Cocrystal Formation of Betulinic Acid and Ascorbic Acid: Synthesis, Physico-Chemical Assessment, Antioxidant, and Antiproliferative Activity

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Betulinic acid (BA) was demonstrated to be a very promising anticancer agent against various tumor cell lines such as breast, colon, lung, and brain. Despite its strong cytotoxic effect, betulinic acid exhibits low water solubility, feature that is reflected in its poor bioavailability. To overcome these drawbacks, numerous strategies were conducted to improve its physicochemical and pharmacokinetic profile, among which cocrystallization emerged as a promising approach. Thus, our work consisted in obtaining slowly grown cocrystals of BA and ascorbic acid (BA+VitC) in isopropyl alcohol obtained in a hydrothermal experiment. The newly formed cocrystals were characterized by physico-chemical methods such as SEM, DSC, XRPD, and FT-IR spectroscopy demonstrating BA+VitC cocrystal formation while their antioxidant activity revealed an additive antioxidant effect. To investigate the biological effect, BA+VitC cocrystals were tested on HaCat (immortalized human keratinocytes), B164A5 and B16F0 (murine melanoma), MCF7 and MDA-MB-231 (human breast cancer), and HeLa (cervical cancer) cell lines. Results of BA upon the tested tumor cell lines, after cocrystallization with vitamin C, indicated a superior cytotoxic effect with the preservation of a good selectivity index assumably due to an improved BA water solubility and consequently an optimized bioavailability.

Keywords: cocrystal, betulinic acid, vitamin C, antioxidant activity, antiproliferative activity

INTRODUCTION

The field of pharmaceutical formulations is similar to a living organism, constantly changing, and adapting to new therapeutic needs. Numerous newly reported active chemical compounds exhibit low water solubility and dissolution rate which may correlate to poor bioavailability, especially via oral administration (Gadade et al., 2016). Among the current options to mitigate these flaws (Prasad et al., 2012), crystal engineering provides several possibilities to develop...
single- or multi-component alterations of an active compound, including the synthesis of pharmaceutical cocrystals (Pathak et al., 2013). Cocrystals are crystalline complexes of active/neutral compounds that form a unique crystalline lattice through non-covalent bonds, in particular hydrogen bonds; the main benefit associated with cocrystallization consists in the preservation of the intrinsic pharmacological properties of the active ingredients while the physicochemical profile (i.e., melting point, solubility, dissolution, etc.) changes (Prasad et al., 2012). Drug-drug cocrystals represent a promising line of research in light of the fact that combined therapies are frequently prescribed for the effective treatment of numerous pathologies (Sekhon, 2012); cocrystals of multiple active compounds might overcome the drawbacks of conventionally combined drugs.

Betulinic acid [BA, 3β-hydroxy-20(19)-lupan-28-oic acid] is a pentacyclic triterpene of lupan skeleton with a wide range of pharmacological activities such as: anti-inflammatory, antitumor (Faujan et al., 2009), anti-angiogenic (Mukherjee et al., 2004), anti-viral (Baltina et al., 2003), and antiplasmodial (Ziegler et al., 2004); its first anticancer effect against melanoma was reported by Pisha et al. (1995). The antitumor activity of betulinic acid is currently known to be selectively exerted on numerous tumor cell lines (breast, colon, lung, brain) mainly by apoptosis induction (Fulda and Kroemer, 2009). The favorable therapeutic index suggests betulinic acid as promising antitumor agent (Pisha et al., 1995). It also possesses significant antioxidant properties as revealed by Peng et al. (2015).

Betulinic acid is characterized by high hydrophobicity and limited aqueous solubility leading to poor bioavailability; therefore, it qualifies as candidate for cocrystallization in order to achieve superior pharmacokinetic properties.

Ascorbic acid, vitamin C, (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one (VitC), is a natural highly water-soluble vitamin which acts as a potent reducing and antioxidant agent; its biomedical uses reside in fighting bacterial infections, in detoxifying reactions, and in the formation of collagen in fibrous tissue, teeth, bones, connective tissue, skin, and capillaries. VitC was previously used as cocrystal former for several nutraceuticals in a patent issued in 2008 for the purpose of providing improved properties, in particular as oral formulations (Zaworotko et al., 2008). It also acted as cocrystal former for several zwitterion structures (i.e., sacrosine, nicotinic acid, betaine) leading to cocrystals that exhibit carboxylate-hydroxyl supramolecular heterosynthons (based on intermolecular hydrogen bonds) (Kavuru et al., 2010).

Up until recently, a significant number of computational methods that can predict new cocrystal formation from various structures have been developed. Such an approach consists in the determination of molecular electrostatic potential surfaces (MEPS) for molecular complementarity assessment, as well as the assessment of potential cocrystal formation based on an electrostatic model described by intramolecular interactions as a set of specific contact interaction points (SSIPs) on the molecular surfaces (Karagiani and Malamataris, 2018).

A synergistic antioxidant effect of betulinic acid and ascorbic acid mixture was described by Adesanwo et al. (2013) who suggested the ascorbic acid-mediated chain-breaking electron transfer to DPPH (2,2-diphenyl-1-picrylhydrazyl) followed by ascorbic acid regeneration due to proton transfer from betulinic acid, thus leading to a resonance stabilized betulinic acid radical (Adesanwo et al., 2013).

This paper is the first approach to obtain and characterize slowly grown cocrystals of betulinic acid and ascorbic acid, using as solvent isopropyl alcohol. Given the already reported significant antioxidant activity of both compounds and that a Cambridge data base search was made and the a BA+VitC cocrystal has yet to be reported, we also aimed to assess the synergistic/additive result of cocrystallization in terms of this particular biological effect. To the best of our knowledge, no previous studies of betulinic acid cocrystals were reported. In addition, the biological in vitro activity of the cocrystals was tested on both normal and cancer cell lines.

**MATERIALS AND METHODS**

**Materials**

Betulinic acid (99%), L-ascorbic acid (99.7–100.5%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Germany; isopropyl alcohol and ethanol 96% (v/v) from Chemical Company SA, Iasi, Romania. All substances were used without further purification.

**Gas Phase ab initio DFT Calculations for BA-VitC Cocrystal Formation**

This method is based on the calculation of gas phase MEP for the proposed structures, followed by the conversion of local MEP maxima and minima into SSIPs, α and β, that describe the potential H-bond interaction sites (Musumeci et al., 2011; Grecu et al., 2013). Structure geometry optimization and DFT calculations were employed by using the GAMESS software while MEPS were generated using MacMolPlt and Avogadro. Geometry optimization and MEP for both structures were achieved by *ab initio* gas phase DFT calculations, at the B3LYP 6-31G(d) level of theory. Local MEP maxima and minima were converted in SSIPs using the following equations (Equations 1, 2) (Musumeci et al., 2011);

\[
\alpha = 0.0000162\text{MEP}_{\text{max}}^{2} + 0.00962\text{MEP}_{\text{max}}
\]

\[
\beta = 0.000146\text{MEP}_{\text{min}}^{2} - 0.00930\text{MEP}_{\text{min}}
\]

where:

- MEP<sub>max</sub> and MEP<sub>min</sub> represent local maxima and minima (energy values were given in Hartrees)
- Calculated SSIPs were paired for the pure structures and the cocrystal as follows: the highest α values interact with the highest β values, the second highest α value interact with the second highest β and so on. After α/β pairing, the total interaction site energy for the pure solids and cocrystal was estimated using Equation (3) (Musumeci et al., 2011). Energy values were converted from Hartrees into kJ/mol.

\[
E = -\sum_{ij} \alpha_{i}\beta_{j}
\]

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Betulinic/Ascorbic Acid Cocrystals as Anti-cancer Agents
The calculated energy difference between the interaction site pairing energies of the cocrystal and the two pure forms (Equation 4) provides a probability measure for a cocrystal formation (1:1) based on the assumption that within the cocrystal more favorable interactions will be formed.

\[
\Delta E = E_{\text{cocr}} - xE_a - yE_b
\]

where:

- \( E_{\text{cocr}} \) is the calculated interaction energy of the cocrystal,
- \( E_a \) and \( E_b \) are the calculated interaction energies for the pure structures,
- \( x,y \) represent the molar ratio of the two structures within the cocrystal.

**Synthesis of BA+VitC Cocrystals From Isopropyl Alcohol**

30.3 mg BA (MW 456.71 g/mole) and 11.7 mg VitC (MW 176.12 g/mole) were solubilized in 1:1 molar ratio using 4 ml isopropyl alcohol. The mixture was heated at 55°C and then allowed to cool slowly at room temperature, protected from light. The formation of the first crystals was recorded after 10 days.

**Scanning Electron Microscopy (SEM)**

The morphology of the sample was investigated by SEM–scanning electron microscopy (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan) at 10 kV. The samples were gold-palladium coated (90 s) with a sputter coater (Bio-Rad SC 502, VG Microtech, Uckfield, UK) using an electric potential of 2.0 kV at 10 mA for 10 min. The air pressure was 1.3–13.0 mPa.

**Differential Scanning Calorimetry (DSC)**

The thermal response of each product was measured using a differential scanning calorimeter (Mettler Toledo TG 821 e DSC Mettler Inc., Schwerzenbach, Switzerland). About 3–5 mg of powder was precisely weighed into DSC sample pans which were hermetically sealed and lid pierced. Samples were measured in the temperature range of 25–350°C at a heating rate of 10°C/min under constant argon flow of 150 ml/min. Data analysis was performed using the STAR® software.

**X-Ray Powder Diffraction (XRPD)**

The XRPD measurement was carried out with a BRUKER D8 Advance X-ray powder diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) with Cu K\(\lambda\)1 radiation (\(\lambda = 1.5406 \text{ Å}\)) and a VÁNTEC-1 detector. The powder samples were loaded in contact with a plane quartz glass sample slide with an etched square, and measured. Samples were scanned at 40 kV and 40 mA. The angular range was 3–40° 20, at a step time of 0.1 s and a step size of 0.007°. All manipulations, including K02-stripping, background removal, and smoothing of the area under the peaks of the diffractograms were performed using the DIFFRACTPLUS EVA software.

Rietveld analysis on the obtained X-ray diffraction pattern, was carried out using the MAUD software. The refinement was carried out using available structural crystallographic information of the two constitutive phases, namely BA and VitC.

**Fourier-Transform Infra-Red Spectroscopy (FTIR)**

FTIR spectra were recorded on a JASCO 670+ instrument after KBr pelleting. The data were collected in 4,000–400 cm\(^{-1}\) spectral range. Spectra were built up after a number of 24 acquisitions for each spectrum, with a resolution of 2 cm\(^{-1}\).

**Antioxidant Activity Assay**

An amount of cocrystal material was dissolved in 1 ml isopropanol in order to obtain a final concentration of 1.04 mg/ml. The antioxidant activity (AOA) was evaluated by DPPH radical scavenging assay, which was originally described by Blois (1958). Briefly, 1 mmol L\(^{-1}\) solution of DPPH was prepared and stored at 4°C, in the dark, and was used as a standard antioxidant stock solution. A reference solution of 0.167 mmol L\(^{-1}\) ascorbic acid in ethanol 96% (v/v) was also prepared; 0.5 mL of the cocrystal and ascorbic acid solutions, were each added to a 2.5 mL diluted stock solution of DPPH (2 mL ethanol 96% (v/v) + 0.5 mL DPPH 1 mM). The mixture was then analyzed using an Uvi Line 9400 Spectrophotometer from SI Analytics at 516 nm for 20 min.

Antioxidant activity was calculated using the following equation:

\[
\text{AOA} \% = 100 - \frac{A_{516\text{(sample)}}}{A_{516\text{(DPPH)}}} \times 100
\]

where:

- AOA = antioxidant activity [%];
- \(A_{516\text{(sample)}}\) = absorbance of the sample measured at 516 nm at a specific time;
- \(A_{516\text{(DPPH)}}\) = absorbance of the standard solution measured at 516 nm (without sample).

**Cell Culture**

HaCat (immortalized human keratinocytes), B164A5, and B16F0 (murine melanoma) cells were cultured in specific culture medium–Dulbecco’s modified Eagle Medium (DMEM) with high glucose (4.5 g L\(^{-1}\)), L-glutamine and sodium bicarbonate, supplemented with 100 U ml\(^{-1}\) penicillin, 100 μg mL\(^{-1}\) streptomycin, and 10% fetal bovine serum (FBS). The number of cells used in the experiments was determined in the presence of Trypan blue using a Neubauer counting chamber (Coricovac et al., 2017). HaCat and B16F0 cells were purchased from ATCC (American Type Cell Collection) and B16A5 from Sigma-Aldrich Chemie GmbH (Munich, Germany).

Human breast cancer (MCF-7, MDA-MB-231) and cervical (HeLa) cell lines were purchased from European Collection of Cell Cultures (Salisbury, UK). The cells were grown in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids, and 1% anti-biotic-antimycotic mixture. All media and supplements were obtained from Lonza Group Ltd. (Basel, Switzerland). The cells were cultured in a humidified atmosphere with 5% CO\(_2\) at 37°C and were passaged at every two-three days.
Determination of Cell Viability
The antiproliferative activities of the tested substances were determined by standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann, 1984) and Alamar blue assays. MTT: briefly, cells were plated into 96-well plates at a density of 5,000 cells/well and pre-incubated overnight. After incubation for 72 h with test compounds (BA+VitC cocrystal—3, 10, and 30 μM), 20 μl of MTT solution was added and the plates were incubated for another 4 h. The formed formazan crystals were dissolved in 100 μl dimethyl sulfoxide and the absorbance was determined at 545 nm. Wells with untreated cells were utilized as control.

Alamar blue: The cells (1 × 10^4/200 μL medium/well) were seeded in a 96-well plate and allowed to attach. After proper confluence was reached, cells were incubated with different concentrations (3, 10, and 30 μM) of the BA+VitC cocrystal for 48 and 72 h. After the incubation period, the Alamar blue reagent was added to each well (20 μL/well). The plates were incubated for 3 h at 37°C and the absorbance of each well was measured using a xMark™ Microplate Spectrophotometer (Biorad) at 570 and 600 nm (reference) wavelengths. Cell viability was calculated according to the formula described in our previous articles (Soica et al., 2014b).

For both assays, MTT and Alamar Blue, DMSO (dimethyl sulfoxide) was used as negative control. Even at the highest concentration tested—30 μM, the percentage of DMSO in the growth medium was 0.03%, percentage that is considered non-toxic for cells.

RESULTS AND DISCUSSIONS
Cocrystallization emerged as a successful procedure to modulate the properties of solid active ingredients for the purpose of improving drug delivery as well as drug manufacture; cocrystals not only provide optimized physicochemical (i.e., solubility, stability) and mechanical properties of the active drugs but they also offer the possibility of combined therapies that come along with intellectual property opportunities (Sun, 2012). In addition, multdrug cocrystals may exhibit synergistic or additive pharmacological effects. The design of possible cocrystals involves the evaluation of potential non-covalent and non-ionic intermolecular interactions between two or several active ingredients; in general, cocrystal formers bear functional groups that have the ability to form hydrogen bonds (Du et al., 2016).

Betulinic acid was identified as a very promising anticaner agent and included in the Rapid Access to Intervention Development (RAID) NCI program; however, its low water solubility and permeability cause a poor bioavailability which, despite its oral use, imposes the necessity of large oral doses (Godugu et al., 2014). BA solubility and permeability properties may assimilate it as a BCS class IV compound which generally does not reach market development (Chavda et al., 2010). Previous studies reported various attempts to solve BA physicochemical and pharmacokinetic drawbacks through specific formulations: (1) cyclodextrin complexation (Soica et al., 2014a), (2) nanoemulsion (Dehelean et al., 2013), (3) liposomes (Mullauer et al., 2011), carbon nanotubes (Tan et al., 2014), (4) polymer micelles (Das et al., 2016), or conjugates (Lomkova et al., 2016). However, such formulations can be problematic in terms of large scale production as well as storage (Sanphui et al., 2015); therefore, crystal engineering might offer a superior formulation alternative.

In designing potential cocrystals, a key point is the possibility of intermolecular interactions between components, in particular hydrogen bonds, that may trigger the molecular assembly and determine the supramolecular architecture (Venugopalaiah et al., 2016). The formation of a cocrystal depends upon the existence of a coformer that dramatically influences the cocrystal properties, mainly solubility, and dissolution (Tomaszewska et al., 2013). To date, the selection of a suitable coformer is based on several predictive criteria (Schultheiss and Newman, 2009); however, the formation of a real cocrystal can be validated only through valid experimental procedures. The most frequently used coformers are compounds bearing functional groups such as carboxyle, amide, hydroxyle, or amino groups (Jain et al., 2015). We assume that this new compound BA+VitC in isopropanol monosolvate can be linked together by the carboxylic bond.

Vitamin C was previously involved as coformer in the formation of cocrystals (Kovac-Besovic et al., 2009; Meepripruk et al., 2016); moreover, its antioxidant activity could synergistically combine with the intrinsic antioxidant effect of BA thus leading to a more soluble and higher biologically active cocrystal. Another key element in cocrystallization is the selection of solvents which depends on the solubility of both drug and coformer and strongly influences the stoichiometry of the resulting cocrystal (Leyssens et al., 2014).

Gas Phase ab initio DFT Calculations for BA-VitC Cocrystal Formation
Calculated site pairing energy values for the pure forms of the two structures and the expected cocrystal (1:1 ratio) are depicted in Table 1. The obtained ΔE value (−1.79 kJ/mol) suggests that in the new cocrystal more favorable interactions may be formed. Other previously reported ΔE values for confirmed 1:1 ratio cocrystals, such as: caffeine:acetic acid (ΔE = −1), caffeine:adipic acid (ΔE = −3), caffeine:sulfacetamide (ΔE = −3), caffeine:sulproline (ΔE = −3) (Musumeci et al., 2011), fall in the same range as our reported value. We can therefore conclude that a BA-VitC 1:1 ratio cocrystal was formed as a result of our crystallization process.

| Compound       | Calculated site pairing energy (kJ/mol) | Calculated ΔE (kJ/mol) |
|----------------|-----------------------------------------|------------------------|
| VitC           | −22.76                                  | −1.79                  |
| BA             | −20.71                                  |                        |
| VitC+BA proposed cocrystal (1:1) | −45.26                                  |                        |
Scanning Electron Microscopy (SEM) Analysis

SEM images of pure BA, pure VitC, and of their 1:1 cocrystal resulted from isopropanol solution are depicted in Figure 1. The scanning electron microscopy images show morphological differences between the pure ingredients and their cocrystallization product, thus indicating the formation of a new compound, formed through layer by layer deposition.

X-Ray Powder Diffraction Pattern

In order to provide an exhaustive structural characterization at atomic level, a single crystal structure would be needed; however, this single crystal might not be representative for the polycrystalline product used as source. Moreover, single crystal analysis requires a large enough and flawless crystal which is difficult to achieve. In light of these facts, the more readily available X-ray powder diffraction pattern (XRPD) is generally used to assess a powder crystalline material in terms of crystal structure, chemical composition, and physical properties.

The XRPD spectra for BA, VitC, and 1:1 BA + VitC cocrystal, respectively, are presented in Figure 2. The BA + VitC profile exhibits significant differences as compared to the ones recorded for the pure compounds, thus suggesting the formation of the cocrystal; in case of a simple physical mixture, the XRPD spectrum of the sample would be the sum of the diffraction peaks recorded for the pure compounds (BA and VitC) lacking any new diffraction pattern.

The new formed peaks of the BA + VitC cocrystals are present at 2θ, the most intense peaks are depicted at the following values: 5.04/8.82/8.94/9.86. The XRPD of the pure compounds revealed the existence of crystal lattices; in the cocrystal diffractogram, the peaks of starting materials are absent thus indicating the formation of a new crystalline phase and supporting the formation of an actual cocrystal.

Rietveld refinement of experimental XRPD pattern corresponding to the proposed BA + VitC cocrystal is depicted in Figure 3. The values obtained as a result of quantitative analysis indicate the following composition, BA weight (%): 64.2742 and VitC weight (%) 35.7252, although calculated analysis parameters such as the GOF (goodness of fit) = 2.24, Rwp = 11.24%, and Rexp = 4.65% are slightly above the values that ascertain a good fit, as indicated by the software developer. Nevertheless, the refined profile, calculated according to the corresponding data of the two phases, as seen in Figure 3, shows a visual overall good fit. The high values of the analysis parameters, mentioned above and a visual analysis of the refined profile line, revealing that some peaks are not well-fitted, could indicate polycrystalline forms (excess crystal forms of the components) present in the analyzed powder. Taking all of this in consideration and the fact that there is little crystallographic structural information regarding the two phases (BA and VitC), for obtaining a better fit, we can conclude that the formation of the cocrystal was achieved but proposing a structure for the new cocrystal is very difficult and it will constitute a research purpose for future studies.

Differential Scanning Calorimetry (DSC) Analysis

DSC is a thermal analysis that quantifies the difference between the heat amounts required to increase the temperature of the analyzed sample and the reference, respectively, as a function of temperature or time.

Characteristic and comparable thermograms were recorded for both pure ingredients BA and VitC and for their cocrystal as well. The DSC curves for BA, VitC, and BA + VitC cocrystal are presented in Figure 4.

For BA two small endothermic peaks at 241.23 and 255.56°C and a strong endothermic peak at 313.63°C can be noticed, corresponding to the melting of the compound accompanied by decomposition. For VitC a sharp endothermic peak is present around 192.8°C, attributed to the melting point of the compound, followed by a small exothermic peak correlated with the beginning of chemical degradation. For BA + VitC cocrystal: 3 endothermic peaks appear at 150.85, 173.17, and 186.14°C, respectively, followed by an exothermic process that may indicate the formation of a new structure that starts to decompose at 291.39°C; the process is finished above 300°C. The W-shaped thermogram (Yamashita et al., 2013; Stoler and Warner, 2015) such as the one obtained for BA + VitC proved to be characteristic for the cocrystals, different from the V-shaped specific for the eutectic mixtures.

Fourier-Transform Infra-Red Spectroscopy (FT-IR) Analysis

The FTIR spectra of the analyzed samples (Figure 5) reveal the binary adduct formation between the components, by the shifting
of bands from the crystal to lower or higher wavenumbers, outside the range of ±5 cm$^{-1}$. At high wavenumbers (spectral range 3,600–2,500 cm$^{-1}$), the stretching of simple bonds such as O-H and C-H occurs. The broad band observed in the spectral range 3,600–3,400 cm$^{-1}$ reveals the presence of intermolecular bonds, both in the case of pure BA, as in the case of the BA+VitC cocrystal. This observation leads to the conclusion that the formation of cocrystal occurs by hydrogen bonding.

The fingerprint region (spectral range 1,800–400 cm$^{-1}$) contains numerous bands, due to the complex mode of vibration for both precursors (BA and VitC), as well as for the cocrystal. The modification of wavenumbers in this region is not easy to assess and is generally associated with bending and wagging (Schrader, 2008; Kovac-Besović et al., 2009; Ahmad et al., 2010; Joshi et al., 2013; Wang et al., 2014; Stoler and Warner, 2015; Zhang et al., 2015; Yang et al., 2016). The experimental wavenumbers, expressed in cm$^{-1}$, obtained in this study for the cocrystal of BA+VitC/VitC/BA present absorption bands at: 748/756/789 that could be assigned to C-C bending vibration; 883/-/884 assigned to =CH$\text{2}$ wagging vibration; 916/821/918 attributed to C-H and C-C bending vibration. The bands located at 982/869/983, 1010/989/1009, -/1078/1083, 1131/1143/1106 can be assigned to C-H, C-C and C-O bending vibrations. At 1235/1222/1239 is the band corresponding to C-H, C-C and O-H bending vibration. The bending vibration for C-H$_2$ is located at -/1274/1298 and for C-CH$_3$ is located at 1374/1388/1376. The bending vibration of C-H$_2$ in the ring is present at 1453/1498/1450. The band present on the spectra at 1642/-/1643 correspond to C=C stretching vibration. At 1685/1679/1685 is the band that could be assigned to C=O stretching vibration in the ring. Vitamin C also presents a band at 1753 that can be attributed to C=O stretching vibration. C-H stretching vibration is present at 2869 for BA+VitC and at 2868 for BA. At 2942/2916/2940 is present the band that can be assigned to –CH$_2$ stretching vibration. The band characteristic for the O-H stretching vibrations are 3075/3219/3203 and 3437/3315/3471.
The Antioxidant Activity (AOA) Assessment
The AOA of the BA+VitC cocrystal and ascorbic acid, used as standard are exhibited in Figure 6. One can notice that the cocrystal shows as lightly increased AOA compared to that of pure ascorbic acid.

It can be assumed that the BA used in the preparation of the cocrystal has contributed to the reaction with DPPH thus leading to a higher AOA value compared to the one recorded for ascorbic acid. One can notice that values vary by 2% (ascorbic acid—95%, cocrystal—97%). The antioxidant activity of vitamin C was preserved in the 1:1 combination with BA and even increased by the presence of the triterpenic acid; however, the slight increase in antioxidant effect can be due to the intrinsic antioxidant activity of BA itself. Therefore, we can state at most that the antioxidant activities of both compounds was maintained in their cocrystal.

In vitro Analysis
The cytotoxicity assessment of BA+VitC cocrystal solution was performed on healthy (HaCaT-immortalized human keratinocytes) and cancer (B16F10, B164A5–murine melanoma; MCF-7, MDA-MB-231–human breast carcinoma and HeLa–human cervix carcinoma) cell lines using three different concentrations—3, 10, and 30 µM by means of both MTT and Alamar blue assays. DMSO had no impact on cells viability, the inhibition values being similar with the ones obtained for control cells (unstimulated).

The stimulation of HaCaT cells with BA+VitC cocrystal at 3 and 10 µM for 72 h did not reveal a significant cytototoxic effect, the cellular inhibition being maintained below 10% (Figure 7A). The lack of toxic effects of betulinic acid on normal cells has been reported since the first research paper concerning its anti-melanoma activity (Pisha et al., 1995) although the respective paper analyzed its toxicity on in vivo animal models; therefore, betulinic acid was used as scaffold for various chemical derivatives with the purpose of preserving its good selectivity index (Waechter et al., 2017). Our study revealed however a rather significant cell inhibition (~40%) for BA alone at 30 µM that is strongly attenuated by the presence of vitamin C, being reduced at ~15%.

A significant cell inhibition was noted at the lowest concentration (3 µM) against HeLa cells (45%) for both BA and BA+VitC (Figure 7C); the inhibition increased with the applied dose for both samples thus presenting a dose-dependent profile: 70% cell inhibition at 10 µM and ~100% cell inhibition at 30 µM. The inhibitory effect was virtually equal for BA and BA+VitC at all concentrations.

Insignificant cell inhibition values were noted at 3 µM against MCF-7 and MDA-MB-231 cells (<10%) for BA and BA+VitC (Figure 7C). At 10 µM a 25% cell inhibition was reported for BA alone against MCF-7 cells while the inhibitory activity almost doubled for the cocrystal at the same concentration thus indicating a strong antiproliferative activity induced by the presence of VitC. When concentration increased at 30 µM a total cell inhibition was recorded for both BA and its cocrystal with VitC; therefore, it can be stated that the cell inhibition profile evolves in a dose-dependent manner.

Interestingly, the MDA-MB-231 cell line was moderately sensitive to 10 µM BA (25% cell inhibition) but did not react to the same concentration of BA+VitC cocrystal, when cell inhibition was practically null. The use of the highest tested concentration, 30 µM, induced an inhibitory activity of 60% for BA alone while in the presence of VitC cell inhibition reached
90% thus revealing a highly effective antiproliferative activity of the cocrystal.

Gao et al. reported in 2017 an important inhibition of the MDA-MB-231 cell line by various concentrations of betulinic acid ranging between 10 and 160 \( \mu \)M; however, in his study, cellular viability only decreased to around 50% when 40 \( \mu \)M BA was used, due to ultrastructural and morphological changes (Gao et al., 2018). In the current study, a similar cell inhibition was caused by a lower concentration of BA (30 \( \mu \)M) which significantly improves, reaching 90% cell inhibition, when the BA+VitC cocrystal was used. Our results are in line with the ones reported by Weber et al. who determined the IC_{50} value of betulinic acid against MDA-MB-231 cells as 21.9 \( \mu \)M thus revealing a strong cytotoxic effect (Weber et al., 2014). For HeLa cells, a 50% cellular viability was reported after 48h stimulation with 30 \( \mu \)M BA, with a time- and dose-dependent inhibitory activity and an IC_{50} value of 30.42 ± 2.39 \( \mu \)M (Xu et al., 2017). The same group of authors reported in 2014a 50% viability of HeLa cells after 48h stimulation with 50 \( \mu \)M BA (Xu et al., 2014). By comparison, our results showed similar inhibition percentages at much lower concentrations (3–10 \( \mu \)M) while at 30 \( \mu \)M the cellular inhibition was almost complete both for BA and its cocrystal with VitC. An important antiproliferative activity was also previously reported by our group for BA against MCF-7 and HeLa cell lines (Soica et al., 2012).

In the case of murine melanoma cells–B16F10 (Figure 7B), the lowest concentration of both samples (3 \( \mu \)M) induced a negligible cell inhibition, whereas the higher concentrations—10 and 30 \( \mu \)M—exerted significant cytotoxic effects, the highest inhibition index being recorded after the 30\( \mu \)M stimulation (around 50% for both samples); at 10 \( \mu \)M, a stronger cytotoxic activity was noted for BA+VitC cocrystal when compared to BA alone. The cell inhibition activity followed a dose-dependent profile. BA was previously reported as anti-melanoma agent (Pisha et al., 1995) and was revealed to increase in a synergistic manner the antitumor activity of vincristine in an in vitro/in vivo study on B16F10 cells (Sawada et al., 2004); BA caused the arrest of B16F10 melanoma cells in the G1 phase.

A visibly higher cytotoxic activity was recorded against B164A5 murine melanoma cells after stimulation with BA+VitC cocrystal as compared to BA alone, in all concentrations, respectively; the cell inhibition activity evolved in a dose-dependent manner. The highest inhibitory activity for BA alone (20%) was recorded at its highest used concentration (30 \( \mu \)M); in a similar manner, the strongest cell inhibition was exhibited by BA+VitC cocrystal at 30 \( \mu \)M but the value of cell inhibition was more than twice compared to BA alone (~45%).

Our group reported in 2014 the cytotoxic activity of BA against B164A5 cells leading to 50% cell viability at 10 mM; BA was solubilized by inclusion in octakis-[6-deoxy-6-(2-sulfanyl ethanesulfonic acid)]-\( \gamma \)-CD (Soica et al., 2014a). By
Vitamin C was successfully preserved. Therefore, we presume that vitamin C with quercetine is synergistic in actions (Zhu et al., 2015). Guerriero et al. tested the cytotoxic effect of vitamin C, mitoxantrone, and their combination, respectively, against MCF-7 and MDA-MB-231 breast cancer cells; they noticed a dose-dependent cell inhibition on both cell lines for both tested compounds while their combination provided a synergistic effect and allowed lower chemotherapeutic doses (Guerriero et al., 2014). The association of vitamin C with quercetin is controversial. Most papers revealed the potentiation of the cytotoxic effects of various drugs following combination with vitamin C. Bober et al. reported in 2016 the up- and down-regulation of numerous proteins (229) by the combination of doxorubicin and vitamin C that resulted in the augmentation of the antiproliferative effect of doxorubicin (Bober et al., 2017). Guerriero et al. tested the cytotoxic effect of vitamin C, mitoxantrone, and their combination, respectively, against MCF-7 and MDA-MB-231 breast cancer cells; they noticed a dose-dependent cell inhibition on both cell lines for both tested compounds while their combination provided a synergistic effect and allowed lower chemotherapeutic doses (Guerriero et al., 2014). The association of vitamin C with quercetin is exerted antiproliferative effects against several breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468) through the induction of Nrf2-mediated oxidative stress in cancer cells in a synergistic manner as well (Mostafavi-Pour et al., 2017); the combination of vitamin C and quercetin with doxorubicin and paclitaxel caused a dramatical decrease of the IC_{50} of drugs and induced apoptosis in early stages (Ramezani et al., 2017). The administration of non-cytotoxic doses of vitamin C increased the chemotherapeutic response and apoptosis of HeLa cells treated with etoposide and cisplatin (Reddy et al., 2001). High concentrations of ascorbic acid (7–10 mM) administered intravenously were found to induce apoptosis of HeLa cells through the intrinsic and extrinsic pathways (Roberts et al., 2015); also, high doses of vitamin C inhibited energy metabolism in MCF-7 cancer cells through NAD depletion, thus inducing cell death (Uetaki et al., 2015). In counterpart, vitamin C was found to dose-dependently protect MCF-7 cancer cells against lipid peroxidation caused by tamoxifen thus causing a dose-dependent attenuation of cytotoxicity and diminishing the therapeutic response (Subramani et al., 2014).

Taking into account all these data, we can state that the co-crystallization of BA with vitamin C significantly improved the cytotoxic effect of BA against the tested tumor cell lines while preserving its good selectivity index. In part, we can assume that the increase of the biological activity of BA could be due to the intrinsic cytotoxic activity of vitamin C. However, the existing reports that incriminate vitamin C as cancer cell protector cast a shadow of doubt upon this assumption; in this regard, we may assume that vitamin C exhibited a protective effect on the MDA-MB-231 cancer cells when the 10 \mu M concentration was used, concentration at which BA alone caused a 25% cell inhibition. The mechanism behind this reported protective activity is yet to be investigated.

In the meantime, an important parameter in the investigation of poorly soluble drugs such as BA is solubility, parameter that can be greatly improved through co-crystallization (Qiao et al., 2011). As an example, the apparent solubility of vitamin K3 was greatly increased by co-crystallization with naphthoic acids and sulfamerazine (Zhu et al., 2015). Vitamin C was successfully employed as coformer in the co-crystallization of acetylovir for the purpose of improving the latter’s water solubility and bioavailability (Meepripruk et al., 2016). Therefore, we presume that the experimental data reported here that revealed a very strong cytotoxic effect of BA+VitC cocrystals as compared to previously and currently reported data for BA alone are partially due to an increased water solubility of BA as a result of vitamin C co-crystallization; moreover, the presence of vitamin C as coformer may lead to an optimized bioavailability, subject that needs however further investigations.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the experiments. MN, RG, CS, AD, and RR conducted the experiments of obtaining the cocrystals and characterization by optical microscopy. MM performed DFT calculations. RA, RMR, MN, and MV performed XRD, DSC, and SEM experiments. MN, RG, and RMR conducted IR experiments. IZ, DC, CF, CD, and C-AD contributed with in vitro tests. EM and C-AD performed antioxidant activity. All authors analyzed the obtained data. All authors contributed with reagents, materials, and analysis instruments. All authors wrote the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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