





## Article

# Optimization of Extraction Conditions and Cytotoxic Activity of Rapanone in Comparison to Its Homologue, Embelin

Dagmara Wróbel-Biedrawa <sup>1</sup>, Agnieszka Galanty <sup>1</sup>, Paweł Zagrodzki <sup>2</sup> and Irma Podolak <sup>1,\*</sup>

<sup>1</sup> Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Medyczna 9, 30-688 Cracow, Poland

<sup>2</sup> Department of Food Chemistry and Nutrition, Pharmaceutical Faculty, Medical College, Jagiellonian University, Medyczna 9, 30-688 Cracow, Poland

\* Correspondence: irma.podolak@uj.edu.pl

**Abstract:** Rapanone is a plant-derived simple alkyl-dihydroxybenzoquinone structurally close to embelin, a well-known cytotoxic agent. The pharmacological characterization of rapanone is still incomplete, and to fill the data gap, a good source for its acquisition is required to conduct further research. This study aimed to optimize the conditions for the extraction of rapanone from the leaves of white-berried *Ardisia crenata* Sims. For this purpose, three methods were employed: heat reflux (HRE), shaking (SE), and ultrasound-assisted extraction (UAE), and such parameters as the extraction time, solvent, and the number of extractions from the same sample were set as experimental variables. Furthermore, cytotoxic activity toward prostate cancer, thyroid cancer, and colorectal carcinoma cell lines was investigated and compared with doxorubicin and embelin. The most effective and economical method for the extraction of rapanone was shown to be 20 min UAE with ethyl acetate or chloroform. Rapanone exhibited high cytotoxic activity against PC3 (IC<sub>50</sub> = 6.50 µg/mL), Du145 (IC<sub>50</sub> = 7.68 µg/mL), FTC133 (IC<sub>50</sub> = 6.01 µg/mL), 8505C (IC<sub>50</sub> = 7.84 µg/mL), and Caco-2 (IC<sub>50</sub> = 8.79 µg/mL) cell lines after 24 h and against the HT29 cell line after 48 h (IC<sub>50</sub> = 11.67 µg/mL). Furthermore, it revealed a more favorable safety profile than either its homologue, embelin, or doxorubicin. The set of optimal extraction parameters obtained may be utilized for scientific and industrial purposes to achieve the best rapanone yield. Moreover, this benzoquinone revealed a high cytotoxic activity with good selectivity.

**Keywords:** rapanone; embelin; cytotoxic; extraction; optimization; cancer



**Citation:** Wróbel-Biedrawa, D.; Galanty, A.; Zagrodzki, P.; Podolak, I. Optimization of Extraction Conditions and Cytotoxic Activity of Rapanone in Comparison to Its Homologue, Embelin. *Molecules* **2022**, *27*, 7912. <https://doi.org/10.3390/molecules27227912>

Academic Editor: Halina Ekiert

Received: 14 October 2022

Accepted: 14 November 2022

Published: 16 November 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

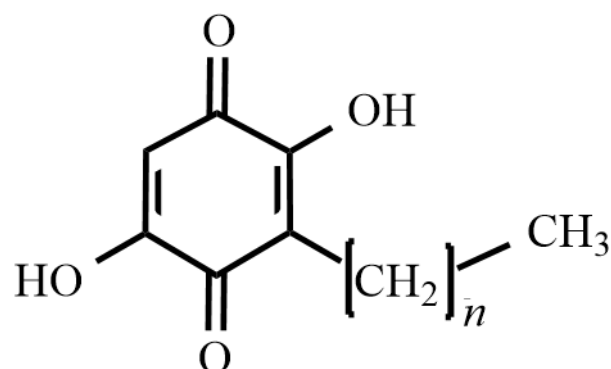


**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Rapanone (Figure 1) represents simple alkyl-dihydroxybenzoquinones that occur naturally in plants. Hydroxybenzoquinone derivatives are considered typical secondary metabolites for some species belonging to the *Primulaceae* family, especially those that were previously classified to the *Myrsinaceae*. Rapanone, along with its structural homologue, embelin, is often concomitant in some plant species, e.g., *Ardisia crenata* Sims [1], *Lysimachia punctata* [2], *Myrsine guianensis* [3]. These two alkyl-dihydroxybenzoquinones differ structurally in only two carbon atoms in the side chain (Figure 1). Most published reports refer to embelin, which is an active ingredient of *Embelia ribes*, a well-known Ayurvedic medicinal plant used as a wound healer, anti-inflammatory, antimicrobial, or contraceptive agent [4,5]. Recent studies not only supported some of the traditional uses of the plant in relation to embelin, but also showed new directions of its pharmacological activity with potential applications: cytotoxic, anti-diabetic, anxiolytic, antidepressive, neuroprotective, and cardioprotective [6–8]. The compound was also shown to be an XIAP (X-linked inhibitor of apoptosis protein) inhibitor [9]. The pharmacological properties of rapanone, in turn, are poorly investigated in comparison to those of embelin, although the similarity of their structures allows us to assume a high biological potential of rapanone, as well. The results

of recent studies revealed anti-inflammatory [10,11], antifertility [3], cytotoxic [11–14], and antioxidant [11,15] activity.



$n = 10$ , Embelin

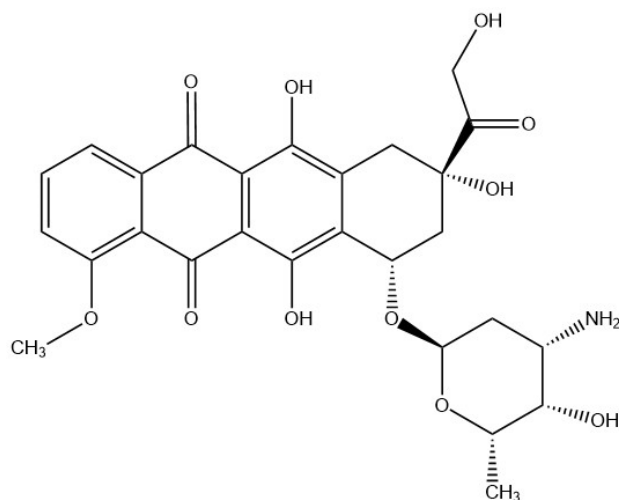
$n = 12$ , Rapanone

**Figure 1.** Structure of rapanone and embelin.

Rapanone was isolated for the first time from *Rapanea maximowiczii* (Koidz) by Kawamura and Hokoku in 1937, just five years after embelin was evaluated as an active compound of *E. ribes* [16]. As mentioned above, both compounds often occur together, with embelin as the predominant benzoquinone constituent. Our team found that *A. crenata* Sims may be a good source to obtain rapanone solely [1]. As with other phytochemicals, the yield greatly depends on the applied extraction procedure. Although there are a couple of studies optimizing the extraction conditions for embelin from *E. ribes* [17,18], no data on the relationship between the selection of extraction parameters and the efficiency of the process are available for rapanone. Taking this into account, we aimed to optimize the extraction conditions of this benzoquinone from the leaves of the white-berried variety of *A. crenata* Sims.

In 2020, almost 20% of all deaths worldwide were caused by cancer [19]. Among the different types of cancer, colon and rectum cancers, as well as prostate cancers, are the most common [19]. Furthermore, the incidence of thyroid cancer is increasing and recently has become the fifth most common neoplastic change in the USA [20]. Given these alarming statistics, it is clear that preventive measures are not enough and the need for more effective and safer pharmacotherapeutics is still unmet. That motivates a continued search for new therapeutic options. According to the investigations performed previously and the structural similarities of rapanone and embelin, we assumed that the former should also possess interesting cytotoxic activity.

Taking the abovementioned into account, the aims of our study were to optimize the extraction conditions of rapanone and to discuss the influence of different variables of the extraction process on the yield of rapanone as well as to evaluate its cytotoxic potential by checking the impact on cancerous cell lines from the prostate, thyroid, and gastrointestinal panel. The activity of rapanone was compared to the reference drug, doxorubicin, which is a quinone derivative (Figure 2), as well as to embelin, as a structural homologue. Doxorubicin is a cytostatic drug used in chemotherapy of many types of cancers, for example, in prostate, thyroid, gastric, or hepatic cancer. Furthermore, it is widely used as a standard in testing substances for cytotoxic potential.



**Figure 2.** Structure of doxorubicin.

## 2. Results

### 2.1. Optimization of Rapanone Extraction from Plant Material

To optimize the extraction conditions, we have checked the influence of the following variables in our study: the method (heat reflux extraction (HRE), shaking (SE), and ultrasound-assisted extraction (UAE)), the extraction time (30, 60, and 120 min (HRE, SE), or 10, 20, and 30 min (UAE)), the number of extractions from the same plant material sample (1–3 repetitions of extraction from the same sample and every repetition was conducted with the new portion of the solvent), and the solvent (chloroform, ethyl acetate, and acetone) (Table 1). As a result, we obtained the yields of rapanone (counted as milligrams of rapanone per 1 g of dried plant material) in every combination of parameters (Table 2).

**Table 1.** Experimental design and codes for each set of parameters.

Set Code	HRE			Set Code	SE			Set Code	UAE		
	Solvent	Time of Extraction [min]	Number of Extraction Repetitions		Solvent	Time of Extraction [min]	Number of Extraction Repetitions		Solvent	Time of Extraction [min]	Number of Extraction Repetitions
1H	chloroform	30	1	1S	chloroform	30	1	1U	chloroform	10	1
2H	chloroform	30	2	2S	chloroform	30	2	2U	chloroform	10	2
3H	chloroform	30	3	3S	chloroform	30	3	3U	chloroform	10	3
4H	chloroform	60	1	4S	chloroform	60	1	4U	chloroform	20	1
5H	chloroform	60	2	5S	chloroform	60	2	5U	chloroform	20	2
6H	chloroform	60	3	6S	chloroform	60	3	6U	chloroform	20	3
7H	chloroform	120	1	7S	chloroform	120	1	7U	chloroform	30	1
8H	chloroform	120	2	8S	chloroform	120	2	8U	chloroform	30	2
9H	chloroform	120	3	9S	chloroform	120	3	9U	chloroform	30	3
10H	ethyl acetate	30	1	10S	ethyl acetate	30	1	10U	ethyl acetate	10	1
11H	ethyl acetate	30	2	11S	ethyl acetate	30	2	11U	ethyl acetate	10	2
12H	ethyl acetate	30	3	12S	ethyl acetate	30	3	12U	ethyl acetate	10	3
13H	ethyl acetate	60	1	13S	ethyl acetate	60	1	13U	ethyl acetate	20	1
14H	ethyl acetate	60	2	14S	ethyl acetate	60	2	14U	ethyl acetate	20	2
15H	ethyl acetate	60	3	15S	ethyl acetate	60	3	15U	ethyl acetate	20	3
16H	ethyl acetate	120	1	16S	ethyl acetate	120	1	16U	ethyl acetate	30	1

Table 1. Cont.

Set Code	HRE			Set Code	SE			Set Code	UAE		
	Solvent	Time of Extraction [min]	Number of Extraction Repetitions		Solvent	Time of Extraction [min]	Number of Extraction Repetitions		Solvent	Time of Extraction [min]	Number of Extraction Repetitions
17H	ethyl acetate	120	2	17S	ethyl acetate	120	2	17U	ethyl acetate	30	2
18H	ethyl acetate	120	3	18S	ethyl acetate	120	3	18U	ethyl acetate	30	3
19H	acetone	30	1	19S	acetone	30	1	19U	acetone	10	1
20H	acetone	30	2	20S	acetone	30	2	20U	acetone	10	2
21H	acetone	30	3	21S	acetone	30	3	21U	acetone	10	3
22H	acetone	60	1	22S	acetone	60	1	22U	acetone	20	1
23H	acetone	60	2	23S	acetone	60	2	23U	acetone	20	2
24H	acetone	60	3	24S	acetone	60	3	24U	acetone	20	3
25H	acetone	120	1	25S	acetone	120	1	25U	acetone	30	1
26H	acetone	120	2	26S	acetone	120	2	26U	acetone	30	2
27H	acetone	120	3	27S	acetone	120	3	27U	acetone	30	3

HRE—heat reflux extraction, SE—shaking extraction, UAE—ultrasound-assisted extraction.

Table 2. The means ( $\pm$ standard deviation, SD) and medians of rapanone content in the extracts obtained in various experimental systems, calculated as milligrams of rapanone per 1 g of dried weight of plant material.

Set Code	HRE		Set Code	SE		Set Code	UAE	
	Mean [mg/g]	Median [mg/g]		Mean [mg/g]	Median [mg/g]		Mean [mg/g]	Median [mg/g]
1H	1.69 $\pm$ 0.17	1.70	1S	2.23 $\pm$ 0.14	2.24	1U	17.42 $\pm$ 1.38	17.29
2H	1.86 $\pm$ 0.20	1.92	2S	2.75 $\pm$ 0.03	2.76	2U	17.93 $\pm$ 0.51	18.01
3H	1.72 $\pm$ 0.15	1.70	3S	2.45 $\pm$ 0.35	2.54	3U	19.77 $\pm$ 1.30	20.15
4H	1.70 $\pm$ 0.32	1.61	4S	2.32 $\pm$ 0.28	2.22	4U	20.54 $\pm$ 0.91	20.18
5H	1.73 $\pm$ 0.30	1.71	5S	2.83 $\pm$ 0.33	2.75	5U	19.48 $\pm$ 1.10	19.25
6H	1.83 $\pm$ 0.33	1.74	6S	3.06 $\pm$ 0.32	2.93	6U	17.54 $\pm$ 1.22	17.19
7H	2.15 $\pm$ 0.21	2.16	7S	2.30 $\pm$ 0.14	3.06	7U	20.09 $\pm$ 1.63	21.11
8H	2.53 $\pm$ 0.71	2.37	8S	2.99 $\pm$ 0.27	3.03	8U	17.16 $\pm$ 1.63	16.86
9H	2.00 $\pm$ 0.20	2.01	9S	3.28 $\pm$ 0.47	3.20	9U	17.86 $\pm$ 0.54	17.88
10H	11.39 $\pm$ 0.90	11.52	10S	11.76 $\pm$ 1.08	11.82	10U	12.21 $\pm$ 3.09	12.30
11H	11.16 $\pm$ 5.08	9.37	11S	18.42 $\pm$ 3.86	19.27	11U	12.99 $\pm$ 3.79	11.82
12H	10.33 $\pm$ 1.86	10.80	12S	17.79 $\pm$ 0.22	17.85	12U	13.06 $\pm$ 1.39	13.29
13H	13.18 $\pm$ 0.45	13.28	13S	7.16 $\pm$ 0.93	7.05	13U	21.39 $\pm$ 1.21	21.06
14H	14.10 $\pm$ 1.74	13.91	14S	11.90 $\pm$ 1.08	12.24	14U	19.40 $\pm$ 3.41	19.36
15H	12.92 $\pm$ 1.97	12.39	15S	12.76 $\pm$ 1.89	12.75	15U	14.74 $\pm$ 2.29	14.70
16H	14.80 $\pm$ 1.65	15.09	16S	8.11 $\pm$ 1.25	8.68	16U	18.28 $\pm$ 1.37	18.04
17H	14.18 $\pm$ 1.27	14.46	17S	6.43 $\pm$ 1.28	6.86	17U	18.71 $\pm$ 2.46	17.99
18H	17.00 $\pm$ 2.67	16.42	18S	8.34 $\pm$ 2.69	8.41	18U	10.71 $\pm$ 2.13	10.77
19H	2.68 $\pm$ 1.05	2.43	19S	0.05 $\pm$ 0.05	0.06	19U	3.34 $\pm$ 0.31	3.27
20H	2.79 $\pm$ 1.56	2.83	20S	0.25 $\pm$ 0.27	0.15	20U	2.92 $\pm$ 0.66	3.07
21H	2.75 $\pm$ 0.88	2.26	21S	0.02 $\pm$ 0.01	0.02	21U	2.18 $\pm$ 0.71	2.23
22H	2.05 $\pm$ 0.80	2.10	22S	0.13 $\pm$ 0.13	0.07	22U	0.04 $\pm$ 0.05	0.01
23H	4.40 $\pm$ 2.46	4.12	23S	0.14 $\pm$ 0.06	0.117	23U	0.10 $\pm$ 0.09	0.04
24H	2.24 $\pm$ 0.85	2.55	24S	Nd	Nd	24U	Nd	Nd
25H	2.50 $\pm$ 0.81	2.14	25S	0.11 $\pm$ 0.06	0.12	25U	0.17 $\pm$ 0.12	0.11
26H	1.97 $\pm$ 1.22	1.61	26S	0.01 $\pm$ 0.02	0.000	26U	0.01 $\pm$ 0.01	0.00
27H	1.35 $\pm$ 0.77	1.49	27S	Nd	Nd	27U	0.10 $\pm$ 0.09	0.09

Nd—not determined; plant material—leaves of white-berried *A. crenata* Sims.

Based on the analysis carried out separately in each experimental system (HRE, SE, and UAE), it could be concluded that the only factors significantly affecting the amount of rapanone in the extracts obtained in the UAE system were the linear ( $p = 0.000$ ) and quadratic effects ( $p = 0.000$ ) related to the type of solvent. Their corresponding regression coefficients in the multinomial model, provided in the ANOVA procedure, for the normalized values of the input parameters, were  $-8.84$  and  $-5.66$ , respectively. The constant value in

the same model was 16.32 and  $R^2 = 0.909$ . In the other two systems, the factors significantly influencing the amount of rapanone were: the square effect related to the solvent and the linear effect related to the extraction time (system HRE), and both the square and linear effects related to the solvent and the linear effect related to the time (system SE) (detailed data were not shown as the results obtained in these systems were significantly lower than in the UAE system).

The results obtained for the various experimental systems are presented in Table 2.

The rapanone content in the extracts obtained by different methods varied from a close to zero amount up to  $21.39 \pm 1.21$  mg per 1 g of dry weight (d.w.) of the plant material (white-berried *A. crenata* leaves) (median = 21.06 mg/g). The highest content was determined after ultrasound-assisted extraction, performed once for 20 min, using ethyl acetate as a solvent (Table 1). Of these highest results, four revealed the largest number of significant differences from the rest of the results: 13U, 4U, 7U, and 14U. They were all obtained by the UAE, with chloroform or ethyl acetate; however, two extractions demanded a longer extraction time (7U) or repeated extraction (14U), so they required more time and solvent investment. Taking that into account, extractions 4U and 13U seem to be more favorable.

Generally, the lowest rapanone content was obtained for the acetone extracts in the three methods used to prepare the extracts (Table 2); however, among them, the HRE-prepared extracts gave the best results, as only in this case the rapanone content was noticeable.

## 2.2. Cytotoxic Activity of Rapanone and Embelin

Our study revealed a high cytotoxic activity of rapanone with low, that is, below 10  $\mu\text{g}/\text{mL}$ ,  $\text{IC}_{50}$  values for almost all cancer cell lines tested (except HT29, which was still below 20  $\mu\text{g}/\text{mL}$ ) (Tables 3–5). For embelin, all  $\text{IC}_{50}$  values were below 25  $\mu\text{g}/\text{mL}$ . In the case of thyroid cancer cell lines, the values were higher than 10  $\mu\text{g}/\text{mL}$ , which showed a weaker cytotoxic effect compared to rapanone (Table 5). Interestingly, doxorubicin activity was low in metastatic grade IV prostate cancer PC3 and thyroid cancer 8505C, with  $\text{IC}_{50}$  values greater than 50 and 40  $\mu\text{g}/\text{mL}$ , respectively (Tables 3 and 5). It should be noted that in the case of rapanone, after 24 h the  $\text{IC}_{50}$  values were, respectively, 6.50 and 7.84  $\mu\text{g}/\text{mL}$ , and for embelin 9.27 and 18.86  $\mu\text{g}/\text{mL}$ .

**Table 3.**  $\text{IC}_{50}$  values of rapanone, embelin, and doxorubicin in prostate cancer panel.

Compound	$\text{IC}_{50}$ [ $\mu\text{g}/\text{mL}$ ]					
	PC3		Du145		PNT2	
	24 h	48 h	24 h	48 h	24 h	48 h
Rapanone	6.50	4.92	7.68	5.07	16.72	12.65
Embelin	9.27	5.24	8.02	4.18	10.14	4.61
Doxorubicin	>50.00	NE	3.18	NE	1.38	NE

NE—not examined; PC3 grade IV prostate carcinoma, derived from metastatic site: bone, ATCC CRL-1435, Du145 prostate carcinoma, derived from metastatic site: brain, ATCC HTB-81; PNT2 prostate epithelial cells, and ECACC 95012613.

Both rapanone and embelin revealed high cytotoxicity in Caco-2 cells, as well as the other cell line from the gastrointestinal panel, HT29; however, the activity of both compounds was still lower compared to doxorubicin (Table 4).

Notable is that the activity of rapanone in non-neoplastic cell lines, prostate epithelial cells, PNT2, and hepatocellular carcinoma, HepG2 was always lower than for cancerous cells, with  $\text{IC}_{50}$  values greater than 10  $\mu\text{g}/\text{mL}$ , at least twice as high than for cancer cell lines (Tables 3 and 4). After 24 h of incubation at 10  $\mu\text{g}/\text{mL}$  of rapanone, the mean percentage of dead Du145 cells was nine times higher than for PNT2 (82.33% vs. 9.30%), while for embelin it was 61.67% vs. 49.00% and the reference, doxorubicin, was even more toxic to normal cells, killing almost all of them (88.38% vs. 99.21%) (Figure 3).

**Table 4.** IC<sub>50</sub> values of rapanone, embelin, and doxorubicin in colorectal carcinoma panel.

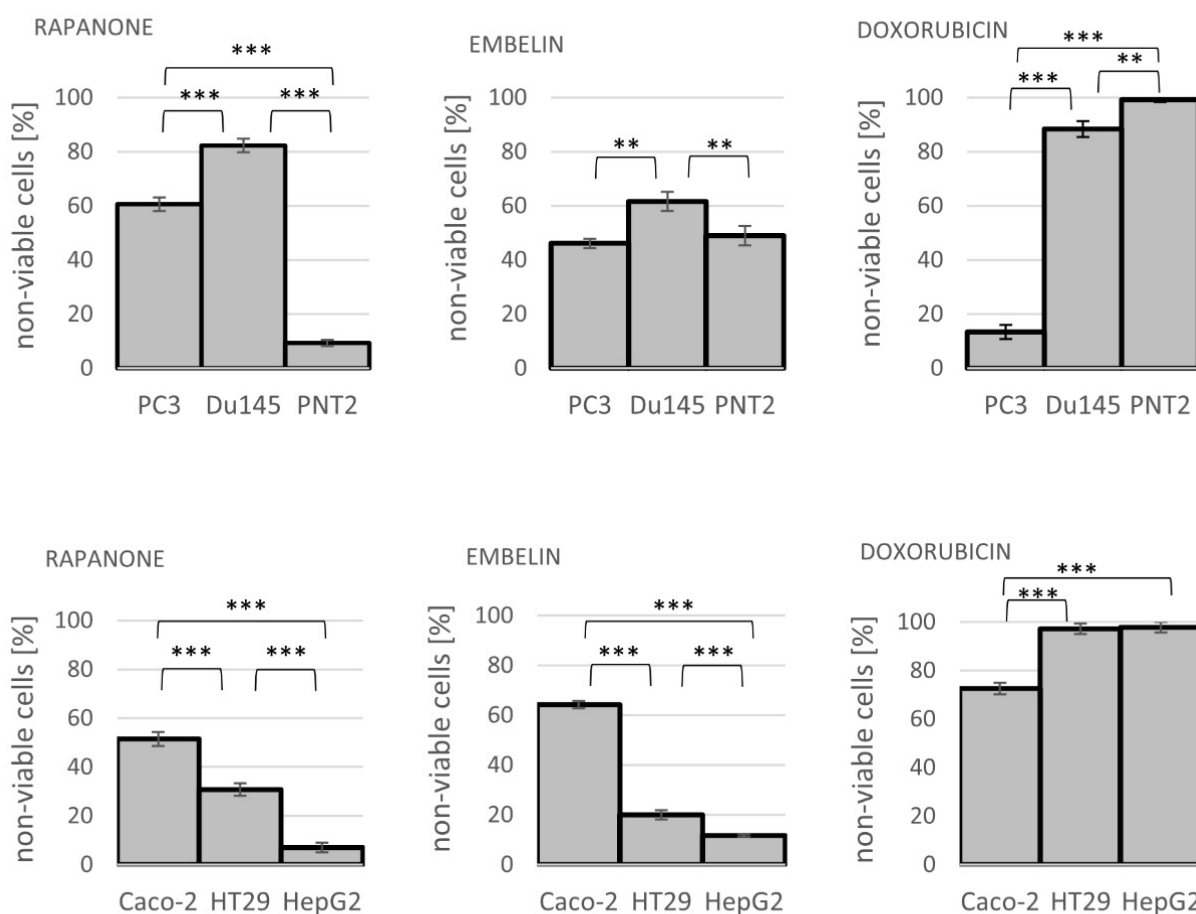
Compound	IC <sub>50</sub> [µg/mL]					
	Caco-2		HT29		HepG2	
	24 h	48 h	24 h	48 h	24 h	48 h
Rapanone	8.79	5.66	16.91	11.67	36.27	16.70
Embelin	6.12	3.37	24.70	13.72	15.98	6.77
Doxorubicin	3.44	NE	1.53	NE	1.03	NE

NE—not examined; Caco2 colorectal adenocarcinoma, ATCC HTB-37, HT29 colorectal adenocarcinoma, ATCC HTB-38, HepG2 hepatocellular carcinoma, and ATCC HB-8065.

**Table 5.** IC<sub>50</sub> values of rapanone, embelin, and doxorubicin in thyroid cancer panel.

Compound	IC <sub>50</sub> [µg/mL]			
	FTC133		8505C	
	24 h	48 h	24 h	48 h
Rapanone	6.01	4.42	7.84	5.50
Embelin	10.51	6.48	18.86	13.84
Doxorubicin	4.02	NE	>40.00	NE

NE—not examined; follicular thyroid carcinoma FTC133, ECACC 94060901; undifferentiated thyroid carcinoma 8505C and ECACC 94090184.



**Figure 3.** Cytotoxic activity of rapanone, embelin, and doxorubicin at 10 µg/mL towards cell lines of prostate and gastrointestinal panels (see Material and Methods section for cell line descriptions); \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Noticeably, increasing the incubation time (24 and 48 h) resulted in a decrease in  $IC_{50}$  values for rapanone and embelin (Tables 3–5), indicating a higher number of dead cells with longer exposure to each benzoquinone.

### 3. Discussion

Rapanone is a plant-derived benzoquinone that possesses promising biological potential. To investigate its pharmacological activities more thoroughly, it is vital to provide the substance in a proper amount. Our previous studies indicate that the leaves of white-berried *A. crenata* Sims, a plant that can be cultivated under greenhouse conditions, may serve as a good source for the isolation of rapanone [1,11]. In search of an effective extraction procedure, we decided to explore this issue in more detail.

To define the most efficient conditions for the extraction of rapanone, we chose variable parameters for the study based on the literature and our own experience. As data on rapanone isolation are limited, the literature search focused on embelin, a structural homologue of the former (Figure 1). The first isolation of embelin was made in 1932 by Paranjpe and Gokhale from *E. ribes* fruits [16]. Subsequently, it was isolated from *n*-hexane extracts of *E. ribes* berries (obtained by cold maceration or heat extraction) [21–24], dichloromethane extract [25], ethanol extract [26], methanol extract of leaves of *E. ribes* [27,28], or from petroleum ether and chloroform extracts of *M. africana* berries (as reported by Kiprono [16] based on previous works).

In the available literature, in which the isolation of rapanone was reported, this compound was derived from ethyl acetate extracts obtained by simple maceration, e.g., from the stem bark of *Connarus venezuelanus* Baill [14,15], or the root bark of *C. suberosus* [29]. Furthermore, chloroform [2] or petroleum ether [3,30] was used for further fractionation of these extracts.

Taking these data into account, we selected five solvents: *n*-hexane, chloroform (as it is often recognized to possess separation properties better than dichloromethane), ethyl acetate, acetone, and methanol. A preliminary study (data not published) revealed that chloroform, ethyl acetate, and acetone had the best extraction ratio, so these were chosen for further experiments. Other optimized parameters were the method (heat reflux extraction (HRE), shaking (SE), and ultrasound-assisted extraction (UAE)), the extraction time (30, 60, and 120 min (HRE, SE), or 10, 20, and 30 min (UAE)), and the number of extractions from the same plant material sample (1–3 repetitions of extraction from the same sample and every repetition was conducted with the new portion of the solvent). The volume of the solvent (20 mL) and the degree of fragmentation (fine grounded) were experimentally fixed by us (data not shown).

Generally, our study has shown that chloroform and ethyl acetate appear to have better rapanone-extracting properties than acetone. However, in the case of HRE and SE, which are conventional methods but still commonly used in laboratories due to their simplicity, in most variants of extraction, the level of rapanone in acetone extracts was similar to that of chloroform ones. Keeping this in mind, even though chloroform is used to extract benzoquinones from different plant materials [1,16], in the case of *A. crenata* leaves, this solvent may not be the best choice for conventional techniques. In our experiment, only in the case of the UAE, chloroform was a significantly more efficient extractant compared to other solvents tested. Shaking, in turn, which requires very mild conditions of the process (see the Materials and Methods section), is the safest method for tissues and active substances because the temperature is low, and the extraction is based on simple diffusion. In this case, only using ethyl acetate as an extractant, the mean rapanone content was significantly high, but, contrary to HRE, during the shortest extraction (30 min repeated twice or three times).

The conventional liquid-solid extraction methods (HRE, SE) are time-consuming, laborious, and require a lot of solvents to obtain the expected yield of the substance [31], whereas sonication (UAE) represents a green approach for the process, consuming less time, solvent, and work. Its higher effectiveness is due to very locally generated high pressure

and temperature, causing a faster and better penetration into plant tissue, as well as the transportation of compounds [32].

In our study, when the UAE was applied, the highest rapanone content was achieved for chloroform and ethyl acetate. Interestingly, increasing the number of extraction repetitions did not bring any significant benefits. Furthermore, in a couple of cases, a decreasing trend in terms of rapanone content was observed in the variant of three repetitions for two longer extraction times (20 and 30 min). Generally, in the UAE, the extraction efficiency is highest at the beginning of the sonication process [32]. Exceeding that optimal time can result in the degradation of the cellular structures of the plant material or simply limiting the yield of the process, which is no longer beneficial. For *A. crenata* leaves, 20 or 30 min UAE, once or twice, is enough to obtain optimal efficiency with either chloroform or ethyl acetate. As there was no significant gain in increasing the number of extractions or extending the time over 20 min, it is more economical to choose more time-, solvent- and energy-sparing parameters. Good results were obtained in a 10 min process with chloroform. Here, a trend of increasing the content of rapanone with an increase in the number of repetitions was observed. However, repeating the extraction from the same sample requires additional portions of the solvents, which makes this option less favorable.

All in all, it seems that chloroform and ethyl acetate are both good extractants for rapanone from the leaves of *A. crenata*. The best results were obtained by the UAE method, in which the shortest time (20–30 min) was required and the volume of solvent used was low. An alternative may be maceration with shaking for 30 min with ethyl acetate, but twice or three times, which is definitely more time- and solvent-consuming. The classical heat reflux method (HRE) seems not to be an economically attractive option, as it involved much more time and repetitions of the process to obtain the best yield. Extraction with ethyl acetate in all three methods resulted in a significantly high rapanone content.

The second part of our experiment concerned the assessment of the cytotoxic activity of rapanone. Its potential was compared with embelin, a structural homologue of rapanone, which is a known cytotoxic agent, and an XIAP inhibitor [9]. Embelin activity has been previously reported not only in vitro but also in vivo models (see recent reviews [7,33,34]). Doxorubicin was used as a reference because it is a cytostatic, commonly used in the investigation of potential anticancer agents, and a clinical chemotherapeutic for the treatment of different cancers, e.g., prostate, thyroid, gastric, or hepatic. Interestingly, its structure contains a quinone moiety; however, instead of the simple benzene configuration with two carbonyl groups, as in the case of rapanone or embelin, there is a more complex configuration of tetracene (Figures 1 and 2).

Previously, we reported that rapanone was a selective cytotoxic agent against the melanoma cell line, WM793 [11]. Other studies showed its cytotoxic potential towards several cancer cell lines: Hep-2 (larynx cancer), MCF-7 breast cancer), MKN-45 (gastric cancer), SPC-212 (human mesothelioma), A549 (lung carcinoma), and weak activity for DLD-1 (colorectal adenocarcinoma) [12,13]. In the current study, we evaluated the in vitro cytotoxic potential of rapanone against cancers that are an overwhelming therapeutic challenge affecting a high percentage of the population. The compound was found to be cytotoxic against all cancer cell lines studied: prostate, Du145 and PC3, thyroid, FTC133 and 8505C, colorectal, Caco-2, and HT29, in a dose- and time-dependent manner. Our study not only proved the in vitro anti-cancer potential of rapanone itself, but also demonstrated some interesting differences between its activity and those of embelin and doxorubicin. Doxorubicin is known to be a very potent cytotoxic agent; however, in our study, it was not in the case of 8505C thyroid cancer or PC3 prostate cancer cell lines. Other authors reported  $IC_{50}$  of doxorubicin towards PC3 lines at 0.51  $\mu$ M [35] which still was about 20 times lower activity than towards Du145 [36]. Nevertheless, the doxorubicin resistance of PC3 or 8505C cell lines is a well-known phenomenon [37,38]. Rapanone, in contrast, revealed a high anti-cancer potency in both cases, which was even slightly stronger than that of embelin. Previously, embelin was shown to inhibit the proliferation of castration-resistant prostate cancer cells from PC3 and Du145 lines with an  $IC_{50}$  value of 13.6  $\mu$ M



and 21.3  $\mu\text{M}$ , respectively [39]. It induced G1 cycle arrest and apoptosis. The Akt/NF- $\kappa\text{B}$ /survivin signaling pathway was found to be involved in the mechanism of action of this benzoquinone [39]. Park et al. [40] also indicated that embelin caused apoptosis in the PC3 cell line. Furthermore, it was shown to participate in an inhibition of Akt and GSK-3 $\beta$  activation in prostate cancer cells. Kim et al. [41], in turn, demonstrated an involvement of the Akt/mTOR/S6K1 signaling cascade in the mechanism of pro-apoptotic action of embelin. Heo et al. [42] indicated the STAT3 pathway as a target of a possible mechanism assigned to benzoquinone anticancer activity towards the Du145 cell line.

Furthermore, rapanone activity was similar against both metastatic prostate cancers: Du145 and PC3. It should be noted that PC3 is a grade IV prostate carcinoma, very differentiated from normal cells, and highly aggressive. This is the first report on the antiprostata cancer potential of rapanone, and such a high activity of rapanone against this malignant type of cancer is a tremendously hopeful and promising result.

In addition, rapanone was highly active in both thyroid cancer cell lines, FTC133 and 8505C. This is the first report on its antithyroid-cancer potential. Again, its activity seemed to be even better than that of embelin. The latter was previously shown to induce apoptosis in different thyroid cancer cell lines: BRAF V600E-mutant KTC-1 and 8505C, and wild-type FTC-133 and CAL-62 [43]. Furthermore, it inhibited the cell growth of the PTC (papillary thyroid carcinoma) cell line, causing apoptosis, and induced tumor regression in the PTC xenograft in nude mice [44].

In the case of gastrointestinal adenocarcinoma, the Caco-2 cell line, rapanone was slightly less active than embelin (IC<sub>50</sub> values: 8.76 vs. 6.12  $\mu\text{g}/\text{mL}$ ), but had a better effect on the HT29 cell line (IC<sub>50</sub> values: 16.91 vs. 24.70  $\mu\text{g}/\text{mL}$ ). However, the IC<sub>50</sub> value for rapanone obtained in our study is slightly higher than that reported by Cordero et al. [12], 29.4  $\mu\text{M}$ , which is equal to 9.3  $\mu\text{g}/\text{mL}$ . Embelin, in turn, induced oxidative stress (lipid peroxidation, reduction in reduced glutathione and glutathione S-transferase) in HT29 cells, causing their apoptosis in the study conducted by Sumalatha [45]. The IC<sub>50</sub> calculated by that team was slightly higher than in our experiment, that is 35  $\mu\text{g}/\text{mL}$ .

Excitingly, in our study, rapanone showed a good selectivity profile that was more beneficial than that of both doxorubicin and embelin. Embelin was only a bit less toxic to normal PNT2 cells than to cancerous ones, PC3 and Du145, while doxorubicin already at 10  $\mu\text{g}/\text{mL}$  killed almost all normal cells, whereas cancer cells lines exhibited higher resistance towards the reference cytostatic. However, in the case of HepG2 cells, chosen as the second non-neoplastic cell line in our study, Kuete et al. [13], Cordero et al. [12], and Andreu et al. [14] reported a lower IC<sub>50</sub> for rapanone. However, the activity against Caco-2 reported by Kuete et al. [13] was comparable to our results. Park et al. [40] demonstrated that the IC<sub>50</sub> value for embelin was higher than 200  $\mu\text{M}$  after 24 h and then after 48 h it was 91.6  $\mu\text{M}$ . This large difference may be the result of different study conditions, as Park's team used the EZ-CyTox kit to investigate cell viability [40]. Doxorubicin exhibited cytotoxicity toward control cells at the same or much higher level compared to cancer cell lines. Embelin was slightly safer, as it was less or similarly toxic to that of both control and cancer cells. In our previous study, similar relationships were observed with respect to melanoma and keratinocyte cell lines [11].

Data from the current study revealed for the first time the *in vitro* antiprostata and antithyroid cancer activity of rapanone. Furthermore, rapanone was highly active against an aggressive type of prostate cancer, PC3. Additionally, it seems to be simultaneously selective toward cancerous cells and less toxic against normal cells. Our conclusion on the beneficial selectivity profile is consistent with the conclusions of some other researchers [13,14].

## 4. Materials and Methods

### 4.1. Standards and Reagents

The standards of embelin (98% pure) and rapanone (98% pure) were obtained from Sigma-Aldrich and Sequoia Research Products, respectively. HPLC purity grade acetonitril, chloroform, orthophosphoric acid, and deionized water were obtained from Chempur

Poland. Doxorubicin, cell culture medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, DMEM/F12, Dulbecco's Modified Eagle Medium low glucose, DMEM low glucose, Modified Eagle Medium, MEM with non-essential amino acids NEAA), Triton X-100, penicillin-streptomycin solution, foetal bovine serum FBS, sodium phosphate buffer (pH 7.0) were purchased from Sigma-Aldrich. All reagents used were of analytical grade. The LDH viability test was purchased from Clontech.

#### 4.2. Plant Material

Plant specimens of the *Ardisia crenata* Sims white-berried variety from Van den Bos Premium Ardisia'a B.V. (Steenenburchweg 11, s-Gravenzande, The Netherlands) were purchased in a local florist store (Świat Roślin, Cracow, Poland). Botanical identification was performed by Dr. E. Skrzypczak-Pietraszek of the Department of Pharmaceutical Botany, Jagiellonian University Medical College, Cracow, Poland. The voucher sample (ACRE-W-2017) is deposited in the Department of Pharmacognosy, Jagiellonian University Medical College, Cracow, Poland. *Lysimachia punctata* L. was collected from controlled cultivation in The Garden of Medicinal Plants, Jagiellonian University, Cracow, Poland (GPS coordinates: latitude 50.011298, longitude 19.994175). The voucher specimen (KFg/2010031) is deposited in the Department of Pharmacognosy, Pharmaceutical Faculty, Medical College, Jagiellonian University, Cracow, Poland.

#### 4.3. Optimization of Extraction Conditions and Preparation of Samples and Study Design

Aliquots of 0.2 g finely ground leaves of *A. crenata* Sims were transferred to round bottom glass flasks. For extraction by each of the three methods, each sample was poured with 20 mL of solvent. To choose the best extractant for the tested plant material, three solvents, differing in polarity, were chosen: chloroform, ethyl acetate, and acetone. Three time levels of the processes were implemented. HRE and SE were conducted for 30, 60, and 120 min, while the UAE was conducted for 10, 20, and 30 min. To study the influence of the number of extraction repetitions on its efficacy, another parameter was introduced, and samples were single- (one portion of the solvent), double- (the sample was twice extracted, every time with a new portion of the solvent), or triple-extracted (the sample was three times extracted, every time with a new portion of the solvent). Heat reflux extraction (HRE) was performed in a water bath (90 °C), shaking (SE), and ultrasonic-assisted extractions (UAE), at room temperature. The ultrasonic bath (a Polsonic Palczyński Sp. J., Sonic—3 type) was used for the UAE. The power of the device was 310 W, while the frequency was 50 Hz. Maceration with stirring (SE) was conducted with a laboratory shaker type 358S at a speed of 150 c.p.m. Each combination of experimental parameters was tested in six replicates.

Combinations of parameters with corresponding codes are presented in Table 1.

#### 4.4. Quantitative HPLC Analysis

The procedure was carried out with reference to Podolak and Strzałka [2]. All prepared extracts were transferred to 10 mL volumetric flasks, then the solutions were filtered through 0.45 µm membrane filters into the HPLC 1.5 mL vials and the rapanone content was assessed with an HPLC Dionex apparatus equipped with a Hypersil BDS C-18 column, with mobile phase water with 0.1% v/v H<sub>3</sub>PO<sub>4</sub> (A) and acetonitrile (B), A:B 10:90, a column temperature of 25 °C, a flowrate of 1 mL/min, and a detection wavelength of 286 nm. A rapanone calibration curve was determined with the following concentrations of the substance: 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/mL. Based on the rapanone content in the extracts, its amount in dried plant material was calculated and shown in milligrams of rapanone per 1 g of dried weight of leaves of white-berried *A. crenata*. Representative chromatograms are provided in "Supplementary Materials" (Figure S1).

#### 4.5. Extraction, Isolation, and Identification of the Benzoquinones

For cytotoxic testing, rapanone and embelin were isolated from leaves of the white-berried variety of *A. crenata* Sims and the roots of *L. punctata* L., respectively. The procedure

was described by Wróbel-Biedrawa et al. [11] and Podolak et al. [46]. The purity and identity were checked by the LC–MS method compared to authentic standards, as we previously showed [11]. NMR spectra are provided in Supplementary Materials (Figures S2–S5).

#### 4.6. Cell Culture and In Vitro Cytotoxic Assay

In the study, three human cancer and normal cell lines were used. They were grouped as follows: prostate panel (prostate carcinoma Du145, derived from the metastatic site: brain, ATCC HTB-81; grade IV prostate carcinoma, PC3, derived from the metastatic site: bone, ATCC CRL-1435; prostate epithelial cells PNT2, ECACC 95012613), gastrointestinal panel (colorectal adenocarcinomas Caco2, ATCC HTB-37, and HT29, ATCC HTB-38; hepatocellular carcinoma HepG2, ATCC HB-8065), thyroid panel (follicular thyroid carcinoma FTC133, ECACC 94060901; undifferentiated thyroid carcinoma 8505C, ECACC 94090184). Cells were grown at 37 °C in a 5% CO<sub>2</sub> atmosphere, with relative humidity, with culture medium DMEM/F12 (PC3, PNT2, FTC133, 8505C, HT29, and HepG2), DMEM low glucose (Du145), MEM with NEAA (Caco-2), supplemented with 10% fetal bovine serum (FBS), and appropriate antibiotics. Cells were seeded in 96-well plates ( $1.5 \times 10^4$  cells/well) and kept for 24 and 48 h. Subsequently, the culture medium was replaced with a fresh medium containing different concentrations of the tested substances from 4 to 50 µg/mL and the cells were incubated for 24 and 48 h. As a reference drug, doxorubicin was used. Cell viability was examined using the LDH assay, as previously described [46,47]. The absorbance was measured at 490 nm (the reference wavelength 600 nm) with a Biotek Synergy microplate reader. Each experiment was performed in triplicate. Cytotoxicity of the samples was measured using the formula:

$$\% \text{cytotoxicity} = \frac{A_{\text{sample}} - A_{\text{spont}}}{A_{\text{max}} - A_{\text{spont}}} \times 100$$

where:

$A_{\text{sample}}$  is the absorbance value for cells treated with the tested substances

$A_{\text{spont}}$  is the value for spontaneous LDH release

$A_{\text{max}}$  the value in cells lysed in the presence of Triton X100

The half-maximal effective concentration value  $IC_{50}$ , defined as the substance concentration necessary to obtain 50% of dead cells, was determined.

The half-maximal effective concentration value  $IC_{50}$ , defined as the substance concentration necessary to obtain 50% of dead cells, was determined.

#### 4.7. Statistical Analysis

In the study evaluating optimization extraction conditions, the outlying values of rapanone amount in each set of parameters were removed. Thirty-two outlier values were determined using “Extreme Outlier” software (MP System Co., Kraków, Poland), with an algorithm implemented by Shoemaker and based on a robust technique proposed by Tukey [48]. The ANOVA procedure was used to assess the impact of changing individual parameters on the amount of rapanone obtained in various extraction systems. The same statistical procedure was used to determine the regression coefficients of the primary parameters in the respective multinomial models (describing the relationship between the amount of rapanone and the experimental conditions) and to establish the statistical significance of these coefficients. Then, the results obtained for all combinations of parameters were compared using a Kruskal–Wallis test with a Dunn’s posthoc test (by GraphPad 5 Prism, demo version) to identify the results differing significantly from the rest sets of parameters. The results with the highest number of differences were chosen as representing the combinations of parameters, (i.e., the particular variants of experimental systems), that were used giving the highest efficacy of the extraction process.  $IC_{50}$  values in cytotoxicity analysis were estimated using GraphPad 5 Prism. A comparison of the means in a cytotoxicity analysis was performed using one-way ANOVA followed by a posthoc Tukey test.

Differences with  $p < 0.05$  were considered statistically significant.

## 5. Conclusions

The most efficient extraction of rapanone from the leaves of white-berried *A. crenata* Sims can be achieved with the UAE method, using ethyl acetate or chloroform. However, the method is time-, solvent-, and energy-sparing, and requires special resources. The SE and HRE methods are more time-consuming and require more solvents, but they can be simpler alternatives. For the first time, a high cytotoxic potential of rapanone with a simultaneous beneficial safety profile against prostate cancer cell lines, Du145 and PC3, and thyroid cancer cell lines, FTC133 and 8505C, was revealed.

Our study has provided further confirmation of the anti-cancer activity of rapanone and its potential for further development as a lead compound in the search for antitumor agents. Pre-clinical studies require access to substantial amounts of pure compounds. The optimal conditions for the extraction of rapanone from the leaves of white-berried *A. crenata* Sims. that were proposed in this study may be helpful to obtain the best yield of this bioactive compound and aid further exploration of its antitumor potential.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules27227912/s1>, Table S1. Significant differences between pairs of medians of rapanone content in dried plant material. Figure S1. Representative HPLC chromatograms showing content of rapanone in extracts from leaves of white-berried *A. crenata* Sims, obtained by different methods (HRE, SE, UAE). Figure S2.  $^1\text{H}$  NMR data for rapanone. Figure S3.  $^{13}\text{C}$  NMR data for rapanone. Figure S4.  $^1\text{H}$  NMR data for embelin. Figure S5.  $^{13}\text{C}$  NMR data for embelin.

**Author Contributions:** Conceptualization, D.W.-B., A.G., I.P. and P.Z.; investigation, D.W.-B. and A.G.; data curation, D.W.-B. and P.Z.; writing—original draft preparation, D.W.-B.; writing—review and editing, D.W.-B., I.P., A.G. and P.Z.; visualization, D.W.-B.; supervision, I.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by the Polish Ministry of Science and Higher Education (project: N42/DBS/000292).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are available on request from the authors.

**Acknowledgments:** The authors thank Aleksandra Gdowska, Safiye Nur Yaman, and Sena Gülbahar for technical support in preparing extractions.

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

**Sample Availability:** Samples of the compounds are not available.

## References

1. Podolak, I.; Mynarski, A.; Wróbel, D.; Grabowska, K.; Galanty, A. Bioactive benzoquinones content variability in red-berry and white-berry varieties of *Ardisia crenata* Sims. and assessment of cytotoxic activity. *Nat. Prod. Res.* **2019**, *35*, 157–161. [[CrossRef](#)] [[PubMed](#)]
2. Podolak, I.; Strzałka, M. Qualitative and quantitative LC profile of embelin and rapanone in selected *Lysimachia* species. *Chromatographia* **2008**, *67*, 471–475. [[CrossRef](#)]
3. Calle, J.; Olarte, J.; Pinzon, R.; Ospina, L.F.; Mendoza, M.C.; Orozco, M.J. Alterations in the reproduction of mice induced by rapanone. *J. Ethnopharmacol.* **2000**, *71*, 521–525. [[CrossRef](#)]
4. Lal, B.; Mishra, N. Importance of *Embelia ribes*: An update. *Int. J. Pharm. Sci. Res.* **2013**, *4*, 3823.
5. Souravi, K.; Rajasekharan, P.E. Ethnopharmacological uses of *Embelia ribes* Burm. F. A review. *IOSR J. Pharm. Biol. Sci.* **2014**, *9*, 23–30. [[CrossRef](#)]
6. Kundap, U.P.; Bhuvanendran, S.; Kumari, Y.; Othman, I.; Shaikh, M.F. Plant derived phytochemical, embelin in CNS disorders: A systematic review. *Front. Pharmacol.* **2017**, *8*, 76. [[CrossRef](#)] [[PubMed](#)]
7. Ko, J.H.; Lee, S.G.; Yang, W.M.; Um, J.Y.; Sethi, G.; Mishra, S.; Shanmugam, M.K.; Ahn, K.S. The application of embelin for cancer prevention and therapy. *Molecules* **2018**, *23*, 621. [[CrossRef](#)]

8. Othman, S.N.N.; Lum, P.T.; Sekar, M.; Mazlan, N.A.; Yusri, P.Z.S.; Ghazali, N.F.; Mohd, I.M.; Shazalyana, A.; Ismail, M.; Noor, A.A.M. Molecules of interest—embelin—A review. *Res. J. Pharm. Technol.* **2020**, *13*, 3485–3493. [CrossRef]
9. Nikolovska-Coleska, Z.; Xu, L.; Hu, Z.; Tomita, Y.; Li, P.; Roller, P.P.; Wang, R.; Fang, X.; Guo, R.; Zhang, M.; et al. Discovery of embelin as a cell-permeable, small-molecular weight inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine three-dimensional structure database. *J. Med. Chem.* **2004**, *47*, 2430–2440. [CrossRef]
10. Ospina, L.F.; Calle, J.; Arteaga, L.; Pinzón, R.; Alcaraz, M.J.; Payá, M. Inhibition of acute and chronic inflammatory responses by the hydroxybenzoquinonic derivative rapanone. *Planta Med.* **2001**, *67*, 791–795. [CrossRef]
11. Wróbel-Biedrawa, D.; Grabowska, K.; Galanty, A.; Sobolewska, D.; Żmudzki, P.; Podolak, I. Anti-melanoma potential of two benzoquinone homologues embelin and rapanone—a comparative in vitro study. *Toxicol. Vitro.* **2020**, *65*, 104826. [CrossRef]
12. Cordero, C.P.; Gómez-González, S.; León-Acosta, C.J.; Morantes-Medina, S.J.; Aristizabal, F.A. Cytotoxic activity of five compounds isolated from Colombian plants. *Fitoterapia* **2004**, *75*, 225–227. [CrossRef]
13. Kuete, V.; Omosa, L.K.; Tala, V.R.S.; Midiwo, J.O.; Mbaveng, A.T.; Swaleh, S.; Karaosmanoglu, O.; Sivas, H. Cytotoxicity of plumbagin, rapanone and 12 other naturally occurring quinones from Kenyan flora towards human carcinoma cells. *BMC Pharm. Toxicol.* **2016**, *17*, 1–10. [CrossRef]
14. Andreu, G.L.P.; Dos Reis, F.Z.; González-Durruthy, M.; Hernández, R.D.; D'Vries, R.F.; Berghe, W.V.; Alberici, L.C. Rapanone, a naturally occurring benzoquinone, inhibits mitochondrial respiration and induces HepG2 cell death. *Toxicol. Vitro.* **2020**, *63*, 104737. [CrossRef]
15. de la Vega-Hernández, K.; Antuch, M.; Cuesta-Rubio, O.; Núñez-Figueroa, Y.; Pardo-Andreu, G.L. Discerning the antioxidant mechanism of rapanone: A naturally occurring benzoquinone with iron complexing and radical scavenging activities. *J. Inorg. Biochem.* **2017**, *170*, 134–147. [CrossRef] [PubMed]
16. Kiprono, P.C. Chemistry and Some Biological Activities of Benzoquinones of *Embelia schimperi*. Ph.D. Thesis, University of Nairobi, Nairobi, Kenya, 1997.
17. Ganesan, B.; Perumal, P.; Manickam, V.; Gotteti, S.D.; Srikakolapu, S.R.; Thirumurthy, L.S. Optimization of extraction conditions for embelin in *Embelia ribes* by UV spectrophotometry. *Arch. App. Sci. Res.* **2010**, *2*, 49–53.
18. Alam, M.S.; Damanhour, Z.A.; Ahmad, A.; Abidin, L.; Amir, M.; Aqil, M.; Khan, S.A.; Mujeeb, M. Development of response surface methodology for optimization of extraction parameters and quantitative estimation of embelin from *Embelia ribes* Burm by high performance liquid chromatography. *Pharm. Mag* **2015**, *11*, 166.
19. World Health Organization. Available online: <https://www.who.int/news-room/fact-sheets/detail/cancer> (accessed on 29 October 2022).
20. Cabanillas, M.E.; McFadden, D.G.; Durante, C. Thyroid cancer. *Lancet* **2016**, *388*, 2783–2795. [CrossRef]
21. Mahendran, S.; Thippeswamy, B.S.; Veerapur, V.P.; Badami, S. Anticonvulsant activity of embelin isolated from *Embelia ribes*. *Phytomedicine* **2011**, *18*, 186–188. [CrossRef]
22. Thippeswamy, B.S.; Mahendran, S.; Biradar, M.I.; Raj, P.; Srivastava, K.; Badami, S.; Veerapur, V.P. Protective effect of embelin against acetic acid induced ulcerative colitis in rats. *Eur. J. Pharm.* **2011**, *654*, 100–105. [CrossRef]
23. Kaur, V.; Hallan, S.S.; Nidhi, A.N.; Mishra, N. Isolation of embelin from and evaluation of its anti-cancer potential in *Embelia ribes* breast cancer. *Asian J. Pharm. Pharm.* **2015**, *1*, 33–39.
24. Durg, S.; Kumar, N.; Vandal, R.; Dhadde, S.B.; Thippeswamy, B.S.; Veerapur, V.P.; Badami, S. Antipsychotic activity of embelin isolated from *Embelia ribes*: A preliminary study. *Biomed. Pharm.* **2017**, *90*, 328–331. [CrossRef]
25. Feresin, G.E.; Tapia, A.; Sortino, M.; Zacchino, S.; de Arias, A.R.; Inchausti, A.; Yaluff, G.; Rodriguez, J.; Theoduloz, C.; Schmeda-Hirschmann, G. Bioactive alkyl phenols and embelin from *Oxalis erythrorhiza*. *J. Ethnopharmacol.* **2003**, *88*, 241–247. [CrossRef]
26. Swamy, H.K.; Krishna, V.; Shankarmurthy, K.; Rahiman, B.A.; Mankani, K.L.; Mahadevan, K.M.; Harish, B.G.; Naika, H.R. Wound healing activity of embelin isolated from the ethanol extract of leaves of *Embelia ribes* Burm. *J. Ethnopharmacol.* **2007**, *109*, 529–534. [CrossRef] [PubMed]
27. Afzal, M.; Gupta, G.; Kazmi, I.; Rahman, M.; Upadhyay, G.; Ahmad, K.; Imam, F.; Pravez, M.; Anwar, F. Evaluation of anxiolytic activity of embelin isolated from *Embelia ribes*. *Biomed. Aging Pathol.* **2012**, *2*, 45–47. [CrossRef]
28. Gupta, G.; Kazmi, I.; Afzal, M.; Upadhyay, G.; Singh, R.; Habtemariam, S. Antidepressant-like activity of Embelin isolated from *Embelia ribes*. *Phytopharmacology* **2013**, *4*, 87–95.
29. da Costa, R.C.; Santana, D.B.; Araújo, R.M.; de Paula, J.E.; do Nascimento, P.C.; Lopes, N.P.; Braz-Filho, R.; Espindola, L.S. Discovery of the rapanone and suberonone mixture as a motif for leishmanicidal and antifungal applications. *Bioorg. Med. Chem.* **2014**, *22*, 135–140. [CrossRef]
30. Reguero, M.T.; Mata, R. De la Corteza de *Rapanea guianensis* Aubl Det. wordsck. *Rev. Colomb. Cien. Quím.-Farm.* **1989**, *17*, 17–23.
31. Wang, L.; Weller, C.L. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol.* **2006**, *17*, 300–312. [CrossRef]
32. Esclapez, M.D.; García-Pérez, J.V.; Mulet, A.; Cárcel, J.A. Ultrasound-assisted extraction of natural products. *Food Eng. Rev.* **2011**, *3*, 108–120. [CrossRef]
33. Prabhu, K.S.; Achkar, I.W.; Kuttikrishnan, S.; Akhtar, S.; Khan, A.Q.; Siveen, K.S.; Uddin, S. Embelin: A benzoquinone possesses therapeutic potential for the treatment of human cancer. *Future Med. Chem.* **2018**, *10*, 961–976. [CrossRef] [PubMed]

34. Devi Daimary, U.; Girisa, S.; Parama, D.; Verma, E.; Kumar, A.; Kunnumakkara, A.B. Embelin: A novel XIAP inhibitor for the prevention and treatment of chronic diseases. *J. Biochem. Mol. Toxicol.* **2022**, *36*, e22950. [[CrossRef](#)] [[PubMed](#)]
35. Zhao, W.; Ning, L.; Wang, L.; Ouyang, T.; Qi, L.; Yang, R.; Wu, Y. miR-21 inhibition reverses doxorubicin-resistance and inhibits PC3 human prostate cancer cells proliferation. *Andrologia* **2021**, *53*, e14016. [[CrossRef](#)] [[PubMed](#)]
36. Tyagi, A.K.; Singh, R.P.; Agarwal, C.; Chan, D.C.; Agarwal, R. Silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G2-M arrest, and apoptosis. *Clin. Cancer Res.* **2002**, *8*, 3512–3519. [[PubMed](#)]
37. Massart, C.; Poirier, C.; Fergelot, P.; Fardel, O.; Gibassier, J. Effect of sodium butyrate on doxorubicin resistance and expression of multidrug resistance genes in thyroid carcinoma cells. *Anti-Cancer Drugs* **2005**, *16*, 255–261. [[CrossRef](#)] [[PubMed](#)]
38. El-Zawahry, A.; McKillop, J.; Voelkel-Johnson, C. Doxorubicin increases the effectiveness of Apo2L/TRAIL for tumor growth inhibition of prostate cancer xenografts. *BMC Cancer* **2005**, *5*, 1–9. [[CrossRef](#)]
39. Xu, T.; Li, T.; Zhang, Y.; Huan, S.; Cui, T.; Sun, L. Inhibition of Akt/NF- $\kappa$ B/survivin pathway by embelin on castration-resistant prostate cancer cells. *Int. J. Clin. Exp. Med.* **2017**, *10*, 4386–4397.
40. Park, N.; Baek, H.S.; Chun, Y.J. Embelin-induced apoptosis of human prostate cancer cells is mediated through modulation of Akt and  $\beta$ -Catenin signaling. *PLoS ONE* **2015**, *10*, e0134760. [[CrossRef](#)]
41. Kim, S.W.; Kim, S.M.; Bae, H.; Nam, D.; Lee, J.H.; Lee, S.G.; Shim, B.S.; Kim, S.-H.; Ahn, K.S.; Choi, S.-H.; et al. Embelin inhibits growth and induces apoptosis through the suppression of Akt/mTOR/S6K1 signaling cascades. *Prostate* **2013**, *73*, 296–305. [[CrossRef](#)]
42. Heo, J.Y.; Kim, H.J.; Kim, S.M.; Park, K.R.; Park, S.Y.; Kim, S.W.; Nam, D.; Jang, H.-J.; Lee, S.-G.; Ahn, K.S.; et al. Embelin suppresses STAT3 signaling, proliferation, and survival of multiple myeloma via the protein tyrosine phosphatase PTEN. *Cancer Lett.* **2011**, *308*, 71–80. [[CrossRef](#)]
43. Han, J.M.; Kim, W.B.; Yim, J.H.; Kim, W.G.; Kim, T.Y.; Choi, H.J.; Kim, E.Y.; Shong, Y.K. The effect of a XIAP inhibitor, embelin, on apoptosis of thyroid cancer cell lines. *Cancer Res.* **2012**, *72*, 1709. [[CrossRef](#)]
44. Hussain, A.R.; Bu, R.; Ahmed, M.; Jehan, Z.; Beg, S.; Al-Sobhi, S.; Al-Dayel, F.; Siraj, A.K.; Uddin, S.; Al-Kuraya, K.S. Role of X-linked inhibitor of apoptosis as a prognostic marker and therapeutic target in papillary thyroid carcinoma. *J. Clin. Endocrinol. Metab.* **2015**, *100*, E974–E985. [[CrossRef](#)]
45. Sumalatha, K.; Gowda, M.; Meenakshisundaram, S. ROS-mediated induction of apoptosis by benzoquinone embelin in human colon adenocarcinoma cells HT-29. *J. Complement. Integr. Med.* **2017**, *14*. [[CrossRef](#)]
46. Podolak, I.; Galanty, A.; Janeczko, Z. Cytotoxic activity of embelin from *Lysimachia punctata*. *Fitoterapia* **2005**, *76*, 333–335. [[CrossRef](#)]
47. Grabowska, K.; Podolak, I.; Galanty, A.; Żmudzki, P.; Koczurkiewicz, P.; Piska, K.; Pékala, E.; Janeczko, Z. Two new triterpenoid saponins from the leaves of *Impatiens parviflora* DC. and their cytotoxic activity. *Ind. Crop. Prod.* **2017**, *96*, 71–79. [[CrossRef](#)]
48. Shoemaker, J. Robust Outlier Identification Using SAS<sup>®</sup>. Available online: <https://support.sas.com/resources/papers/proceedings/proceedings/sugi24/Infovis/p161-24.pdf> (accessed on 15 September 2022).