still explored as potential food colorants [8].

Amongst all cancers, Colorectal cancer (CRC) is one of the most diagnosed cancers in the world with over 700,000 mortalities per year. Environmental and lifestyle factors (unhealthy diets, lack of physical exercise, increase in alcohol consumption, and sleep

disruption) have contributed to this toll. The potential implications of nutraceuticals such as prebiotics and probiotics in the prevention and treatment of CRC has been recently reviewed [9]. Overall, the expectations for chemoprotective nutraceuticals is high and reported in many studies [10].

Anthraquinone derivatives are naturally occurring pigments and the largest quinone group [11]. They are found in fungi, lichens, insects, and in the plant kingdom and occur mainly in dicotyledon families including Hypericaceae, Polygonaceae, Rhamnaceae and Rubiaceae [12]. Liliaceae is the only family of monocotyledons that contains this chemical class (aloe) [13]. Approximately 90% of Anthracene occurs naturally as derivatives of 9,10 anthracenedione (anthraquinones) with different functional groups such as hydroxy, carboxy, methyl, and hydroxymethyl groups [14]. Their biosynthetic pathways are still somewhat unknown [15].

They are the largest group of naturally different dyes with their number in the region of 700 compounds [16,17]. Approximately 200 of these compounds can be produced by flowering plants, whereas the remainder can be produced by fungi and lichens, with the most frequently reported being rhein, catenarin, and emodin [18].

Apart their ecological role, anthraquinones have a significant economic value, and the anthraquinone market is predicted to account for $2.2 billion by 2025 [19]. Most of them are used as pigments, but they also play a role in bioactivities such as anticancer, anti-inflammatory, antimicrobial, laxative, antioxidant, and diuretic activities [20]. They can be extracted from natural sources but also synthesised at an industrial scale. Essentially, six methods exist for manufacturing anthraquinone: oxidation of naphthalene, synthetic preparation from benzene and phthalic anhydride, oxidation of anthracene with nitric acid, dimerization of styrene, oxidation of anthracene with chromic acid, and finally, condensation of 1,4-naphthoquinone with butadiene [21].

Hydroxyanthraquinones are organic compound derivatives from anthraquinone where one hydrogen atom is replaced by a hydroxyl group [22]. Therefore, they may be included under the wider term of "polyphenolic compounds," which are generally endowed with health protecting properties [23]. In this regard, and apart from their classical use as laxatives, hydroxyanthraquinones have been increasingly explored for both their pharmacology, as possessors of anticancer, [24] antioxidant, and immunosuppressive properties [25], and their toxicology, as genotoxic compounds and agents in tumour promotion [26,27]. Derivatives of Hydroxyanthraquinones have been found to exhibit cytotoxic activity in cancer cell lines such as L1210 and have structural similarities to some antitumour agents [28,29]. Thus, hydroxyanthraquinone derivatives can also have mutagenic and cytotoxic activities and are therefore promising anticancer leads [24].

Although several permeability studies using Caco-2 cells are reported in the literature for these compounds, there is an apparent lack of reports for their IC_{50}s on this well-known cell line. We will study the cytotoxicity and purity of selected anthraquinones, namely, aloe-emodin, barbaloin, rhein, catenarin, chrysazin, rugulosin, and helminthosporin (Figure 1) that were stored in a chemical library for more than 50 years by chromatographic and spectroscopic methods. We will frame this research with a succinct review of their cytotoxic/antitumor properties.

1.1. Aloe-Emodin

Aloe-emodin (hereafter AE) (1,8-dihydroxy-3-(hydroxymethyl) anthracene-9,10-dione) is a natural hydroxyanthraquinone found in various traditional medicinal plants, for example, *Aloe vera* leaves and *Rheum palmatum* L. (Polygonaceae) [30]. It possesses laxative, antibacterial, antiviral, and hepatoprotective effects, and many studies have reported that AE exhibited an apoptosis effect in several cancer cell lines such as human hepatoma cells, human tongue squamous cancer cells, gastric carcinoma cells, and cervical cancer cells [31–33]; they have also shown that AE inhibits tumour growth via the targeting of multiple molecules responsible for angiogenesis and cellular invasion [31].

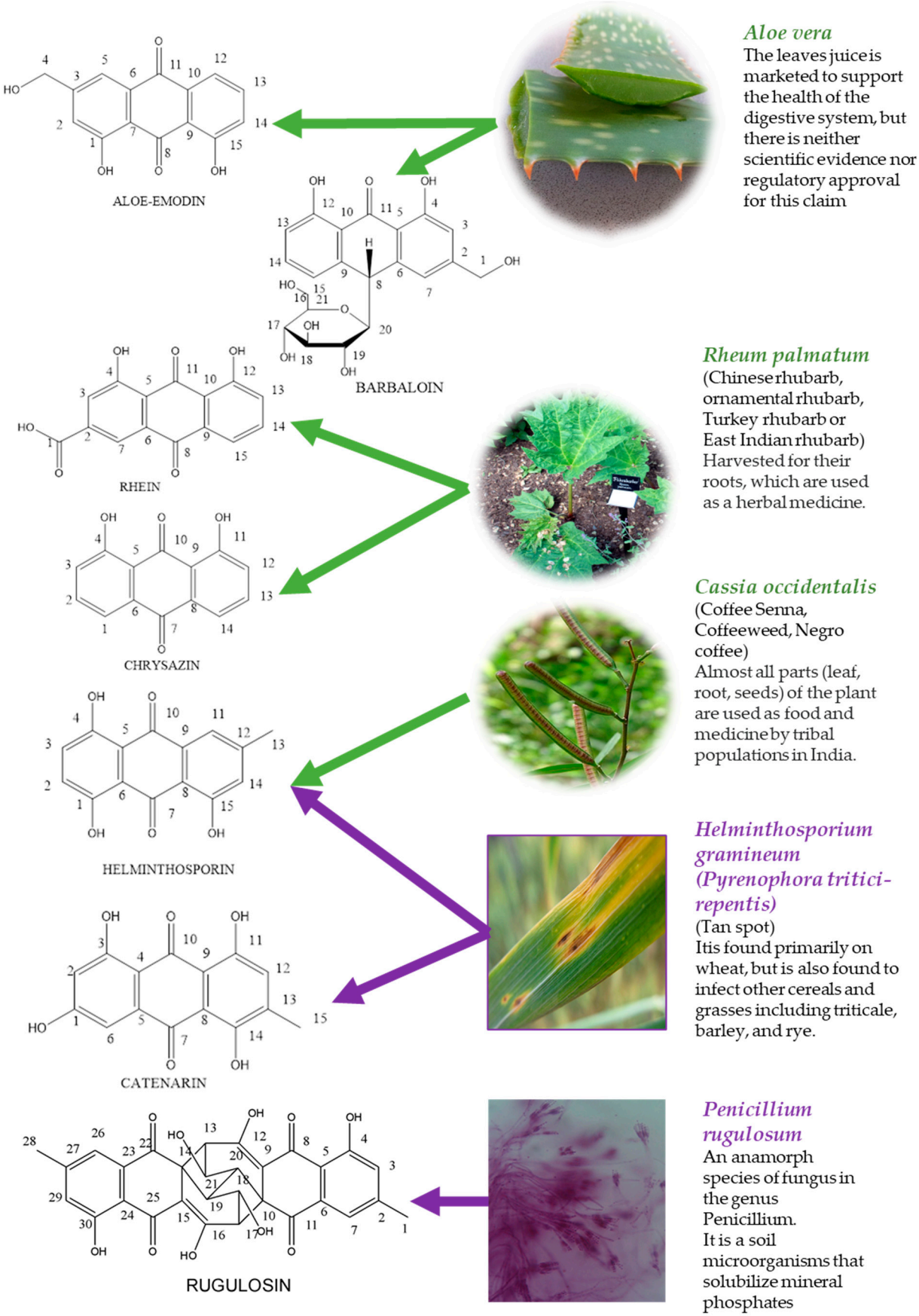


Figure 1. Chemical structures and natural sources of the selected hydroxy anthraquinones. Green names denote plants, while purple denotes fungi.

In in vitro studies, AE induced apoptosis in many cancer cell lines such as the human nasopharyngeal carcinoma cells, lung squamous carcinoma cells, and lung non-small carcinoma cells [34–37]. Additionally, AE showed higher cytotoxic activities against human oral squamous cell carcinoma (HSC-2), salivary gland tumour (HSG), and normal human gingival fibroblast (HGF) cell lines [38]. Moreover, AE has a role in inducing an apoptosis effect in human nasopharyngeal carcinoma cell lines modulated by both caspase-8 and mitochondria [35,37]. Furthermore, it has been reported that AE has a cytotoxic effect on neuroblastoma and Ewing's sarcoma cell causing growth inhibition of the neuroectodermal tumour cell line [31,39,40]. AE shows exceptional antineoplastic activity on cancer cells lines such as colon and hepatic tumours [35–37]. This occurs via multiple molecular mechanisms including disruption of the cell cycle, which improves immune function, induction apoptosis, and anti-metastasis effects [36]. Interestingly, AE possesses an antineoplastic effect in both melanospheres and metastatic human melanoma cell lines by inducing intracellular tissue transglutaminase [41–43]. Alternatively, AE did not show activity when treated with some epithelial cancers, for instance, colon carcinoma cells, T-cell leukaemia cells, cervix carcinoma cells, and normal fibroblasts cell lines [44]. To complicate matters further, Mijatovic and co-workers warn of "two opposing actions of the drug: its capacity to directly kill tumour cells, but also to protect them from NO-mediated toxicity" [45]. Furthermore, neither a toxicity profile nor the pharmacokinetics of AE are completely known [36].

1.2. Barbaloin

Barbaloin (10β-D-glucopyranosyl-1,8-dihydroxy-3-hydroxymethyl-9(1OH)-anthracenone) occurs in over 80 species of aloe [46]. Known more colloquially as "aloin", it indicates the crude material from which barbaloin is isolated [47]. It has been shown to have a laxative effect in humans and animals [46]. Barbaloin did not show any cytotoxic activity in vitro in a MTT colorimetric assay on two human colon cancer cells lines (DLD-1 and HD-29) [48].

1.3. Catenarin

Catenarin (1,4,5,7-tetrahydroxy-2-methylanthracene-9,10-dione) was first separated and isolated as a metabolic product from the fungi *Helminthsporium gramineum* Rabenhorst [49]. Subsequently, catenarin (orange pigment) was found to be the main colouring agent of *Helminthsporium catenarium* (current accepted name *Pyrenophora tririci-repentis*) [50]. In addition, catenarin can occur in leaf and wheat kernels which are infected by *Pyrenophora tririci-repentis* or *Aspergillus glaucus* ([50,51]. It has anthraquinone derivatives, including one methyl group and (OH) groups at positions 1, 3, 5 and 8 [50]. Yet, no studies have reported on its cytotoxic activity.

1.4. Chrysazin

Chrysazin (1,8-dihydroxyanthraquinone) is known as danthron. It was used for the relief of acute and chronic inflammation of the oral cavity and oropharynx, but after reports of genotoxocity, it was discontinued [52]. It is produced in the roots of *Rheum palmatum*, has laxative properties [53], and has been isolated from dried leaves and stems of *Xyris semifuscata* [54]. Moreover, chrysazin has been shown to be carcinogenic to rats [3] and significantly induces both intestinal tumours in rats and hepatocellular carcinoma in mice [26,55]. Although chrysazin shows similar cytotoxic activity compared to AE in GLC4 and GLC4/ADR cell lines using an MTT assay [56], no significant cytotoxic activity was reported in human solid tumour cell lines in vitro [57].

1.5. Helminthosporin

Helminthosporin (1,5,8-trihydroxy-3-methylanthraquinone) is a secondary metabolite which can be isolated from fungi [58,59]. Moreover, it is possible to isolate it from plants such as *Cassia obtusifolia*, rhizomes, and *Aloe turkanensis* leaves [60]. In an in vitro study, helminthosporin exhibited anticancer properties in human extra hepatic bile duct (TFK-1)

and liver (HuH7) cancer cell lines [61]. However, helminthosporin did not inhibit liver (HuH7) cancer cell lines in the in vitro study [61].

1.6. Rhein

Rhein (5-dihydroxyanthraquinone-2-carboxylic acid) is a naturally occurring anthraquinone derivative found in rhubarb roots, such as *Rheum palmatum*, and various plant species, for example, Leguminosae and Polygonaceae [62–64]. Research has shown the biological activities of rhein, including antitumour, anti-inflammatory, anti-angiogenic, and antibacterial activities [63,65]. It promotes in vitro apoptosis in several cancer cell lines such as the promyelocytic leukaemia cell (HL-60), human colonic adenocarcinoma cells, and cervical cancer Ca SKi cells [33,66]. It also induces apoptosis in nasopharyngeal carcinoma cells and human tongue cancer cells by both caspase and mitochondrial death pathways [33]. Similarly, it elicits in vitro cytotoxicity in primary human liver hl-7702 cells by inducing apoptosis through a mitochondria-mediated pathway [67]. Additionally, rhein has cytotoxic activity versus HCT-116 human colon cancer cells by enhancing the intrinsic apoptotic pathway [68]. Moreover, rhein can suppress cancer cell growth in rat livers in vivo [33,66]. Contrarily to AE, Rhein seems to inhibit autophagy in cancer cells, but such a prolonged effect eventually induces apoptosis [62,69].

1.7. Rugulosin

Rugulosin has a bisanthraquinone skeleton with a yellow colour [70]. It was primarily isolated from fungi as diverse as *Aschersonia samoensis* and *Penicillium rugulosum* but also identified in moulds, lichen, and various endophytic fungi [70]. It shows intriguing biological properties, such as anti-HIV, insecticidal, and antibiotic effects, and displays hepatotoxicity and carcinogenic activities because of high liver accumulation [71]. In in vivo, rugulosin displays weak toxic activity against mammalian cells, and in human hepatoma cells (HepG2), rugulosin did not exhibit any cytotoxic activity [72].

1.8. Overall Mechanisms of Action

From the above succinct review, it seems that this class of compounds is able to eventually trigger "programmed cell death" in cancer cells mainly via the mitochondrial-dependent intrinsic apoptotic pathway with effects on caspases [35,37,68]. The role of oxidative stress in the cytotoxicity of natural hydroxyanthraquinones has been determined for rhein, emodin, and chrysazin. They act react/compete with the single-electron-transferring flavoenzyme ferredoxin, NADP+ reductase and NADPH–cytochrome P450 reductase, causing oxidative stress and an increased lipid peroxidation [53]. Several studies suggest that numerous nutraceuticals have epigenetic targets in cancers [73,74]. There is already preliminary evidence that this is the case in regard to substituted anthraquinones (similar to chrysazin) that can act as DNA methyltransferase 1-specific inhibitors [75]. Figure 2 summarises the mechanisms for aloe-emodin, chrysazin, and rhein; the other compounds have not been found to be active. We prefer not to discuss molecular mechanisms following what Dietersen and co-workers reported: that there is a high variability depending on the structure, time schedule of treatments, and type of cell that does not allow for clear consensus [69].

1.9. Aims

Previous studies have evaluated anthraquinones for their anti-proliferative activity against various tumour cell lines and bioavailability in Caco-2 cells. However, there are scarce data for their cytotoxicity in the later cell line and long-term stability. Therefore, this study will check the purity of a number of 'aged' samples using mutually complementary analytical techniques such as HPTLC and NMR assays as well as evaluate the anti-proliferative activity of the purest of these samples using the Caco-2 cell line.

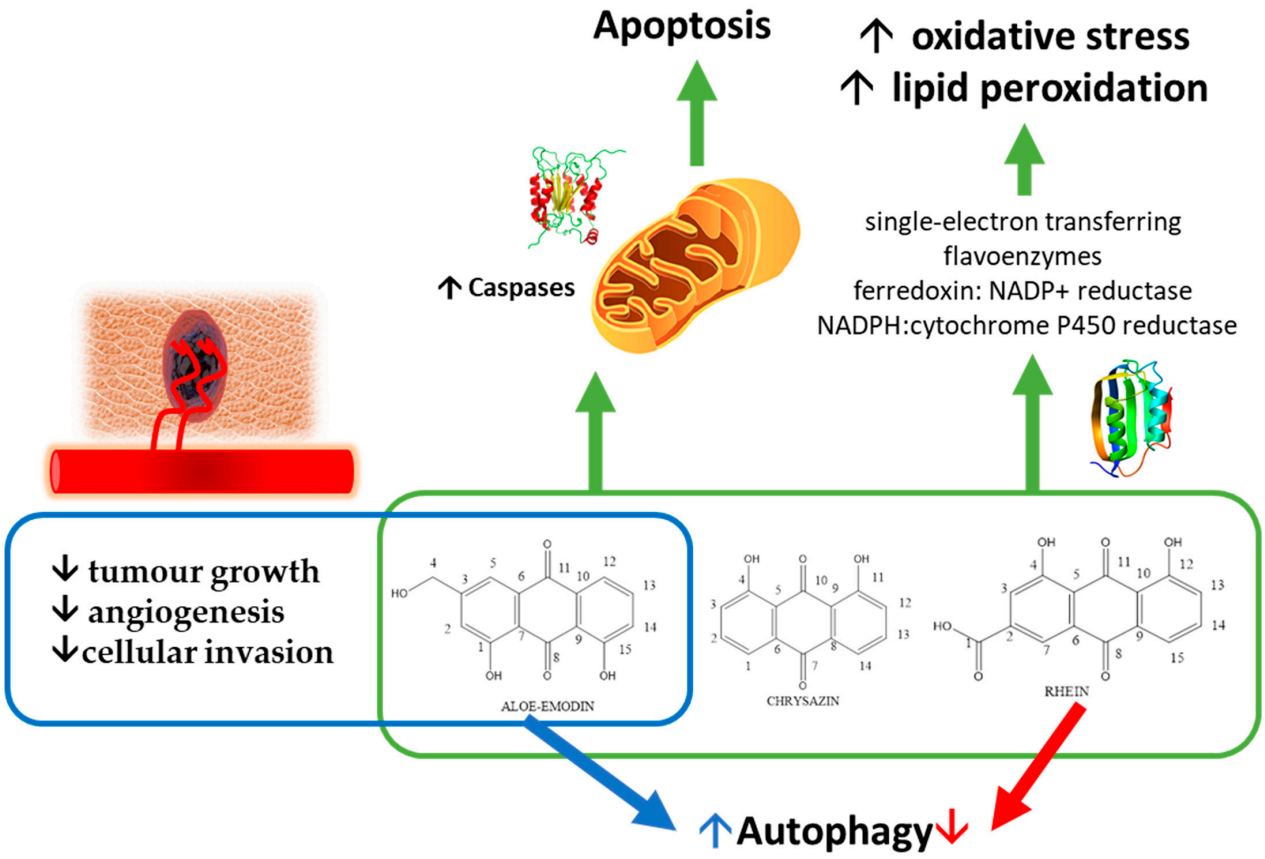


Figure 2. Main mechanisms of cytotoxicity for aloe-emodin, chrysazin, and rhein.

2. Material and Methods

2.1. Compounds and Reagents

The compounds were sourced from the chemical library of the former Department of Pharmacognosy (The School of Pharmacy, University of London). These compounds were isolated by the late Prof Fairbairn as described elsewhere [1,76–79].

2.2. Thin Layer Chromatography

Development of mobile phases prior to their use in high-performance thin-layer chromatography was achieved through classic thin-layer chromatography using silica gel TLC plates 60 F 254 (Merck, Darmstadt, Germany) and solvent of HPLC grade (Sigma Aldrich, Darmstadt, Germany) in glass chromatographic tanks saturated with the mobile phase by means of filter paper (Whatman, Darmstadt, Germany).

2.3. High-Performance Thin-Layer Chromatography

To ensure that impurities after long storage were within acceptable limits, we developed and ran two different conditions for high-performance thin-layer chromatography analyses. The equipment consisted of a Linomat 5 (CAMAG, Hungerford, UK) coupled with a 100 µL syringe (CAMAG, Hungerford, UK) and compressed air with 60–90 psi., an Automatic Developing Chamber ADC 2 (CAMAG, Hungerford, UK), and a TLC Visualizer (CAMAG, Hungerford, UK). The compounds were dissolved in 2 µL of 75% methanol, and an extract of each sample was loaded on HPTLC plates silica gel 60 F 254 (Merck, Hungerford, UK). The HPTLC was started after the developing solvent system was saturated for 20 min and pre-conditioned for 5 min. The condition of the tank was held at a humidity of 33% and a temperature of 23 °C. The process stopped once the developing solvent reached the plate at an 80 mm migration distance. After 5 min plate drying, the plates were visualized under white light at 254 and 366 nm wavelengths. VisionCATS, the HPTLC workflow and analytical software, was used in plate analysis and graphic editing.

2.4. Nuclear Magnetic Resonance

Compounds were dissolved in 600 µL of deuterated solvent in an NMR tube (VWR international Ltd., Lutterworth, UK) and submitted to a Bruker Advance 500 MHz spectrometer (Bruker, Coventry, UK). Standard protocols for 1D (^{1}H, ^{13}C, Dept 135, and Dept 90) and 2D (COSY, HMQC, and HMBC) experiments were applied. Spectra were processed using Top Spin software (Bruker, Coventry, UK).

2.5. Anti-Proliferative Activity

Anti-proliferative activity was assessed via an SRB assay. This assay determines the ability of the extract to inhibit cellular growth by measuring the cell density, thereby estimating cell number. This assay was performed according to a previously described method [80]. Caco-2 cells were seeded at a density of 10,000 cells/well in a 96-well plate (Thermo Scientific, Waltham, MA, USA) and left overnight to attach at 37 °C. Afterwards, cells were treated with a serial dilution of the plant extracts. Upon the completion of the incubation period, the cells were fixed with trichloroacetic acid solution for one hour at 4 °C. After washing with water, cellular protein was stained with SRB solution and left at room temperature for one hour; this was followed by washing the plate four times with 1% acetic acid and flicking to remove unbound dye. Then, a Tris base buffer solution was added to each well, and the absorbance was measured at 510 nm (M200 Infinite, Tecan, Männedorf, Switzerland). Cell growth was calculated using the following equation:

$$\%Cell\ growth = \frac{Absorbance\ (sample) - Absorbance\ (blank)}{Absorbance\ (vehicle\ control) - Absorbance\ (blank)} \times 100 \quad (1)$$

Each concentration of samples (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL) was evaluated in triplicate. Each experiment was performed twice. The concentration affecting the "Growth inhibition 50%" (abbreviated GI_{50}) was calculated using a Quest Graph IC50 Calculator [81].

3. Results

3.1. Development of High-Performance Thin-Layer Chromatography Systems for the Determination of the Purity of Hydroxyantharquimones

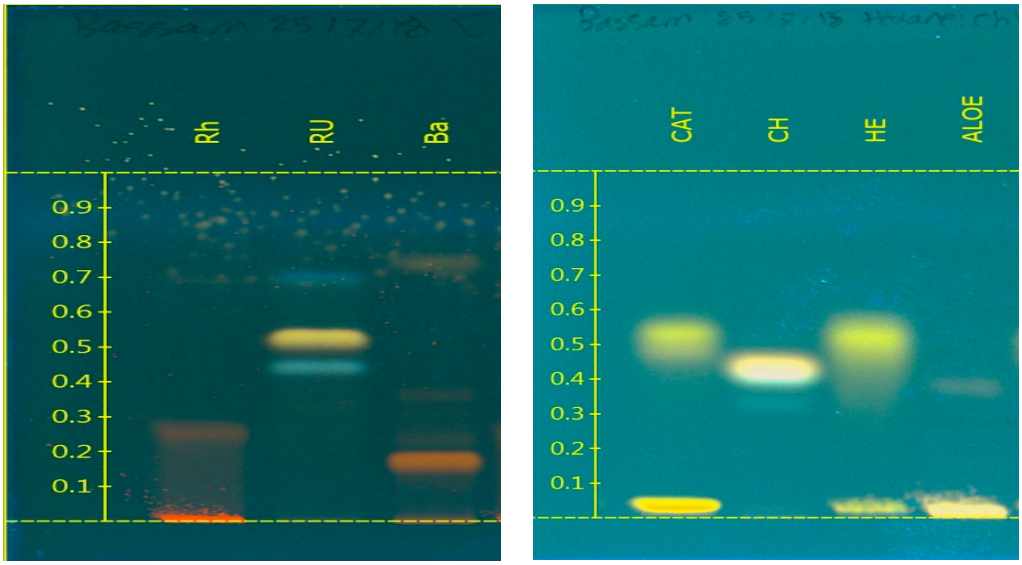
Many mobile phase systems have been trialled in classic TLC according to the literature reviews in order to find a suitable system; these include ethyl acetate-methanol-water (10:2:1), petrol ether-ethyl acetate-formic acid (75:25:1), petrol ether-ethyl acetate-formic acid (75:25:1), and toluene-ethyl acetate-ethanol-formic acid (10:8:1:2) [82–88]. The results of all these systems showed unsuccessful separation and similar *Rf* values, and all the bands migrated and appeared at the top of the plate with polar systems. In addition, once the polarity of the mobile phase decreased, some compounds such as rugulosin, rhein, and barbaloin did not migrate and showed no spots. After testing over 30 different TLC conditions previously reported, we opted for developing two new conditions due to their differences in polarity. Two different mobile phases were finally chosen: (1) ethyl acetate: 2-propanol: formic acid (84:16:1 $v/v/v$) and (2) hexane fraction from petroleum: chloroform: formic acid (80:2:1 $v/v/v$). These are the best for separation of rugulosin/rhein/barbaloin and aloe-emodin/catenarin/chrysazin/helminthosporin, respectively. Table 1 and Figure 3 show the results and *Rf* values of the major bands.

The transfer of the TLC method into HPTLC experiments showed that catenarin and helminthosporin were as seen in TLC (data not shown), but, interestingly, chrysazin and aloe-emodin exhibited one more spot (Figure 3). In the second experiment, barbaloin showed at least four spots, which confirms that it contained some more impurities besides the aglycone. Furthermore, long and large bands were displayed by rugulosin, which may be due to some contaminations. Finally, compared to the TLC plate, rhein displayed approximately the same result with only one spot, suggesting an absence of contamination. This is due to the higher power or separation of the HPTLC over TLC.

Table 1. *Rf* values of the selected anthraquinones in two different mobile phases.

Compounds	System 1	System 2
Rugulosin	0.68, 0.74 and 0.79	-
Rhein	0.38	-
Barbaloin	0.28 and 0.79	-
Aloe-emodin	-	0.10
Catenarin	-	0.35 and 0.44
Chrysazin	-	0.14
Helminthosporin	-	0.22 and 0.30

(1) Ethyl acetate: 2-propanol: formic acid (84:16:1 *v*/*v*/*v*). (2) Hexane fraction from petroleum: chloroform: formic acid (80:2:1 *v*/*v*/*v*).



(**a**) (**b**)

Figure 3. HPTLC plates showing aged (**a**) rugulosin/rhein/barbaloin eluted using System 1 and (**b**) aloe-emodin/catenarin/chrysazin/helminthosporin eluted using System 2.

3.2. NMR Studies

Our experimental data were compared to the data provided by both an in-silico prediction made by the computer application Chemdraw (Perkin Elmer, Bucks, UK) and those reported in the literature to ascertain potential differences between authors, equipment, and the validity of predictive algorithms. In addition, they enabled the observation of the relative purity of the compounds.

3.2.1. NMR Spectroscopic Characteristics of Aged Aloe-Emodin

A comparison of the results of the experiment and literature review published by Sanchez et al., (2011) for ^{1}H and ^{13}C chemical shifts showed approximately the same chemical shifts (see Table 2). On the other hand, the ChemDraw prediction displayed a slightly different chemical shift for ^{13}C, which might be due to the low of frequency at 300 MHz. The proton's chemical shift showed that this compound has six protons at positions 2, 4, 5, 12, 13, and 14 and the Heteronuclear Multiple Quantum Correlation (HMQC) between protons and carbons had been determined (see Table 2). Moreover, the ^{13}C Chemical Shift illustrated that aloe-emodin has nine quaternary carbons and two of them are carbonyl groups at 8 and 11 position (191.62 and 181.46 MHz, respectively) (see Table 2). Additionally, both carbon Dept 135 and Dept 90 exhibited a clear spectrum for CH and CH2. Therefore, it can be deduced that this compound is pure with some minor contaminations.

Table 2. Experimental, predicted, and literature data of the ^{1}H and ^{13}C NMR chemical shifts (ppm) of aloe-emodin.

Position	Experimental (500 MHz, DMSO-*d*6)			Prediction (ChemDraw) (300 MHz, DMSO-*d*6)		Literature Data [89] (500 MHz, DMSO-*d*6)	
	^{1}H	Peak Shape	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–	–	161.62	–	162.1	–	162.3
2	7.29	S	120.64	7.11	120.6	7.29	121.4
3	–	–	153.9	–	153.2	–	154.4
4	4.62	S	62.12	4.61	65.0	4.62	62.7
5	7.69	S	117.01	7.34	118.3	7.69	117.8
6	–	–	133.09	–	133.6	–	133.9
7	–	–	114.48	–	115.2	–	115.2
8	–	–	191.62	–	188.0	–	192.4
9	–	–	115.91	–	116.3	–	116.7
10	–	–	133.31	–	133.1	–	134.1
11	–	–	181.46	–	182.1	–	182.2
12	7.71	D	119.34	7.74	119.4	7.72	120.0
13	7.80	T	137.74	7.65	136.2	7.80	138.0
14	7.38	D	124.38	7.06	124.1	7.38	125.1
15	–	–	161.33	–	161.9	–	162.0

3.2.2. NMR Spectroscopic Characteristics of Aged Barbaloin

Both the ^{1}H and ^{13}C Chemical Shift results exhibited extra proton and carbon peaks (data not shown) in accordance with the results of TLC and HPTLC plates showing the presence of impurities.

3.2.3. NMR Spectroscopic Characteristics of Aged Catenarin

The results of the experiment and the ChemDraw prediction demonstrated similar ^{1}H and ^{13}C Chemical Shift values (See Table 3). However, no research has established catenarin's ^{13}C Chemical Shift. In addition, the results of the ^{1}H Chemical Shift were in agreement with those published by Kalidhar [90]. This compound has four protons and eleven quaternary carbons, two of which are carbonyl groups at position 7 and 10 (186.0 and 187.71 MHz, respectively). The proton and carbon correlation (HMQC) for the two experiments showed similar chemical shifts; small variations were due to the different frequencies that were used (300 and 500 MHz). Moreover, both carbon Dept 135 and Dept 90 exhibited a clear spectrum for CH and CH3 (Supplementary Materials). Finally, as TLC and HPTLC plates for this compound displayed more than one spot and both the ^{1}H and ^{13}C spectrum showed some extra signals, this compound has some minor contamination only.

Table 3. Experimental, predicted, and literature data of the ^{1}H and ^{13}C NMR chemical shifts (ppm) of catenarin.

Position	Experimental (500 MHz, DMSO-*d*6)			Prediction (ChemDraw) (300 MHz, DMSO-*d*6)		Literature Data [90] (400 MHz, DMSO-*d*6)	
	^{1}H	Peak Shape	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–	–	164.43	–	163.3		NO Data
2	6.58	*ds*	108.19	6.48	107.0	6.66	
3	–	–	165.51	–	164.5		
4	–	–	108.97	–	108.9		
5	–	–	134.70	–	136.4		
6	7.13	*ds*	108.30	6.75	108.3	7.13	
7	–	–	186.0	–	186.4		
8	–	–	110.07	–	111.7		
9	–	–	111.43	–	111.7		
10	–	–	187.71	–	188.0		
11	–	–	156.04	–	157.4		
12	7.24	*s*	129.09	6.82	129.4	7.32	

Table 3. *Cont.*

Position	Experimental (500 MHz, DMSO-*d*6)			Prediction (ChemDraw) (300 MHz, DMSO-*d*6)		Literature Data [90] (400 MHz, DMSO-*d*6)	
	^{1}H	Peak Shape	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
13	–	–	139.75	–	140.9		
14	–	–	156.74	–	158.0		
15	2.25	S	15.87	2.15	15.4	2.35	

3.2.4. NMR Spectroscopic Characteristics of Aged Chrysazin

Both the study published by Pullella et al. [91] and the experimental results demonstrated roughly the same ^{1}H and ^{13}C Chemical Shift values as those from the ChemDraw prediction, which showed a slightly different Chemical Shift (see Table 4). As this compound is symmetrical, both ^{1}H and ^{13}C illustrated clear and long signals. This compound has six protons, and eight quaternary carbons appear as long signal. Moreover, among of the quaternary carbons, two of them are carbonyl carbons at position 7 and 10 (181.38 and 192.01 MHz) and two attach to hydroxyl groups at position 4 and 11 (161.30 MHz). Additionally, both carbon Dept 135 and Dept 90 exhibited three large signals as this compound is symmetrical (see Supplementary Materials). Therefore, as per TLC, HPTLC and NMR results, it can be deduced that this compound is pure (See Table 4).

Table 4. Experimental, predicted, and literature data of the ^{1}H and ^{13}C NMR chemical shifts (ppm) of chrysazin.

Position	Experimental (500 MHz, DMSO-*d*6)			Prediction (ChemDraw) (300 MHz, DMSO-*d*6)		Literature Data [91] (500 MHz, DMSO-*d*6)	
	^{1}H	Peak Shape	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	7.71	DD	119.29	7.74	119.4	7.70	120.2
2	7.81	DD	137.44	7.65	136.2	7.80	138.0
3	7.39	DD	124.39	7.06	124.1	7.37	125.1
4	–	–	161.30	–	161.9	–	163.0
5	–	–	115.94	–	116.3	–	116.7
6	–	–	133.28	–	133.1	–	134.4
7	–	–	181.38	–	182.1	–	182.0
8	–	–	133.28	–	133.1	–	134.4
9	–	–	115.94	–	116.3	–	116.7
10	–	–	192.01	–	188.0	–	193.4
11	–	–	161.30	–	161.9	–	163.0
12	7.39	DD	124.39	7.06	124.1	7.37	125.1
13	7.81	DD	137.44	7.65	136.2	7.80	138.0
14	7.71	DD	119.29	7.74	119.4	7.70	120.2

3.2.5. NMR Spectroscopic Characteristics of Aged Helminthosporin

For helminthosporin, all three experiments illustrated nearly the same ^{13}C Chemical Shift values, and the ChemDraw prediction showed slightly different results for ^{1}H Chemical Shift compared to the rest (Table 5). This compound has five protons at position 2, 3, 11, 13, and 15 and ten quaternary carbons, two of which are carbonyl groups at position 7 and 10 (189.94 and 186.36 MHz). The ^{1}H Chemical Shift values demonstrated some small peaks, which implies that it contained minor contaminations (see Supplementary Materials). Moreover, the Dept 135 spectrum indicated that there are four protons attached to the aromatic rings at position 2, 3, 11, and 14 and a methyl group at position 13 (2.46 MHz). As per TLC, HPTLC, and NMR results, it can be deduced that this compound contained some minor compounds or contaminations.

Table 5. Experimental, predicted, and literature data of the ^{1}H and ^{13}C NMR chemical shifts (ppm) of helminthosporin.

Position	Experimental (500 MHz, DMSO-*d6*)			Prediction (ChemDraw) (300 MHz, DMSO-*d6*)		Literature Data [58] (500 MHz, DMSO-*d6*)	
	^{1}H	Peak Shape	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–	–	157.06	–	157.3	–	158.2
2	7.44	*S*	129.43	7.37	129.5	7.44	129.5
3	7.44	*S*	129.69	7.37	129.5	7.44	129.6
4	–	–	156.39	–	157.3	–	157.6
5	–	–	112.71	–	114.7	–	112.8
6	–	–	112.55	–	114.7	–	112.5
7	–	–	189.94	–	188.0	–	190.6
8	–	–	113.84	–	113.3	–	114.0
9	–	–	132.88	-	133.3	–	133.2
10	–	–	186.36	–	185.5	–	186.6
11	7.64	*S*	120.29	7.22	120.2	7.65	120.8
12	–	–	149.14	–	147.6	–	149.1
13	2.46	*S*	21.63	2.36	21.6	2.45	22.3
14	7.26	*S*	124.3	6.66	122.7	7.26	124.6
15	–	–	161.68	–	161.8	–	162.8

3.2.6. NMR Study of Aged Rhein

All three experiments displayed similar ^{1}H and ^{13}C Chemical Shift values with some variations (see Table 6). The ^{1}H Chemical Shift revealed some impurities and extra signals, which indicates that it might have contained some minor compounds. It has five protons and ten quaternary carbons; two of them are carbonyl groups at position 8 and 11 (181.7 and 187.38 MHz). Furthermore, it also has two hydroxyl groups attached to carbon at position 4 and 12 (161.10 and 158.31 MHz) and one carboxylic group at position 1 (165.55). Lastly, even though Dept 135 and Dept 90 displayed clear signals, it also showed small peaks that could not be integrated, which suggested some minor contaminations (see Supplementary Materials).

Table 6. Experimental, predicted, and literature data of the ^{1}H and ^{13}C NMR chemical shifts (ppm) of rhein.

Position	Experimental (500 MHz, DMSO-*d6*)			Prediction (ChemDraw) (300 MHz, DMSO-*d6*)		Literature Data [92] (500 MHz, DMSO-*d6*)	
	^{1}H	Peak Shape	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–	–	165.55	–	169.3	–	165.52
2	–	–	128.01	–	135.0	–	138.20
3	7.76	DS	123.93	7.81	124.5	7.77	124.21
4	–	–	161.10	–	160.1	–	161.51
5	–	–	119.6	–	121.5	–	118.48
6	–	–	132.95	–	131.5	–	133.61
7	8.13	DS	118.03	7.56	120.6	8.14	119.05
8	–	–	181.7	–	182.1	–	181.25
9	–	–	134.8	–	133.1	–	133.41
10	–	–	120.63	–	116.3	–	116.33
11	–	–	187.38	–	188.0	–	191.49
12	–	–	158.31	–	161.9	–	161.27
13	7.74	DD	122.42	7.06	124.1	7.41	124.64
14	7.91	–	136.26	7.65	136.2	7.84	137.63
15	7.90	–	120.57	7.74	119.4	7.75	119.48

3.2.7. NMR Spectroscopic Characteristics of Aged Rugulosin

This compound could not be interpreted due to rugulosin's many extra protons and carbons (data not shown). Rugulosin is a symmetrical compound and has 18 protons and its ^{1}H Chemical Shift values demonstrated an unclear spectrum. In addition, some peaks

were difficult to integrate, and the ^{13}C Chemical Shift values showed 30 carbons because this compound is symmetrical. As already seen in TLC and HPTLC plates, rugulosin displayed more than three spots. Therefore, it can be concluded that this compound had major contamination.

3.3. Anti-Proliferative Activity

Our results (Table 7) point towards a similar mild cytotoxicity of the selected hydroxyanthraquinones that are one or two orders of magnitude higher than the reference anticancer drug paclitaxel.

Table 7. Antiproliferative activity of the anthraquinones in Caco-2 (human colorectal adenocarcinoma) in the SRB assay (48 h).

Compound	GI_{50} (μg/mL)	GI_{50} (μM)	(CI 95%)
Aloe-emodin	55.34	204.8	(174.1–237.6)
Catenarin	27.29	95.3	(81.0–110.5)
Chrysazin	15.26	63.5	(54.0–73.7)
Helminthosporin	52.91	195.8	(166.4–227.1)
Rhein	49.55	174.3	(148.2–202.2)
Paclitaxel	1.78	2.1	(1.8–2.4)

4. Discussion

4.1. Purity and Identity of the Compounds as a Proxy to Determine Their Long Term Stability

The samples used in this project were stored for more than 50 years at room temperature, and our hypothesis was that this may be a means to understand the long-term stability of such compounds. To ascertain both their purity and identity, HPTLC and NMR were applied, respectively.

With regards to the NMR method, each compound has been analysed by means of different experiments to check their purity, namely, ^{1}H, ^{13}C, Dept 135, Dept 90, Cosy, HMQC, and HMBC experiments (see Supplementary Materials for all spectra). All the compounds were consistent with the literature and prediction data except the rugulosin and barbaloin aged samples. These could not be interpreted due to the ^{13}C and the many extra protons and carbons that made it difficult to integrate. As already seen in TLC and HPTLC plates, rugulosin displayed more than three spots. Therefore, it can be concluded that these compounds had major contamination and/or impurities.

Overall, the outcomes of HPTLC and NMR analyses demonstrated that five out of the seven compounds isolated by the late Prof. Fairbairns were intact and pure after decades of storage at room temperature, with only some minor contaminations. Only barbaloin and rugulosin presented major contaminations and were excluded from SRB assay, although their purity may still be at the level of reagent grade. Thus, the stability of these compounds seems to be higher in the case of aglycones and lower in the case of glycosides and/or dimers. Therefore, aglycones can be stored for long periods of time just at room temperature and away from light and humidity.

4.2. General Toxicity of Selected Anthraquinones, Their Activity on Caco-2 Cells and Cytotoxicity in Other Cancer Cells as a Proxy to Determine Their Nutraceutical Interest

Both catenarin and chrysazin exhibited the best anti-proliferative activity towards the Caco-2 cell line among the tested hydroxyanthraquinones. However, this activity remains one factor of magnitude away from potent cytotoxic drugs of reference, such as paclitaxel.

It is difficult to find any obvious structure–activity correlation in terms of functionality. This is not the first time researchers have failed to rationalise the pleiotropic activities of anthraquinones. Deitersen and co-workers described the cytotoxic effects of all anthraquinones as seemingly "dependent on various factors such as cellular model system, parallel stimulation of pathways, concentration of compounds, administration form and incubation times". These results show each compound acting differently depending on the

cell types. However, it is clear that the three rings, two *para*-keto groups, and hydroxylation in positions C1, C3 and C8 are essential for the bioactivity of such compounds and their pleiotropic effects upon multiple pathways [69].

In our case, the presence of some impurities may further complicate the discussion. However, we can discuss how Caco-2 is being used as a proxy model for the toxicology of the intestinal epithelium cells [93]. In this context, our results match some pre- and clinical observations of a general lack of clear toxicity for the consumption of sources of such anthraquinones despite being sold during many years as laxative [54]; these results also match contradictory reports of carcinogenesis (experimental, not clinical) for chrysazin [55,94]. This compound was used in the past as a laxative to relieve constipation in refractory patients under the name "dantron" (in UK) or "danthron" (USA and elsewhere) until animal toxicological studies reported evidence that oral exposure to this substance caused liver cancer (hepatocellular carcinoma) in male mice and benign and malignant intestinal-tract tumours (adenoma and adenocarcinoma of the colon and adenoma of the cecum) in male rats [95]. According to Van Gorkom et al. [56], chrysazin exhibits cytotoxic activity towards drug resistant lung small cell (GLC4 and GLC4/ADR) carcinoma in the MTT assay. However, no other studies have discussed its anti-proliferative activity.

Regarding catenarin, we have shown it to be mildly active against Caco-2 cell line. As far as we know, there is no clear reports on either its cytotoxicity or anti-proliferative activity. Previous reports point towards no activity (GI_{50} > 100 μM) in Vero, Wish, Calu1, Raji, and HeLa tumour cells [96]. Therefore, this might be the first time that this compound is shown as cytotoxic.

The chemo preventative role of anthraquinones as nutraceuticals or their anticancer potential seem to be hampered by several reports of in vitro, in vivo carcinogenesis. They do not seem to be translated into a high incidence of cancer in humans, and the only available report is from a patient with very high chronic consumption [97]. The toxicity of anthraquinone-containing plant preparations is confounded by the co-occurrence of other toxins, chiefly oxalic acid in rhubarb species [98]. Two recent reports point out the potentially negative effects of anthraquinones on the immune system. On the one hand, emodin had an antiproliferative and apoptotic effect in human T cells characterised by acute stress of the endoplasmic reticulum, high intracellular free Ca^{2+}, alteration to mitochondrial membrane potential, leaking of cytochrome C to the cytosol level, and increased caspase-3, -4, and -9 activities resulting in increased levels of radicals and lipoperoxidative damage. The authors propose that concomitant administration of N-acetylcysteine—a popular nutraceutical—protects from these effects [99]. On the other hand, it also caused DNA damage to human peripheral blood lymphocytes, which resulted in oxidative stress [100].

There is, however, a case to harness the cytotoxicity of the hydroxyanthraquinones by creating derivatives with a more positive benefit/risk balance. This approach may afford new molecules that are safe for regular consumption in the context of a varied diet and healthy lifestyle to support gastrointestinal health and may eventually prevent malignancies as "off label nutraceutical". The fact that many fungi (endophytes, in particular) are able to synthesise these molecules, perhaps due to horizontal plant–fungus transfer [101], may allow for scaling up of the production.

5. Conclusions

The outcomes of HPTLC and NMR analyses demonstrated a remarkable stability in the case of aglycones and a somewhat lower stability in the case of their glycosides and/or dimers. In the later group, barbaloin and rugulosin presented major impurities and were excluded from the proliferation assay, although their purity may be still at the level of reagent grade. Thus, the stability of aglycones of monohydroxyanthraquinones seems to be very high and not a concern when stored at room temperature and away from light and humidity. The anti-proliferative activity of these compounds in the Caco-2 cell line after 48 h treatment unveiled better anti-proliferative activity for catenarin and chrysazin (GI_{50} < 30 μg/mL), whereas rhein, aloe-emodin, and helminthosporin are endowed with

lower anti-proliferative activity (30 μg/mL < GI_{50} < 100 μg/mL). Our succinct review of the cytotoxicity of these compounds afforded two results: that this is the first clear report for catenarin being active in colon cancer cells and that this class of compounds needs to be better studied to clearly evaluate their benefit/risk profile towards both new chemo preventative nutraceuticals and anticancer therapies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nutraceuticals1010004/s1; NMR spectra are presented as Supplementary Materials.

Author Contributions: Conceptualization, J.M.P.; Methodology, formal analysis, investigation, writing—original draft preparation, funding acquisition, B.S.M.A.K.; supervision, project administration, writing—review and editing, J.M.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: B.S.M.A-K is grateful for a sponsorship from Najran University (Kingdom of Saudi Arabia) to study an MSc in Medicinal Natural Products and Phytochemistry.

Conflicts of Interest: The authors declare no conflict of interest.

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