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# **ABSTRACT**

***IN VITRO* EVALUATION OF ANTICANCER POTENTIAL AND  
UNDERLYING MECHANISM OF ACTION OF BETULONIC  
ACID DERIVATIVES**

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## 1. INTRODUCTION

Throughout history, plant-based remedies have played a vital role in combating illnesses, forming the basis for many modern medicinal treatments. Today, medicinal plants are processed into various forms such as total extracts, standardized extracts, isolated pure compounds, or chemically modified molecules, contributing significantly to modern pharmacology. Phytocompounds, derived from plants, exhibit a wide range of pharmacological activities and are widely used in both prevention and treatment of various diseases, including infectious diseases, cardiovascular conditions, diabetes, and cancer. These plant-based compounds have great potential in the development of new, more effective medications through structural modifications, offering improved bioavailability, enhanced biological activity, and efficient targeting in the body.

Among these phytocompounds, triterpenes stand out for their pharmacological importance. Over 20,000 triterpenes have been identified, falling primarily into two categories: tetracyclic and pentacyclic triterpenes. Pentacyclic triterpenoids (PTs), specifically, are recognized for their biological activity, with notable subtypes including lupane, oleanane, and ursane structures. Key bioactive compounds in this class include lupeol, betulin, betulinic acid, betulonic acid, oleanolic acid, maslinic acid, and ursolic acid. These compounds possess a range of therapeutic properties, including anti-inflammatory, antimicrobial, antiviral, and anticancer activities, among others.

Betulinic acid (BI), a lupane-type pentacyclic triterpene, is found in plants like birch and eucalyptus. It has garnered significant attention for its diverse biological actions, particularly its anticancer properties. BI shows specific cytotoxicity against tumor cells, making it a promising candidate for cancer treatment. Betulonic acid, another lupane-type triterpene, is found in various plant species and possesses a similar range of therapeutic effects, including anti-inflammatory, antioxidant, antiviral, and anticancer properties. Betulonic acid is also used as a precursor for semisynthetic derivatives, aimed at improving water solubility and enhancing biological effectiveness.

Recent research has shown that both betulinic acid and betulonic acid offer significant potential as foundations for the development of new, more potent drugs, particularly for cancer treatment. Researchers are focused on improving the water solubility of these compounds through chemical modification, aiming to create more effective anticancer therapies. On a global scale, cancer remains a leading cause of death, and the incidence of the disease is

expected to increase by 70% in the next two decades. One of the most aggressive forms of cancer is malignant melanoma, which has a high mortality rate. Although recent advancements in cancer therapies have improved treatment outcomes, there is still a need for new approaches. Natural plant-derived compounds, such as pentacyclic triterpenoids, remain a key source of novel anticancer agents, holding great promise for future drug development.

## 2. AIM AND OUTLINE

The aim of this doctoral thesis was to synthesize four chemicals, including two newly developed compounds and to ascertain how the fused indole moiety at the C-2/C-3 position of betulonic acid core and peptide chain at its carboxylic group (C-28) influences its cytotoxicity against murine and human melanoma. It is important to mention that none of the four derivatives had undergone anticancer investigation against these selected melanoma cell lines.

The initial section of this thesis presents data from the most recently scientific literature regarding medicinal plants, their history, phytotherapy, regulatory frameworks, approved drugs, and anticancer properties. It also discusses pentacyclic triterpenes like betulin, betulonic acid, and betulonic acid, highlighting chemical derivatization and successful anticancer derivatives.

The personal contribution included, primarily the development of two brand-new synthesized semisynthetic derivatives: N-(2,3-indolo-betulinoyl)diglycylglycine (BA1) and N-(2,3-indolo-betulinoyl)glycylglycine (BA2), using betulonic acid as parent compound. In addition, the semisynthetic compounds N-(2,3-indolo-betulinoyl)glycine (BA3) and 2,3-indolo-betulinic acid (BA4), which were previously synthesized and characterized, were also obtained in order to be evaluated for the *in vitro* anti-melanoma potential together with the new synthesized molecules. Subsequently, the detailed structural characterization of the compounds has been validated through the application of <sup>1</sup>H, <sup>13</sup>C, 2D-HC-HSQC Nuclear Magnetic Resonance (NMR), and Fourier Transform Infrared Spectroscopy (FT-IR) spectroscopy techniques.

The subsequent step was assessing the *in vitro* activity of the semisynthetic derivatives of betulonic acid. The *in vitro* antimicrobial activity of betulonic acid (BI) and 2,3-indolo-betulinic acid and its derivatives was determined on Gram-positive and Gram-negative bacterial strains, as well as *Candida* spp. Furthermore, the subsequent endpoint was to assess the impact of

the size of the glycine side chain on the antiproliferative, cytotoxic, anti-migratory activities, and nuclear alterations against B164A5 murine melanoma and A375 human melanoma cell lines of preexisting BA4 and BA3 versus newly designed BA2 and BA1 possessing two and three glycine moieties, respectively. As a reference benchmark, naturally occurring betulinic acid was employed. Furthermore, the third objective of the study was to determine the effect of tested compounds (BI and BA1-BA4) in terms of their selectivity compared to the human keratinocyte cell line HaCaT.

Additionally, the aforementioned experiments used the most recent methodologies endorsed by the scientific community on the chosen experimental line, such as: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) technique, lactate dehydrogenase (LDH) test, neutral red (NR) assessment, the Scratch test, and Hoechst 3342 staining.

### 3. RESULTS

#### 3.1. CHEMICAL SYNTHESIS AND ANALYSIS OF BETULINIC ACID SEMISYNTHETIC DERIVATIVES

The synthesis of the two newly developed betulinic acid compounds has been effectively accomplished by using betulonic acid as the initial source. The newly synthesized derivatives BA1 and BA2 had a yield of more than 90%, nevertheless BA3 and BA4 showed a yield of over 70%. The compounds' structural characterisation has been confirmed using  $^1\text{H}$ ,  $^{13}\text{C}$ , 2D-HC-HSQC NMR, and FT-IR spectroscopic methods.

#### 3.2. EVALUATION OF THE ANTIMICROBIAL AND ANTIFUNGAL PROPERTIES OF BETULINIC ACID SEMISYNTHETIC DERIVATIVES

*Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC 10231, and *Candida parapsilosis* ATCC 22019 were the pathogenic microbes used in the study. The reactivity of the microorganisms was assessed using two techniques: disk diffusion and dilution method. Compounds BA2, BA3, and BA4 demonstrated antibacterial activity against *Streptococcus pyogenes* ATCC 19615 and *Staphylococcus aureus* ATCC 25923 with MIC values in the range of 13–16  $\mu\text{g/mL}$  and 26–32  $\mu\text{g/mL}$ , respectively. Conversely, betulinic acid was inactive against all microbial strains. On the other hand,

antifungal activity toward *Candida albicans* ATCC 10231 and *Candida parapsilosis* ATCC 22019 was found for compound BA3 with MIC 29 µg/mL.

### **3.3. IN VITRO EVALUATION ASSAYS: ASSESSMENT OF THE ANTIPROLIFERATIVE AND SELECTIVITY OF BA1-BA4 AND BI USING MELANOMA CELL LINES (B164A5 AND A375) IN COMPARISON TO HEALTHY HUMAN KERATINOCYTES (HaCaT)**

Using the MTT assay, it was shown that all tested compounds significantly reduced melanoma cell viability in a dose-dependent manner, with BA1 and BA3 demonstrating the highest antiproliferative effects. Results indicate that BA3 and BA2 exhibit the highest cytotoxicity, with significant reductions in cell viability at low concentrations and IC<sub>50</sub> values of 8.1 µM and 9.1 µM, respectively, on B164A5 cells. The novel compounds BA1 (IC<sub>50</sub> = 5.7 µM) and BA2 (IC<sub>50</sub> = 10.0 µM) were three times and two times more active than the parent cyclic structure BA4 and natural BI against A375 cells. Moreover, the obtained data highlighted that the 2,3-indolo-betulinic acid derivatives displayed selective cytotoxicity toward melanoma cells over normal cells. While the compounds significantly reduced the viability of B164A5 and A375 cells, their impact on HaCaT keratinocytes was limited, particularly at concentrations below 25 µM, where no significant effects were observed.

### **3.4. EVALUATION OF THE CYTOTOXIC POTENTIAL OF BA1, BA2, BA3, BA4, AND BI BY EMPLOYING LACTATE DEHYDROGENASE (LDH) ASSAY**

Using the lactate dehydrogenase (LDH) assay, which measures cell membrane integrity by detecting LDH leakage, the research evaluates the cytotoxic effects of the compounds against murine melanoma B164A5 and human melanoma A365 cell lines.

The LDH release assay revealed that the cytotoxic activity of the tested compounds was concentration-dependent in both B164A5 and A375 cells. Notably, BA2 and BA3 demonstrated the highest LDH release in B164A5 cells, with 62.14% and 80.68%, respectively, at a concentration of 75 µM. This suggests significant loss of cell membrane integrity in these cells. The study also confirmed a similar trend in A375 cells, where BA1 exhibited the most substantial LDH release at 59.3% ± 2.3 at the highest concentration of 75 µM, while BI caused a 50.2% ± 1.8 release. At lower concentrations (25 µM), BA2, BA3, and BA4 displayed higher cytotoxicity against A375 cells compared to higher concentrations, indicating a unique cytotoxicity profile where cytotoxic effects decreased slightly at 75 µM.

This phenomenon might be linked to cell cycle arrest mechanisms, wherein proliferative capacity diminishes and limits further LDH release.

### **3.5. EVALUATION OF THE ANTI-MIGRATORY EFFECT OF BA1, BA2, BA3, BA4, AND BI USING SCRATCH ASSAY**

The wound-healing or scratch assay was used to assess the anti-migratory potential of betulinic acid and its derivatives on both melanoma cell lines. Due to the high metastatic potential of melanoma, inhibiting cell migration is crucial for evaluating potential therapeutic effects. For B164A5 cells, BA2 (50  $\mu$ M) showed the highest inhibition of migration, with a wound healing rate of just 10%, followed by BA3 at 21.3%. In comparison, BI inhibited migration with a 34.9% wound healing rate. These results indicate that BA2 and BA3 most effectively reduced the migratory capacity of B164A5 cells.

Similarly, in A375 cells, the derivatives significantly hampered migration, particularly at concentrations of 25  $\mu$ M and 50  $\mu$ M. BI (25  $\mu$ M) inhibited migration by 17.5%, while BA1 and BA2 (50  $\mu$ M) showed potent anti-migratory effects, achieving wound healing rates of 35% and 30%, respectively. Notably, BA4 exhibited the weakest inhibition of migration, which corresponded to its lower cytotoxic profile. In both cell lines, the inhibition of migration was concentration-dependent.

The study also observed that, upon treatment with the derivatives, cells exhibited apoptotic characteristics, including morphological changes and disintegration, further supporting the cytotoxic effects of the compounds. This was visually evident in both B164A5 and A375 cells, particularly after 24 hours of exposure to the compounds.

### **3.6. DETERMINATION OF THE CYTOTOXIC EFFECTS OF BA1, BA2, BA3, BA4 AND BI USING NEUTRAL RED (NR) ASSAY**

This study employed the Neutral Red (NR) uptake assay to measure the cytotoxic effects of BA1, BA2, BA3, BA4, and BI—on B164A5 murine melanoma cells. The dose-dependent nature of the lysosome disruption caused by BI and the betulinic acid derivatives was confirmed. The lysosomal damage and subsequent cytotoxic effects were most pronounced in the case of BA1, followed by BA2 and BA3, indicating a clear ranking of cytotoxic potency among the compounds tested. BA1 exhibited the most potent cytotoxic response, recording a cytotoxicity level of 77.5% at a concentration of 75  $\mu$ M after 72 hours of

exposure. BA2 and BA3 also demonstrated substantial cytotoxic effects, with cytotoxicity levels of 69.9% and 64.2%, respectively, at the same concentration and exposure time.

### **3.7. DETECTION OF THE PRO-APOPTOTIC POTENTIAL OF BA1-BA4 AND BI VIA HOECHST 3342 STAINING**

The present study aimed to evaluate the pro-apoptotic and cytotoxic effects of indolo-betulinic acid derivatives (BA1, BA2, BA3, BA4) and betulinic acid (BI) on B164A5 murine melanoma cells. Using Hoechst 33342 staining, the nuclear-level effects of these compounds at concentrations of 25, 50, and 75  $\mu$ M were assessed after a 24-hour treatment period. The staining revealed apoptotic and necrotic nuclear alterations, with the extent of these changes varying among the compounds and concentrations tested. BA2 induced apoptosis at concentrations of 50 and 75  $\mu$ M, while BA3 exhibited the highest cytotoxicity, inducing necrosis at the lowest concentration (25  $\mu$ M). BA1 triggered apoptosis only at 75  $\mu$ M, while BI and BA4 caused necrosis at 50  $\mu$ M. These findings were consistent with prior cell viability assays (MTT), which demonstrated reduced viability at these concentrations. The results suggest a potent anti-melanoma effect of BA3, BA2, and BA1, with BA3 showing the strongest pro-necrotic activity

## **4. CONCLUSION**

Semisynthetic derivatives have demonstrated superior anti-melanoma and antimicrobial properties in comparison to naturally occurring betulinic acid, as indicated by the assays. The incorporation of an indole framework at the C2 position of betulinic acid enhanced its antiproliferative, cytotoxic, anti-migratory, and nuclear alteration effects against melanoma, as well as improved its antibacterial capabilities. Moreover, the cytotoxicity of the compounds was augmented by conjugating their carboxylic group with an amino acid residue. The findings indicate that BA1, BA2, and BA3 may serve as potential candidates for melanoma treatment.

Further research of the compounds are necessary to elucidate the mechanism of action responsible for this cytotoxic effects.