

**„VICTOR BABEŞ” UNIVERSITY OF MEDICINE
AND PHARMACY FROM TIMIŞOARA
FACULTY OF PHARMACY
DEPARTMENT II**

NISTOR GABRIELA



ABSTRACT

**SYNTHESIS AND BIOLOGICAL EVALUATION OF
TRIAZOLO-TRITERPENE BIOCONJUGATES
WITH ANTICANCER POTENTIAL**

Scientific coordinator

PROF. UNIV. DR. ȘOICA CODRUȚA

PROF. UNIV. DR. DEHELEAN CRISTINA

**Timișoara
2023**

TABLE OF CONTENTS

LIST OF PUBLISHED SCIENTIFIC PAPERS	V
ARTICLES INCLUDED IN THE PHD THESIS	V
COMPLEMENTARY ARTICLES.....	V
LIST OF ABBREVIATIONS AND SYMBOLS	VI
LIST OF FIGURES	VIII
ACKNOWLEDGEMENTS.....	XIII
INTRODUCTION	XV
GENERAL PART.....	1
1. CANCER	1
2. PENTACYCLIC TRITERPENES	3
2.1. BETULINIC ACID.....	5
2.2. HETEROCYCLIC AND NITROGEN BEARING DERIVATIVES OF BETULINIC ACID.....	8
3. 1,2,4-TRIAZOLES AND THEIR DERIVATIVES	12
4. BETULINIC ACID TRIAZOLE DERIVATIVES	15
EXPERIMENTAL PART.....	23
5. SYNTHESIS AND PHYSICO-CHEMICAL CHARACTERIZATION OF BETULINIC ACID DERIVATIVES.....	23
5.1. MATERIALS AND METHODS.....	24
5.1.1. CHEMICAL REAGENTS.....	24
5.1.2. SYNTHESIS OF 1H-1,2,4-TRIAZOL-3-THIOL (TZ1)	24
5.1.3. SYNTHESIS OF 5-SUBSTITUTED-1H-1,2,4-TRIAZOLE-3-THIOL.....	25
5.1.4. SYNTHESIS OF 3 β -O-ACETYL-30-BROMOBETULINIC ACID (BrBA)	25
5.1.5. SYNTHESIS OF 30-TRIAZOLE SUBSTITUTED BETULINIC ACID 26	26
5.2. INSTRUMENTS.....	26
5.2.1. NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY	26
5.2.2. FOURIER TRANSFORM INFRARED SPECTROSCOPY	27
5.2.3. LIQUID CROMATOGRAPHY – MASS SPECTROSCOPY ANALISYS.....	27
5.2.4. MELTING POINT	27
5.2.5. THIN-LAYER CROMATOGRAPHY	27
5.3. RESULTS	28

IV

5.3.1. CHEMISTRY	28
5.3.2. SPECTRAL DATA AND PHYSICO-CHEMICAL PROPERTIES	30
5.4. DISCUSSIONS	48
6. THE BIOLOGICAL ASSESSMENT OF C30 BA-1,2,4-TRIAZOLE DERIVATIVES	51
6.1. INTRODUCTION	51
6.2. MATERIAL AND METHODS	52
6.2.1. REAGENTS.....	52
6.2.2. CELL LINES AND CELL CULTURE CONDITIONS	53
6.2.3. CELLULAR VIABILITY.....	53
6.2.4. IMMUNOFLUORESCENCE ASSAY	54
6.2.5. QUANTITATIVE REAL-TIME PCR.....	54
6.2.6. HIGH-RESOLUTION RESPIROMETRY STUDIES	55
6.2.7. EVALUATION OF THE IRRITATION POTENTIAL USING THE HET-CAM ASSAY	57
6.2.8. STATISTICAL ANALYSIS.....	58
6.3. RESULTS	59
6.3.1. THE EFFECT OF C30 BA-1,2,4-TRIAZOLE DERIVATIVES ON THE VIABILITY OF NORMAL AND VARIOUS CANCER CELL LINES	59
6.3.2. MORPHOLOGICAL EVALUATION WITH DAPI STAINING OF RPMI-7951 CELLS	66
6.3.3. C30 BA-1,2,4-TRIAZOLE DERIVATIVES EFFECT ON PRO-/ANTI- APOPTOTIC MARKERS	70
6.3.4. MITOCHONDRIAL FUNCTION ASSESSEMENT	71
6.3.5. EVALUATION OF C30 BA-1,2,4-TRIAZOLE DERIVATIVES IRRITATIVE POTENTIAL	76
6.4. DISCUSSIONS	77
CONCLUSIONS.....	87
BIBLIOGRAPHY	90
IN EXTENSO PUBLISHED ARTICLES	II

ABSTRACT

Cancer has become one of the most important health issues in modern society, ranking as the second leading cause of death worldwide. Cancer affects human body tissues, and despite advancements in specific and differential diagnosis, it remains a significant challenge in terms of treatment effectiveness.

Despite significant advances in antineoplastic treatment, selecting the right active compound remains difficult, particularly for achieving complete remission. The current anticancer treatment consists of several drug combinations; however, genomic alterations vary, making it difficult to target patients with different tumor types. Personalized treatments could be one promising strategy for future anticancer therapy. Pentacyclic triterpenes are naturally occurring active compounds found in a wide variety of plant products. The number of cycles in a triterpene structure determines its classification: monocyclic, bicyclic, tricyclic, tetracyclic, pentacyclic, and hexacyclic triterpenes (15,16). Pentacyclic triterpenes are secondary metabolites that are commonly found in the bark, leaves, and peels of various plants.

Betulinic acid, a lupane derivative, has captivated the interest of researchers not only for its wide range of pharmacological properties, but also for its specific cytotoxicity against tumour cells. Despite the potential pharmacological benefits, the existing literature data indicated that the primary drawback of betulinic acid was the poor water solubility (6). To address this shortcoming, the synthesis of novel derivatives emerged as a viable option. Several crucial positions in the chemical structure of betulinic acid were of interest: carboxyl (C28), hydroxyl (C3), ring A and the double bond (C20-C29). The chemical modulation in these positions lead to a vast array of triterpenic scaffolded compounds with stronger biological effects than the parent molecule (58). Furthermore, the interaction of betulinic acid with other pharmacologically active molecules results in bioconjugates that retain the features of both parent substances. In medicinal chemistry, nitrogen-containing heterocyclic

compounds are one of the primary building blocks, comprising about sixty percent of the small-molecule drugs compiled in different databases (59). Within these compounds, triazoles are scaffolds with outstanding pharmacological capabilities; due to their structural features, both 1,2,3-triazoles and 1,2,4-triazoles can react with electrophilic and nucleophilic agents, offering the opportunity to create novel biologically active compounds (60). Betulinic acid conjugates with substituted triazoles at C30 and different modulations of the hydroxyl at C3 were produced while leaving intact the carboxyl group at C28 (9). Triazole moieties were also utilized as linkers in the development of betulinic acid - azidothymidine bioconjugates at C2 (61) and at C28 (62) through carboxyl group derivatization.

The current work describes the synthesis and the biological investigations aimed to decipher the cytotoxicity of three novel BA derivatives: BATZ1 – 3 β -O-Acetyl-30-(1H-1,2,4-triazole-3-ylsulfanyl)-betulinic acid, BATZ2 – 3 β -O-Acetyl-30-[5-(4-methoxyphenyl)-1H-1,2,4-triazol-3-ylsulfanyl]-betulinic acid and BATZ3 – 3 β -O-Acetyl-30-{5-[4-(dimethylamino)phenyl]-1H-1,2,4-triazol-3-yl)sulfanyl}-betulinic acid.

The new compounds synthesized present a triazole moiety, namely 1,2,4-triazole and 5-aryl substituted 1,2,4-triazole derivatives of betulinic acid. The heterocycle was grafted on the triterpenic scaffold at C30, while C28-carboxyle was left free and the C3-hydroxyl was acetylated. The 1,2,4-triazole-3-thiol derivatives were synthesized by NaOH cyclization of aroyl-thiosemicarbazides and were later used to react with the 30-bromo derivative of BA. All obtained final compounds and intermediates were physico-chemically assessed by melting point, NMR, FTIR and LC-MS. The synthesis path and reaction conditions are depicted in Figures 1-2. To the best of our knowledge, only the 1,2,3-triazole ring was used in the C30 position of the betulinic acid molecule, while no other molecule containing 1,2,4-triazole or 5-aryl substituted 1,2,4-triazole in this position have been previously synthesised.

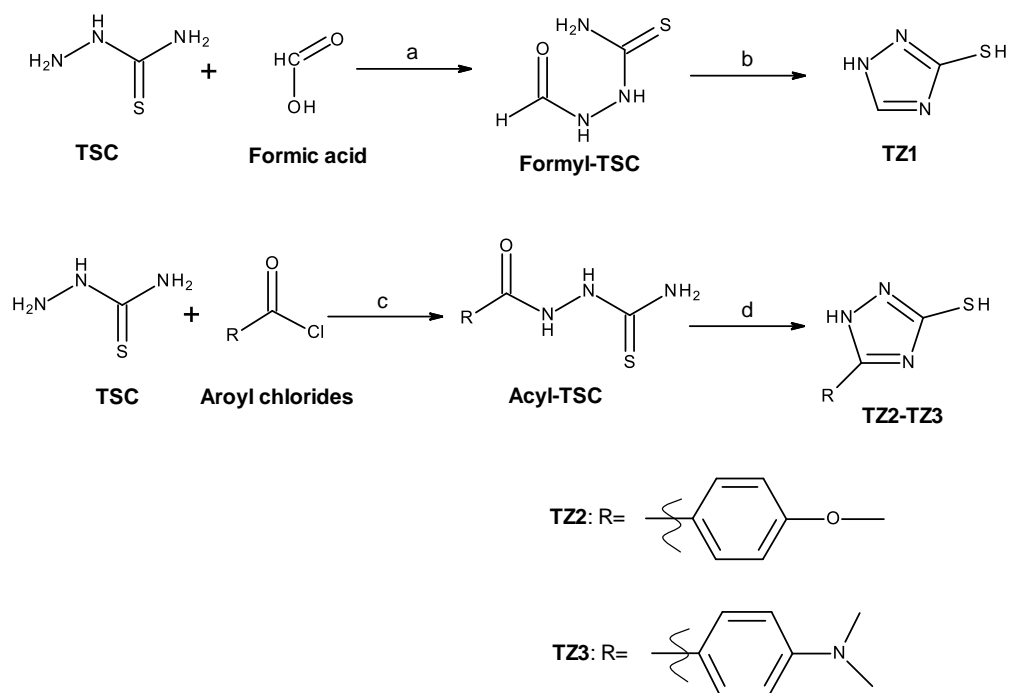


Figure 1. Mechanisms of synthesis for compounds 1H-1,2,4-triazol-3-thiol (TZ1), 5-(4-methoxyphenyl)-1H-1,2,4-triazole-3-thiol (TZ2) and 5-[4-(dimethylamino)phenyl]-1H-1,2,4-triazole-3-thiol (TZ3); TSC = thiosemicarbazide, BA = betulinic acid; reaction conditions: a. reflux, 30 minutes; b. H₂O, NaOH, reflux, 1 hour; c. pyridine/DMF, 1 hour, 50 °C; d. H₂O, NaOH, reflux.

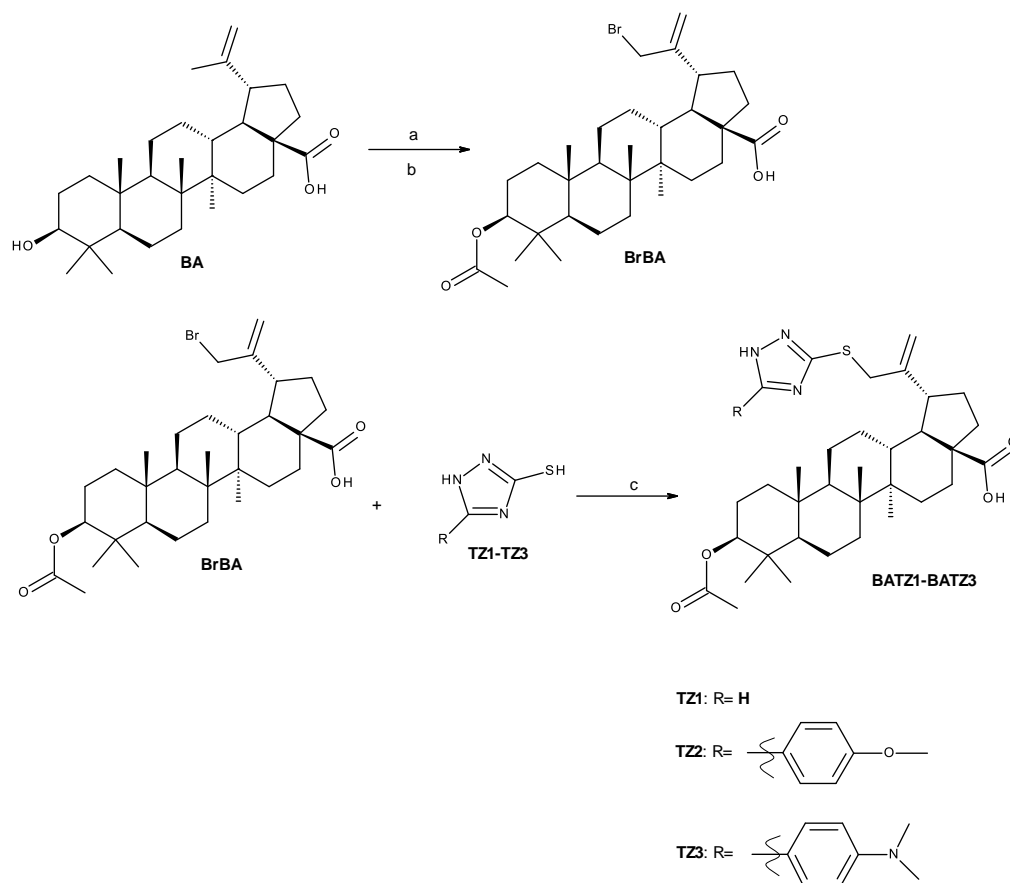


Figure 2. Synthesis of betulinic acid – triazol derivatives; BA – betulinic acid, BrBA – 3β-O-acetyl-30-bromobetulinic acid; TZ1 – 1H-1,2,4-triazol-3-thiol; TZ2 – 5-(4-methoxyphenyl)-1H-1,2,4-triazole-3-thiol; TZ3 – 5-[4-(dimethylamino)phenyl]-1H-1,2,4-triazole-3-thiol; BATZ1 – 3β-O-Acetyl-30-(1H-1,2,4-triazole-3-ylsulfanyl)-betulinic acid; BATZ2 – 3β-O-Acetyl-30-[5-(4-methoxyphenyl)-1H-1,2,4-triazol-3-ylsulfanyl]-betulinic acid; BATZ3 – 3β-O-Acetyl-30-[5-[4-(dimethylamino)-phenyl]-1H-1,2,4-triazol-3-yl)sulfanyl]-betulinic acid; reaction conditions: a. acetic anhydride, pyridine, DMAP, r.t, 12 hours; b. NBS, CCl₄, r.t, 48 hours; c. DMF, K₂CO₃, r.t, 72 hours.

The biological evaluation of the novel synthesized compounds BATZ13, their corresponding triazoles (TZ1-3) and their precursors (BA and BrBA) started with the investigation of their cytotoxic effect on normal keratinocytes HaCaT, RPMI-7951 human melanoma, A549 human lung carcinoma and HT-29 human colorectal adenocarcinoma cell lines. The results from melanoma, lung carcinoma, colorectal adenocarcinoma and normal cells suggests that the newly synthesized BA derivatives exhibit a selective cytotoxic activity at 2 μ M and at 10 μ M against all cancer cell lines tested (Figures 3-5). Compared to BA alone, the derivatives revealed the same level of antitumor selectivity, while the positive control 5-FU, tested at any concentration, did not present any selectivity on the cancer cells. The highest cytotoxic effect exhibited by the novel compounds was against the melanoma cells (IC_{50} : 10.6 μ M for BATZ1, 19.8 μ M for BATZ2 and 20.7 μ M for BATZ3), being more cytotoxically active compared also to their parent compounds.

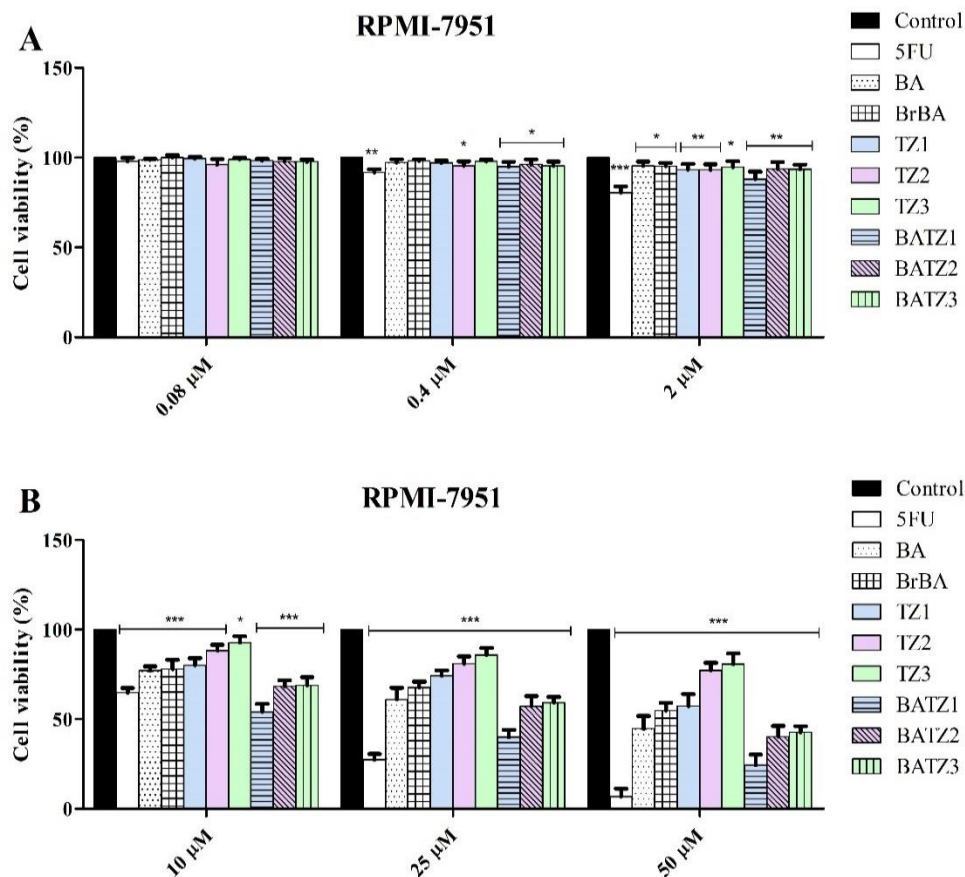


Figure 3. Cell viability of RPMI cells after 48h treatment with 0.08, 0.4 and 2 μ M (A) and 10, 25 and 50 μ M (B) BA, BrBA, TZ1-3 and BATZ1-3, determined using the MTT assay. The results were expressed as cell viability percentage (%) normalized to control (100%). Cell viability of RPMI cells after 48h treatment with 0.08, 0.4 and 2 μ M (A) and 10, 25 and 50 μ M (B) BA, BrBA, TZ1-3 and BATZ1-3, determined using the MTT assay. The results were expressed as cell viability percentage (%) normalized to control (100%) and represent the mean values \pm standard deviation of three independent experiments performed in triplicate. The statistical difference vs. control was determined using two-way ANOVA analysis followed by Bonferroni's multiple comparisons post-test. Values with $p < 0.05$ were considered have a statistically significant difference and were marked with *, as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

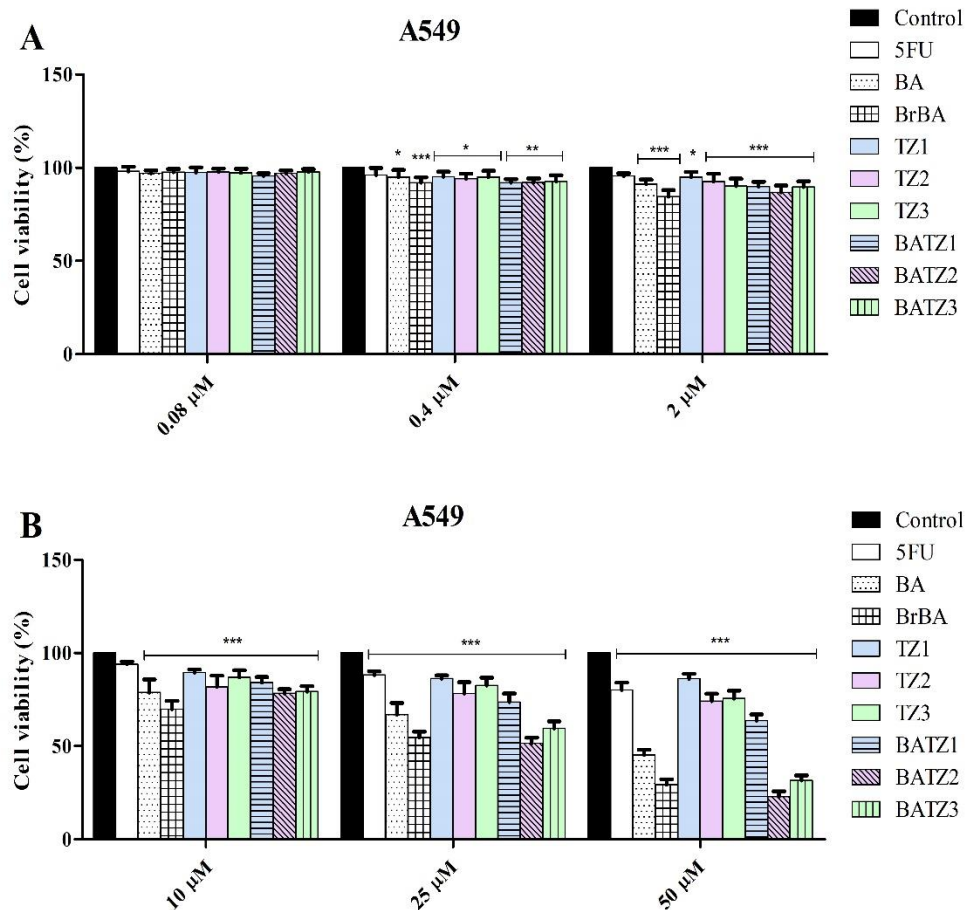


Figure 1. Cell viability of A549 cells after 48h treatment with 0.08, 0.4 and 2 μ M (A) and 10, 25 and 50 μ M (B) BA, BrBA, TZ1-3 and BATZ1-3, determined using the MTT assay. The results were expressed as cell viability percentage (%) normalized to control (100%) and represent the mean values \pm standard deviation of three independent experiments performed in triplicate. The statistical difference vs. control was determined using two-way ANOVA analysis followed by Bonferroni's multiple comparisons post-test. Values with $p < 0.05$ were considered have a statistically significant difference and were marked with *, as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

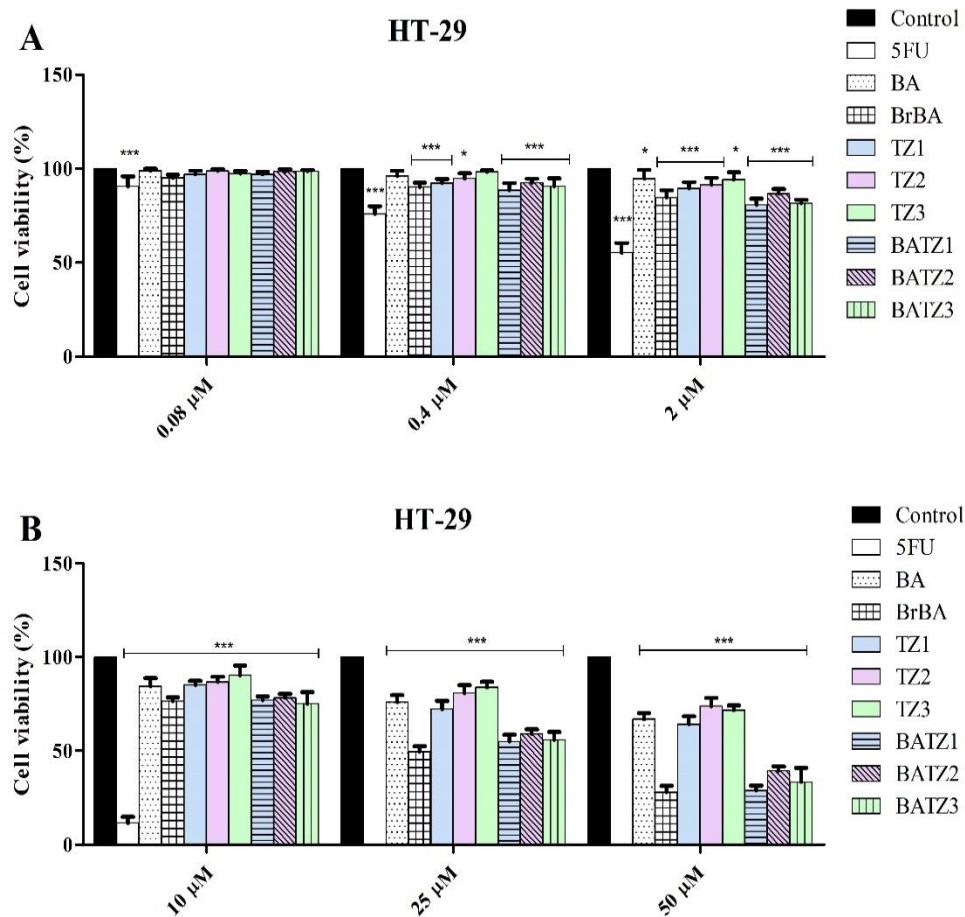


Figure 2. Cell viability of HT-29 cells after 48h treatment with 0.08, 0.4 and 2 μ M (A) and 10, 25 and 50 μ M (B) BA, BrBA, TZ1-3 and BATZ1-3, determined using the MTT assay. The results were expressed as cell viability percentage (%) normalized to control (100%) and represent the mean values \pm standard deviation of three independent experiments performed in triplicate. The statistical difference vs. control was determined using two-way ANOVA analysis followed by Bonferroni's multiple comparisons post-test. Values with $p < 0.05$ were considered have a statistically significant difference and were marked with *, as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Following a more in-depth evaluation of their cytotoxic mechanism, the BA derivatives effect on the nuclear morphology was assessed. The results showed that BA derivative BATZ1 induced apoptotic related nuclear changes that were more marked by increasing concentration in melanoma cells. Moreover, the apoptotic related nuclear changes were observed for all the compounds in all the cancer cell lines tested. Considering their high antiproliferative effect against melanoma cell lines and capacity to induce nuclear changes consistent with apoptosis, the compounds were further investigated on melanoma cells to assess their capacity to influence the gene expression of the Bcl-2/BAX proteins, known to be involved in the intrinsic apoptotic pathway. The results clearly showed that the novel compounds, induced a pro-apoptotic fold change gene expression of Bcl-2/BAX; by upregulating the gene expression of BAX and downregulating the gene expression of Bcl-2, the newly obtained C30 BA-1,2,4-triazole derivatives may trigger the opening of the MPTP and lead to cell death. The cytochrome c addition in the high resolution respirometry protocol revealed that the compounds can impair the OMM, thus supporting the previous statement. Moreover, upon evaluation of mitochondrial function of melanoma cells, the key-player in the intrinsic apoptotic pathway and cancer cell metabolism, all compounds decreased the effectiveness of the electron transport system and impaired the mitochondrial respiration, suggesting that the compounds may lead to inefficient ATP production and thus, can impair mitochondrial function. In the context of cancer cells, mitochondrial dysfunction/inhibition can be viewed as a treatment strategy, considering that some cancer cells display an increased dependency on OXPHOS for migration, proliferation, metastasis and even drug resistance. Further, upon the evaluation of C30 BA-1,2,4-triazole derivatives toxicity and irritation potency it was revealed that the newly synthesised compounds do not exhibit any irritation potential in the HET-CAM assay and are appropriate for mucosal and cutaneous usage.

Collectively, the results show that the newly synthesized betulinic acid derivatives, namely C3 acetylated 1,2,4-triazole and 5-aryl substituted -1,2,4- triazole

are derivatives with improved characteristics in terms of pharmacokinetic profile and pharmacological effects compared to the parent compound. Moreover, the compounds are effective antimelanoma agents with a selective cytotoxic activity at 2 μ M and at 10 μ M against all cancer cell lines tested.