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Isolation, Characterization, and Breast Cancer Cytotoxic Activity of Gyrophoric Acid from the Lichen *Umbilicaria muhlenbergii*

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Abstract: Lichens produce a large variety of secondary metabolites with diverse bioactivities, chemical structures, and physicochemical properties. For this reason, there is a growing interest in the use of lichen-derived bioactive molecules for drug discovery and development. Here, we report on the isolation, identification, and cytotoxic evaluation of gyrophoric acid (GA) from the lichen *Umbilicaria muhlenbergii*, a largely unexplored and scantly described lichen species. A simple purification protocol was developed for the fractionation of lichen crude extracts with silica gel column chromatography using solvents with changing polarity. GA was identified in one of the fractions with Fourier transform infrared spectroscopy (FTIR), ion trap mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (^{1}H-NMR and ^{13}C-NMR). The FTIR spectra demonstrated the presence of aromatic and ester functional groups C=C, C-H, and C=O bonds, with the most remarkable signals recorded at 1400 cm^{-1} for the aromatic region, at 1400 cm^{-1} for the CH_3 groups, and at 1650 cm^{-1} for the carbonyl groups in GA. The MS spectra showed a molecular ion [M-1]$^{-}$ at (m/z) 467 with a molecular weight of 468.4 and the molecular formula $C_{24}H_{20}O_{10.}$ that correspond to GA. The ^{1}H-NMR and ^{13}C-NMR spectra verified the chemical shifts that are typical for GA. GA reduced the cell viability of breast cancer cells from the MCF-7 cell line by 98%, which is indicative of the strong cytotoxic properties of GA and its significant potential to serve as a potent anticancer drug.

Keywords: lichen; *Umbilicaria muhlenbergii*; secondary metabolites; gyrophoric acid; breast cancer; cytotoxicity

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1. Introduction

Cancer is a complex disease with a strong relationship between genetic and environmental factors [1]. One of the hallmarks of cancer is that cancer cells can penetrate adjacent parts of the body and lead to invasion and metastasis [2]. Although cancer research has advanced considerably over the years, cancer mortality remains high [3]. A report showed that the cancer rate has been escalating since 1990, with the most common types being lung, colorectal, and stomach cancers [4]. Between 2012 and 2020, new cancer cases increased by 37% to reach 19.3 million [5,6]. In the U.S. alone, 1.8 million new cancer cases occurred, representing 9% of the global cancer occurrence in 2020 [7]. Among cancer types, breast cancer remains one of the most common lethal types of cancer for women [8], resulting in the deaths of 1 out of 10 women diagnosed with breast cancer [9]. In 2020, there were 2.3 million women diagnosed with breast cancer and 685,000 deaths globally [10]. The

conventional methods of cancer treatment include surgery, radiotherapy, and chemotherapy [11]. In this regard, investigations to identify new chemotherapeutic agents, such as natural compounds with minimum side effects, are gaining importance [12,13].

Natural bioactive molecules are becoming widely recognized for their antifungal, antibiotic, antiviral, anti-inflammatory, and antitumor activities [14]. About 60% of all natural compounds are known as pharmaceutical agents [15], with more than half of all approved drugs for cancer treatment originating from natural sources [16]. Among these natural sources, lichens are symbiotic organisms with diverse secondary metabolites [3,17–19]. These organisms, in addition to their well-known traditional medicinal uses for folk therapeutic and phytochemical purposes [20–23], have been also utilized in other practical areas, including dyes, perfumes, and food [24,25].

More than 1000 secondary metabolites have been identified in different lichens with various biological and pharmacological activities due to the presence of numerous aliphatic, cycloaliphatic, aromatic, and terpenoid compounds [13,26,27]. Lichen crude extracts and lichen secondary metabolites have produced apoptotic and cytotoxic effects against various cancer cell types. For example, lichen extracts and secondary metabolite parietin from *Parmelia sulcate* and *Xanthoria parietina* showed antiproliferative activity against breast cancer cells [28,29]. Similarly, the lichen *Cladonia foliacea* and its secondary metabolites using acid, atranorin, and fumarprotocetraric acid, as well as *Lobaria pulmonaria* and its metabolite static acid, were active against colon cancer cell lines [30,31]. Evidence exists for the promising anti-tumor effects of other lichen-derived secondary metabolites, such as protolichesterinic acid and physodic acid on cervical cancer [32,33], olivetoric acid on liver cancer [34], and atranorin and usnic acid on melanoma [35,36]. Overall, research on lichen-derived secondary metabolites to date has demonstrated their great potential for use as therapeutic agents. Strong evidence has been obtained for their mechanism of action on cancer cells that includes apoptosis, necrosis, or autophagy, with the cell cycle arrest at G0/G1 phases; cell cycle regulation associated with cyclin-dependent kinases (CDK4, CDK6) or cyclin D1; oxidative stress due to superoxide dismutase (SOD) or malondialdehyde (MDA); modulation of inflammatory responses via TNF-α, IL-1β, IL-6, and TGF-β1; and targeting of microRNA molecules, modulation of anti-proliferative effects by regulating signaling pathways (ERK1/2 and AKT), or proliferating protein marker Ki-67 [37,38].

The biological activities and therapeutic potential of secondary metabolites from *Umbilicaria* species have also been investigated. These bioactive molecules had different aromatic, aliphatic, and cyclic structures and were isolated from *Umbilicaria* species, such as *U. crustulosa*, *U. cylindrical* [39], *U. crustulosa*, *U. cylindrical*, *U. polyphylla* [40], and *U. hirsuta* [19]. In the present study, we report on the extraction, fractionation, purification, and cytotoxic evaluation of bioactive fr3actions from the lichen *U. muhlenbergii* against the breast cancer cell line MCF-7. By applying in vitro cell viability MTT assays coupled with an array of spectroscopic, physicochemical isolation, and characterization techniques, we were able to isolate and identify the secondary lichen metabolite GA as a potent and promising cytotoxic molecule. The current work is a continuation of our previous studies on *U. muhlenbergii* [38,41] and provides new evidence for the effects of purified GA on cancer cells. Although GA is known for its anti-tumor activity, reports on its isolation methods are scant. Our objective was to present a systematic study that describes an efficient extraction and purification protocol for the isolation of GA from *U. muhlenbergii*. The protocol that we have developed may be used as an alternative route for the production of GA acid as well as the isolation and purification of other polyphenolic depside molecules.

2. Materials and Methods

2.1. Collection of Lichen Specimens

Lichen samples were collected around the Tamblyn Lake of Northwest Ontario, Canada, and identified as *U. muhlenbergii* based on an organoleptic macroscopic examination. A voucher specimen was deposited in the Claude Garton Herbarium at Lakehead University, Thunder Bay, Ontario, Canada.

2.2. Chemicals

Dulbecco's modified Eagle's medium (DMEM), dimethylsulfoxide (DMSO), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS) containing calcium, magnesium, penicillin/streptomycin, and trypsin-EDTA solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and regents were bought from ThermoFisher Scientific (Waltham, MA, USA).

2.3. Cell Culture and Maintenance

The human breast cancer cell line MCF-7 (purchased from American Type Culture Collection, ATCC, Manassas, VA, USA) was grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cell line was maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.4. Cell Viability Assay and IC_{50}

The MTT method [41] was used to measure cell viability. The MCF-7 cells were seeded in a 96-well plate (Corning@Costar, 96-well plate) at a concentration of 1×10^4 cells/well and incubated in an atmosphere of 5% CO_2 and 37 °C for 24 h to obtain good adherence. The initial medium was then replaced with fresh medium supplemented with 300–500 µg/mL of lichen extract. The MTT substrate, prepared in a physiologically balanced solution, was added to the cell in culture at a final concentration of 5 mg/mL and incubated at 37 °C for 4 h. Thereafter, 50 µL of DMSO was added to each well to dissolve the formazan crystals [13]. The absorbance of each well was measured with a BioTek Microplate Reader Spectrophotometer (San Francisco, CA, USA) at 490 nm (using a reference wavelength of 690 nm). The cells in the medium alone and those in hydrogen peroxide (H_2O_2) served as a negative and positive control, respectively [42]. The percentage cell viability was calculated as per Equation (1):

$$Cellviability(\%) = (At - Ac)/(ADMSO - Ac) \times 100 \quad (1)$$

where At is the absorbance of treated cells, Ac is the absorbance of background controls, and ADMSO is the absorbance of matched DMSO concentration controls. The half-maximal inhibitory concentration (IC_{50}) was determined from the cell viability assay using 300–500 µg/mL of lichen extract [43].

2.5. Extraction and Lichenochemical Analysis

The lichen samples were air-dried and ground using a mortar and pestle. A 50 g dried lichen powder sample was soaked in 500 mL acetone (HPLC grade) and placed in an airtight screw cap bottle on an Innova 44 orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm and at room temperature for 24 h. The crude acetone extract was then dried by vacuum evaporation using a rotary evaporator (Buchi, New Castle, DE, USA). Thereafter, the extract powder was subjected to preliminary lichenochemical screening and analysis using different reagents as described in [35] to identify the presence of phytochemicals (alkaloids, hydrocarbon, terpenoids, proteins, tannins, flavonoids, saponins, phenols, and glycosides).

2.6. Purification and Characterization of GA

As shown in Figure 1, following the initial solvent extraction of the lichen *U. muhlenbergii*, solvents (acetone) were removed by air-drying evaporation in a biosafety cabinet at room temperature, and the dried extracts were redissolved in fresh acetone and subjected to successive extraction on a 60 G silica gel column using solvents with increasing polarity (hexane, ethyl acetate, ethyl acetate: methanol (9:1), ethyl acetate: methanol (8:2), ethyl acetate: methanol (5:5), and pure methanol). Following solvent evaporation, the dried extracts were analyzed spectroscopically to identify the bioactive component (GA) using

UV-Vis spectroscopy; Tensor37 Fourier transformer Infrared (Bruker, Billerica, MA, USA); amazonX ion trap mass spectrometry (Bruker, Billerica, MA, USA) in negative mode; and 1H and 13C NMR spectra at 500 and 125 MHz, with 16 scans using a Bruker Avance III 500 spectrometer (Bruker, Billerica, MA, USA). The 2D NOESY gHSQC and gHMBC methods were employed [19]. The purity validation of fraction F1 was carried out with high-performance liquid chromatography (HPLC, Agilent Technologies 1260) with DAD detection, Kromasil SGX C18 column (7 µm), mobile phase A (5% acetonitrile +1% (*v*/*v*) trifluoracetic acid), and mobile phase B (80% acetonitrile), employing the isocratic program at a flow rate of 0.7 mL/min: 0 min (50% A and 50% B); 25 min (0% A and 100% B); 30 min (50% A and 50% B).

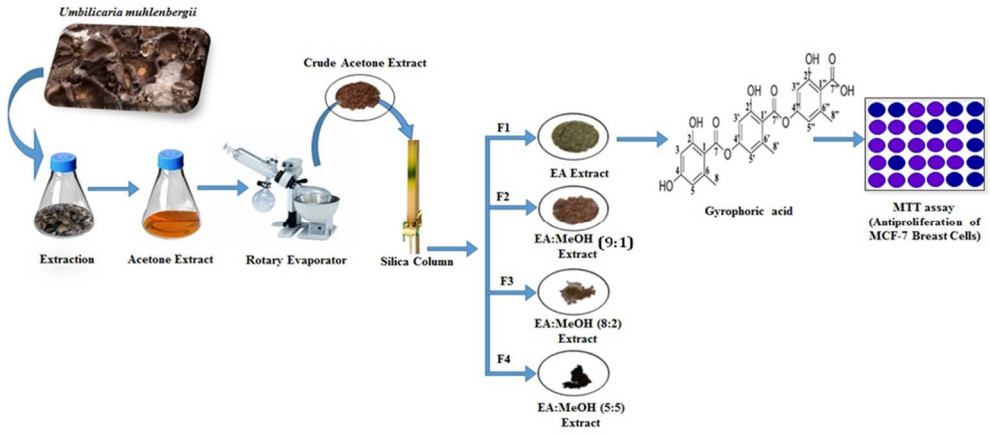

Figure 1. Schematic diagram of purification and MTT characterization of GA from *U. muhlenbergii.* EA, ethyl acetate; MeOH, methanol.

2.7. Statistical Analysis

All experiments were carried out in triplicate, and each result was the average of three independent experiments. Statistical analysis was performed with Microsoft Excel 2010, and the data are presented as the mean standard deviation (±SD) of triplicates. One-way Analysis of Variance (ANOVA) with *p*-values were calculated from https://goodcalculators.com/one-way-anova-calculator/ (accessed on 6 June 2022).

3. Results and Discussion

3.1. Lichen Identification and Anti-Proliferation Screening

A lichen specimen was collected from the area of Tamblyn Lake in Northwestern Ontario, and following organoleptic macroscopic examination, it was identified as *Umbilicaria muhlenbergii*. Acetone extraction of the lichen yielded 14.3% (*w*/*w*) dried crude acetone extract. The cytotoxic activity of acetone extracts from *U. muhlenbergii* against MCF-7 breast cancer cell line has been described in our previous study [41].

3.2. Lichenochemical Analysis

Lichenochemical screening studies on the crude extract of *U. muhlenbergii* revealed the presence of alkaloids and phenols as major components, with tannins, saponins, and hydrocarbons present in smaller quantities, whereas flavonoids, terpenoids, proteins, and glycosides were not detected (data not shown). A qualitative analysis of two Sri Lankan extracts from the lichens *Parmotrema tinctorum* and *Parmotrema rampoddense* revealed the presence of saponins, flavonoids, and polyphenols [44]. Anthracene glycosides were present in *P. tinctorum* but not in *P. rampoddense*, whereas proteins, alkaloids, tannins, reducing sugars, cyanogenic glycosides, and cardenolide glycosides were absent from both lichen extracts [44]. The presence of phenols and alkaloids with strong bioactivities is also in agreement with the published literature [17,45,46]. It has been reported that most of the lichen phenolics isolated from *Parmelia caperata*, *Cladonia convoluta*, *C. rangiformis*,

Platisma glauca, and *Ramalina cuspidata* exhibited strong antioxidant activities and anticancer activities [47]. Lichen-derived compounds have been extensively discussed in previous reviews [48,49], and they provide in-depth information on their bio-therapeutic potential.

3.3. Purification and Characterization of GA

A detailed schematic diagram of purification of GA is shown in Figure 1. The bioactive compound(s) responsible for the anticancer activity of *U. muhlenbergii* was purified with successive solvent elution using nonpolar (hexane and ethyl ether) and polar (methanol, ethyl acetate) solvents as well as their combinations. It appeared that hexane (nonpolar solvent) and methanol (polar solvent) did not produce any bioactive fractions; however, the solvent elution with ethyl acetate alone, and in combination with methanol, yielded chemical containing fractions (F1–F4), as shown in Table 1. In many cases, the purification of secondary metabolites from lichens is tedious and complex. The product yield is affected by the type of solvent used; the presence of other compounds with similar structures and colored substances (pigments); possible interactions; and/or the formation of hydrogen bonding between the targeted molecule, the solvent, and other molecules. All this may create a masking effect due to the overlapping of compounds of different solubility and polarity with the molecule of interest, gyrophoric acid in our case, thereby altering the molecule's polarity and reducing its purification yield [50]. A maximum eluent yield of 0.14% (*w*/*w* of lichen biomass) was obtained in fraction F1 using ethyl acetate as the solvent. Fractions F2–F4 contained extraction yields that decreased as the polarity of the mixed solvent increased.

Table 1. Solvent fractionation and extraction yield of *U. muhlenbergii*.

Fractions	Solvents Used	Eluent Yields (%)
F1	Ethyl acetate	0.14
F2	Ethyl acetate: methanol (9:1)	0.06
F3	Ethyl acetate: methanol (8:2)	0.02
F4	Ethyl acetate: methanol (5:5)	0.02

Fractions F1-F4 were examined for their cytotoxic effect on MCF-7 breast cancer cells. Fractions F1 and F2 showed a maximum of 98% and 96% cytotoxicity, respectively (Figure 2). These results were comparable with the positive control of hydrogen peroxide (H_2O_2). In comparison, fractions F3 and F4 had a much lower antiproliferative activity, thereby reducing the MCF-7 cell viability by only 26% and 21%, respectively. Thus, fraction F1 was selected for further purification and identification studies as it contained the highest eluent yield (Table 1) and highest anti-proliferative activity (Figure 2). These studies included spectroscopic MS, UV, FTIR, and NMR data for F1 [19,51–57].

The UV absorbance of F1 (Figure S1) was measured in the wavelength range of 240–340 nm. The UV absorbance peaks for GA were detected at 210, 240, 310, and 340 nm, with high similarity for the depside groups [48] and in good agreement with previous findings [58–60].

The IR spectrum revealed functional groups and strength binding [13,61]. From the IR spectra at 600–4000 cm^{-1} (Figure S2), it is evident that the peaks at approximately 3300, 3200, and 3665 cm^{-1} reflect the stretching vibration of hydroxyl (OH) groups. The bands at 670–900 cm^{-1} result from the presence of aromatic groups and C-H bending in the compound.

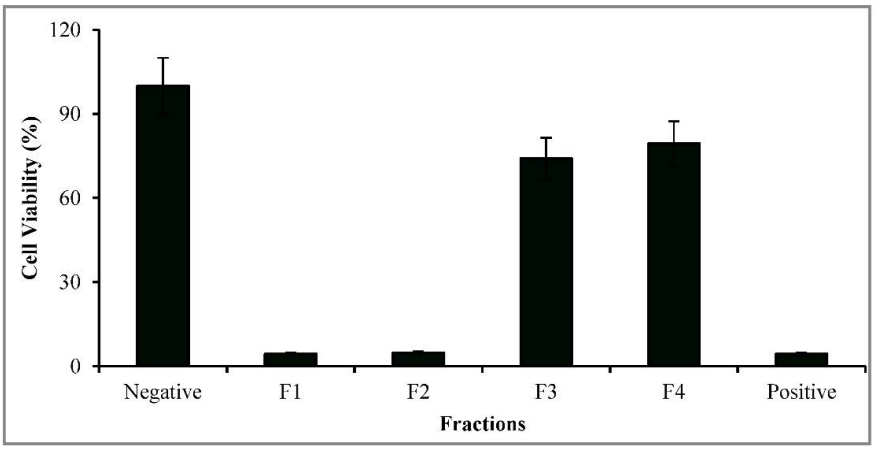

Figure 2. Cell viability assay via MTT for different solvent fractions (1000 µg/mL) on MCF-7 cells with negative (media and cells without substances) and positive (1mM H_2O_2). F1—ethyl acetate, F2—ethyl acetate: methanol (9:1), F3—ethyl acetate: methanol (8:2), and F4—ethyl acetate: methanol (5:5). Data represented in error bars are the mean values of three observations ± SD. Data were significant ($p < 0.001$) as confirmed with ANOVA.

The electron ionization mass spectroscopy (Figure 3) was recorded in the negative ionization mode $[M-1]^-$ (m/z) at 467.0, with an error of 1.4 ppm, corresponding to GA with an exact molecular weight of 468.4 and molecular formula of $C_{24}H_{20}O_{10}$ (Table 2). Its fragmentation produced an ion at (m/z) 316.9 due to ester cleavage, which may indicate lecanoric acid in trace amounts with an error of 1.1 ppm, as presented in Table 2 [55,62,63]. Similar fragmentation patterns for GA were also observed during the rapid identification of lichen compounds based on the structure–fragmentation relationship using ESI-MS/MS analysis [52]. Other studies also demonstrated that GA is present in high concentrations and is the prevalent depside in the Umbilicaria genus [19,53,64–67]. The chemical formula $C_{24}H_{20}O_{10}$ for GA was verified from MS data in several reports [52,68,69].

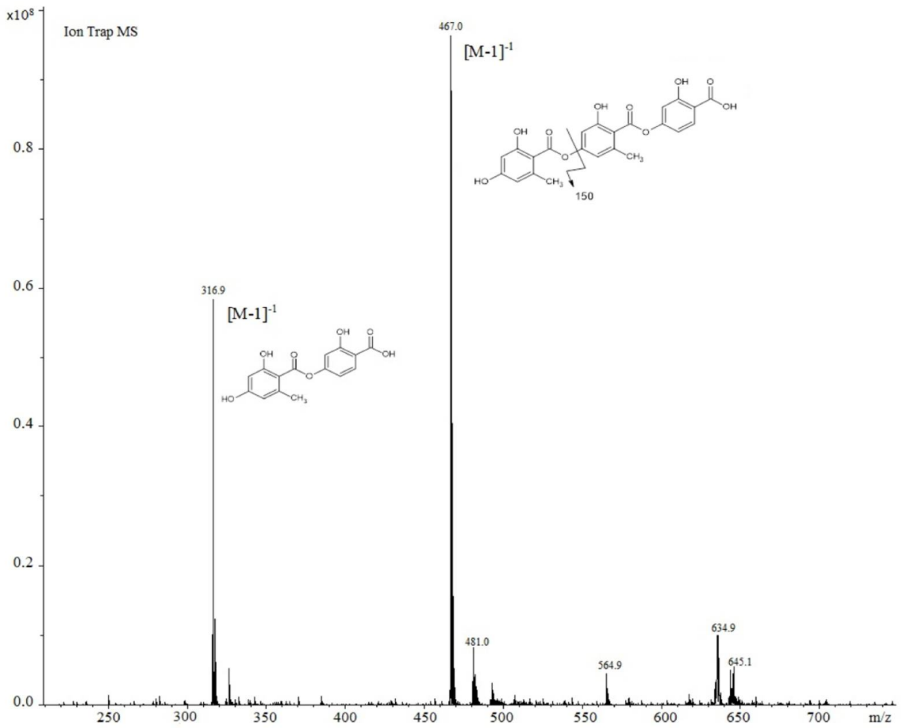

Figure 3. Mass spectrum of fraction F1.

Table 2. Mass spectral identification of fraction F1 with respect to fragment numbers, accurate masses, and formula.

Identified Compound	Accurate Mass (*m*/*z*)	Exact Mass	Error (ppm)	Formula
Molecular ion [−1]	467.0	468.40	1.4	$C_{20}H_{24}O_{10}$
Fragment	316.9	318	1.1	$C_{16}H_{14}O_7$

The ^{1}H-NMR and ^{13}C-NMR spectra verified the chemical shifts that are typical for GA [52,61,69,70] ^{1}H-NMR (Figure S3): (DMSO-d6, 500 MHz) δ 10.52 (hydroxyl protons), 6.66 (1H, d, *J*5″, 3″ = 2.6 Hz) (aromatic protons), 6.69 (1H, d, *J*3′, 5′ = 1.3 Hz), 6.49 (1H, d *J*3″, 5″ = 2.6 Hz), 2.36 (3H, s), 6.44 (1H, d, *J*5′, 3′ = 1.3 Hz), 6.25 (1H, d, *J*3, 5 = 2.6 Hz), 6.23 (1H, d, *J*5, 3 = 2.6 Hz), 2.35 (3H, s), 2.49 (3H,s).

^{13}C-NMR (Figure S4): (DMSO-d6, 125 MHz) δ 108.49 (C-1), 160.0 (C-2), 100.5 (C-3), 161.12 (C-4), 109.84 (C-5), 140.20 (C-6), 167.18 (C-7), 21.28 (C-8), 118.28 (C-1′), 156.30 (C-2′), 107.22 (C-3′), 152.07 (C-4′), 114.21 (C-5′), 137.92 (C-6′), 165.78 (C-7′), 19.35 (C-8′), 116.70 (C-1″), 162.44 (C-2″), 107.17 (C-3″), 151.83 (C-4″), 113.45 (C-5″), 141.17 (C-6″), 171.47 (C-7″), 22.12 (C-8″).

Evidence for the localization of the methyl and hydroxyl groups on the aromatic rings of GA was obtained from heteronuclear multiple bond correlation (HMBC) experiments (Figure S5). The HMBC spectra showed HMBC correlations between the protons of the methyl groups/hydroxyl groups and the corresponding carbon atoms. In addition, the heteronuclear single quantum coherence (HSQC) spectrum provided data of direct determination of carbon and attached protons (hydrogen), which belong to the same spin system (Figure S6). Based on these observations, the molecular structure of GA can be presented as shown in Figure 4. The GA purity was further confirmed by HPLC (data not shown). GA, isolated from *U. hirsuta*, was likewise identified by ^{1}H-NMR and ^{13}C-NMR, and validated with HPLC [19].

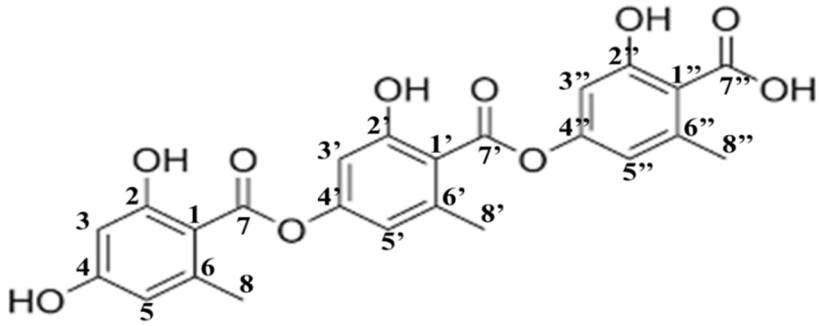

Figure 4. Molecular structure of gyrophoric acid ($C_{24}H_{20}O_{10}$), 4-({4-[(2,4-dihydroxy-6-methylphenoxy)carbonyl]-2-hydroxy-6-methylbenzoyl}oxy)-2-hydroxy-6-methylbenzoic acid.

3.4. Cytotoxicity of GA

Lichen secondary metabolites are known to have diverse biological activities, including antibacterial, antitumor, antiproliferative, and cytotoxic effects [18,48]. Anticancer activities have been reported for several lichen-derived compounds, such as stictic acid [31], physodic acid [13], and parietin, to name a few. As evident from Figure 5 of this study, GA isolated from *U. muhlenbergii* crude extracts decreased the cell viability of MCF-7 cells. At 300 µg/mL GA, the cell proliferation was inhibited by approximately 45%, and thereafter, with a further increase in the GA dose up to 500 µg/mL, it reached a plateau. Keeping in mind the SD of the data presented in Figure 5, no significant difference in the cell viability was observed in the 300–500 µg/mL GA concentration range. This might be due to the fact that the MCF-7 cancer cells were exposed to GA for only 4 h. The low exposure time did not allow for the development of a clear dose-dependent correlation between cell viability and

GA concentration using the MTT assay. Similar observations were reported for the impact of the GA dose on the inhibition of cell proliferation for HDF cells after their incubation with GA for 24 h [19]. Increasing the incubation time to 72 h produced a well-pronounced dose-dependent effect of GA on cell proliferation. The IC_{50} value of 478 μM of GA from *U. muhlenbergii*, calculated from the cell viability data on MCF-7 cells, was comparatively close to the 384 μM reported for GA from *U. hirsuta* on the same cell line [19]. In comparison, a standard GA showed >200 μM on the HT-29 human colon adenocarcinoma cell line [70]. Previous work has identified GA as a major bioactive compound present in 31 of the 33 studied *Umbilicaria* species, including *U. muhlenbergii* [66,71]. The present data are consistent with our previous findings that suggested the inhibition of cellular proliferation and induction of apoptosis of *U. muhlenbergii* crude extracts as possible causes for MCF-7 cell death [41], and they provide further evidence that GA can act as a potent anticancer agent.

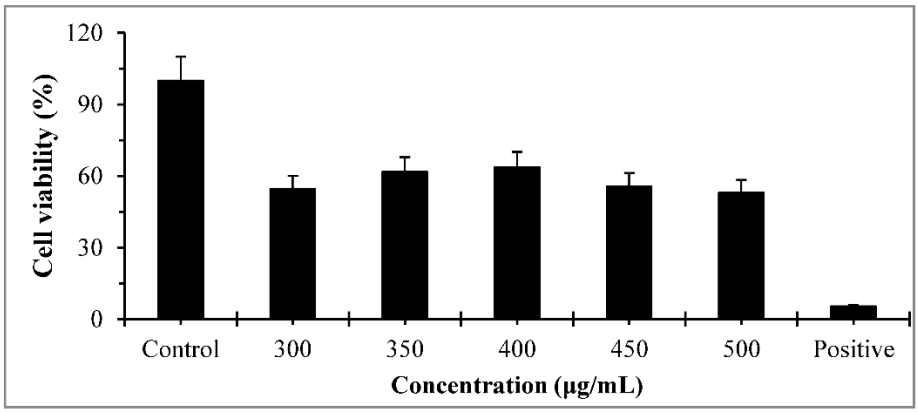

Figure 5. Cell viability assay via MTT for gyrophoric acid on MCF-7 cells at different concentrations (300 to 500 μg/mL), positive control (1mM H_2O_2), negative control (media and cells without substances). Data represented in error bars are the mean values of three observations ± SD. Data were significant ($p < 0.001$) as confirmed with ANOVA.

4. Conclusions

This study demonstrates that the lichen *U. muhlenbergii* is capable of producing GA as a predominant secondary metabolite with strong anticancer activity against MCF-7 breast cancer cells. A simplified extraction and purification protocol to obtain pure GA from *U. muhlenbergii* crude extract was developed. The molecular structure of GA was validated through spectroscopic analyses that included UV, FTIR, MS, NMR, HSQC, and HMBC. GA, 4-({4-[(2,4-dihydroxy-6-methylphenoxy)carbonyl]-2-hydroxy-6-methylbenzoyl}oxy)-2-hydroxy-6-methylbenzoic acid, is a depside molecule that, in addition to the three aromatic rings, contains functional hydroxyl and carboxyl groups. While the polyaromatic structure of GA facilitates free radical scavenging activity, the functional side-groups impart selective reactivity of GA toward biochemical molecules, such as enzymes and cell-bound proteins, thereby promoting a dynamic interaction with different cell types. Due to the unique properties and functionalities that the molecular structure of GA presents, its potential therapeutic applications are expanding beyond the promise it holds as a cytotoxic and antitumor agent to encompass new medicinal uses that take advantage of the antioxidant, antimicrobial, and anti-inflammatory utilities of GA. Further assessment of the cytotoxic effects of GA in clinical studies is needed to establish the potential for anticancer drug development and application.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr10071361/s1, Figure S1: UV spectrum of fraction F1 (gyrophoric acid), Figure S2: IR spectrum of fraction F1 (gyrophoric acid); Figure S3: ^{1}H-NMR spectrum of fraction F1 (gyrophoric acid); Figure S4: ^{13}C-NMR spectrum of fraction F1 (gyrophoric acid); Figure S5: Selected HMBC correlations in fraction F1 (gyrophoric acid); Figure S6: HSQC spectrum of fraction F1 (gyrophoric acid).

Author Contributions: Methodology, M.M. and V.Z.; formal analysis, software and validation, M.M.; conceptualization, writing—original draft preparation, M.M., V.Z. and L.C.; final review, editing and final draft preparation, V.Z., Z.S. and L.C. All authors have read and agreed to the published version of the manuscript.

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

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