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Interaction of the birch-bark terpenoids with human and bovine serum albumins

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Abstract: *Interactions between pentacyclic triterpenes isolated from white birch (Betula pendula Roth.) bark samples from Northeast Bulgaria and bovine serum albumin or human serum albumin were investigated using fluorescence techniques. The experimental results show the formation of complexes between the isolated triterpenes with serum albumins. Quenching of the intrinsic fluorescence of human serum albumins was monitored by emission spectra of varied quencher concentration solutions. By analysing the fluorescence spectra and fluorescence intensity, some parameters of the serum albumins - quencher interaction were determined to evaluate the type of quenching. An extract containing the isolated triterpenes formed complexes with both bovine serum albumin and human serum albumin, leading to quenching the fluorescence of both albumins by a combined quenching mechanism.*

Keywords: Fluorescence quenching, Bovine serum albumin, Human serum albumin, triterpenoids

Introduction

Triterpenes are widely distributed compounds of natural origin that can be isolated from many plants, animals or fungi [1]. Medicinal plants containing triterpenes have been used in folk medicine for the treatment of various diseases by applying decoctions and extracts obtained from natural sources long before it was known which ingredients are responsible for the therapeutic effects. There is a number of ethno-pharmaceutical studies on the triterpene content of diverse natural products and on the relationship between

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their structure and their activity [2, 3]. Numerous scientific studies led to the isolation and determination of various classes of triterpenes and their derivatives reveal and confirm their diverse pharmaceutical activities. [3, 4].

Very important propertiy is their anti-inflammatory effect [1, 5]. Chronic inflammatory processes that remain even after the pathogen's removal lead to series of diseases, such as cancer, arteriosclerosis, rheumatoid arthritis and many others. The antibacterial properties of these compounds have been tested/ against various strains of pathogenic bacteria. The results indicated similar importance to that of the antibiotics [5, 6]. Antiviral properties have been researched intensively especially for those compounds that are potential candidates to treat viruses such as AIDS and hepatitis [7, 8]. The use of biological extracts, as well as natural products, has become popular due to their wide spectrum of immunomodulatory effects and other influence over the immune system's mechanisms. [9]. The outbreak of the novel coronavirus (SARS, CoV-2) has led to numerous studies about identifying compounds for therapeutic drug development [10]. Human body produces free radicals. They are associated with the pathogenesis of a number of diseases, such as cancer, rheumatoid arthritis, Alzheimer's, Parkinson's, etc. [11]. The natural products from the triterpene group have antioxidant activity as well. It has been studied extensively because it reduces the toxic side effects [12]. The antitumor properties of triterpenes are among the most researched compared to their other positive physiological effects on biological objects. Tumor's seeding, development and spreading can be inhibited by their mechanism of action [13].

Since the biochemical and physiological mechanisms by which triterpenes exert their influence on various biological objects are extremely diverse, it is rather difficult to indicate the most important ones and it is impossible to discuss them all. A considerable number of studies are also trying to clarify triterpene's pharmacokinetic mechanisms of action [14, 15].

The lack of widespread application of triterpenes seems irrational given the background of all these proven beneficial properties. Unfortunately, the low solubility in water is a serious obstacle to their bio application, as the body's accessibility to the compounds depends on it. Their non-specific distribution in the body after intravenous administration is also a difficulty. They fail to reach the target cells, but quickly bind to other cells. The conducted research is mostly on cell cultures. There are no thorough clinical studies on the effectiveness of triterpenes, nor on the minimum inhibitory doses, etc. For this reason, research related to chemical [16, 17] and nanotechnological [18, 19] modifications of the triterpenes in order to improve solubility, bioavailability and target specificity in their therapeutic applications is of particular importance.

Recently, significant interest has focused on the group of non-steroidal terpenoid compounds contained in the bark of white birch (*Betula pendula* Roth). They exhibit antitumor activity [20], induce cell

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apoptosis in patients [21] and compromise HIV-virus replication [22], exhibit high antibacterial activity [23] and in general act as an anti-inflammatory agent [24].

The analysis of the available published research allows the conclusion that studying the different aspects of the biochemical and physiological actions of any triterpenes isolated from natural sources is promising. This also determines the purpose of the present work: to study the interaction of triterpenes isolated from white birch bark with serum albumin as a model of interaction with transport proteins.

Materials and Methods

Specimens obtained from birch bark (*Betula pendula* Roth.) in Northeastern Bulgaria were examined. The selected trees for bark harvesting had a trunk diameter of minimum 25 cm, since such trees recover better. The birch bark was extracted by peeling it. The gathered collections were immediately placed to dry in a dark, well ventilated space. The prepared samples were cut into small pieces and then crushed in a blender (A3). The grounded birch bark sample was subjected to continuous 24 hour Soxhlet extraction. As an extraction solvent 400 ml of ethanol (Pharmacopoeia, 96%) was used. After the completion of the procedure 1 L of distilled water was added. The obtained extract was filtered off at normal pressure through a "blue ribbon" filter and washed twice with distilled water. The drying was conducted at temperature not higher than 50°C [25]. The identification of the triterpene's presence in the obtained extract was carried out using gas chromatography.

A spectrofluorometric study of the interaction between the obtained extract containing triterpenes and serum albumins was performed using a Perkin-Elmer LS-3 spectrofluorometer. In order to study the interaction of the extract with the transport proteins (bovine serum albumin (BSA) and human serum albumin (HSA)) solutions were prepared containing equal fractions of water, ethyl alcohol and albumins and by varying the concentration of the extracted product so that the extract concentration ratio to be 0.5; 1; 2; 5; 10; 20 and 50 parts of the serum albumin concentration. In this paper only data acquired by recording the synchronous fluorescence spectra with $\Delta\lambda$ = 60 nm and $\Delta\lambda$ = 20 nm was used, since the spectrum bands are mostly influenced by the microenvironment of the fluorophore group and not by the fluorescence of other fluorophores or from light scattering. Spectrofluorometric spectra were recorded at 20°C and at 30°C.

Results

The results GC-MS analysis of the sample extract confirmed the presence of two compounds of interest: α-amyrin and betulin in approximate ratio 1:7. The measured synchronous fluorescence spectra of

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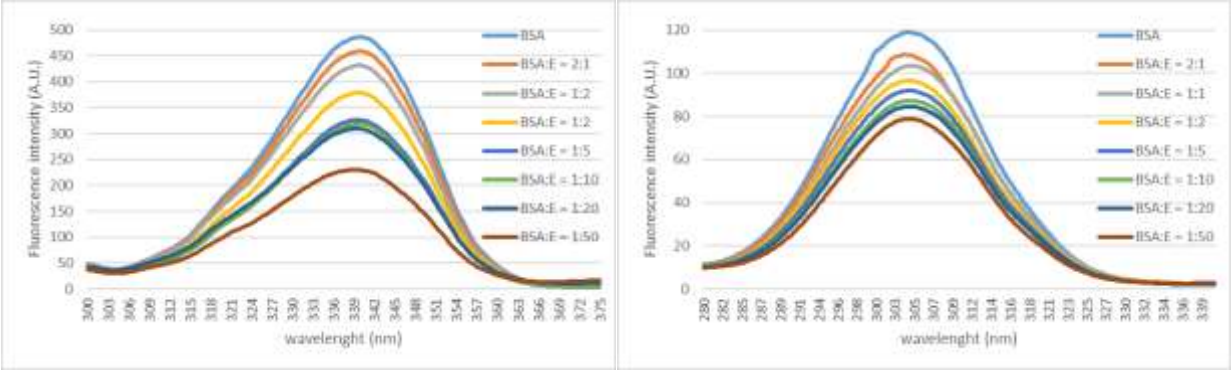
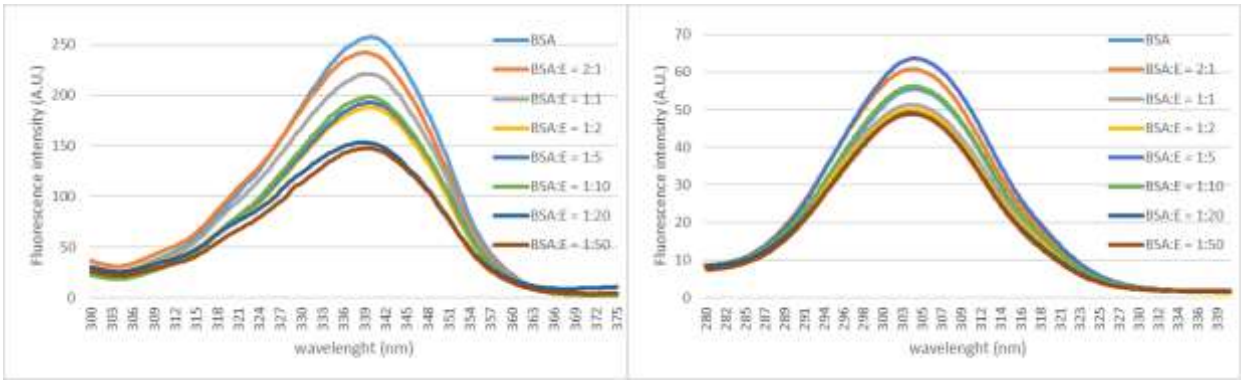
5.25×10^{-7} M BSA and its solutions with the obtained extract in the indicated above proportions at $\Delta\lambda$ = 60 nm and $\Delta\lambda$ = 20 nm at temperature 20ºC and 30ºC are shown on Fig. 1 and Fig. 2.



a. b.

Fig. 1. Synchronous fluorescence spectra of 5.25×10^{-7} M BSA and its solutions with the extract in the indicated approximate ratio 1:7 at 20ºC. a) $\Delta\lambda$=60 nm: b) $\Delta\lambda$=20 nm



a. b.

Fig. 2. Synchronous fluorescence spectra of 5.25×10^{-7} M BSA and its solutions with the extract in the indicated approximate proportions 1:7 at 30ºC: a) $\Delta\lambda$=60 nm; b) $\Delta\lambda$=20 nm

It appears that the fluorescence of the tryptophan residues is significantly intense than that of the tyrosine residues and varies to a much greater extent, from about 230 to 480 and from 150 to 260 fluorescence units at 20ºC and 30ºC respectively. The fluorescence of the tyrosine residues is of lower intensity and ranges

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from 80 to 120 and from 50 to 63 fluorescence units at 20ºC and 30ºC respectively. Therefore the fluorescence of tryptophan residues changes about 1.5-fold, while the changes of tyrosine residues' fluorescence are smaller and range from 30% to 25%, respectively.

The quantitative characteristics of the fluorescence quenching as a result of the BSA interacting with the obtained extract was calculated using the Stern–Volmer equation and modified Stern–Volmer plots as shown on Fig.3. To calculate the Stern-Volmer constants, only the range from 1:10 to 1:50 in which the Stern-Volmer equation is valid was considered. According to these results at 340 nm, the calculated values for K_d were 18275 and 27254 at 20ºC and 30ºC respectively. The segment of the linear relation giving the reciprocal value of f_a indicates that around one fourth of the tryptophan residue's loci were unavailable during the initial stages of the interaction with the extract used.

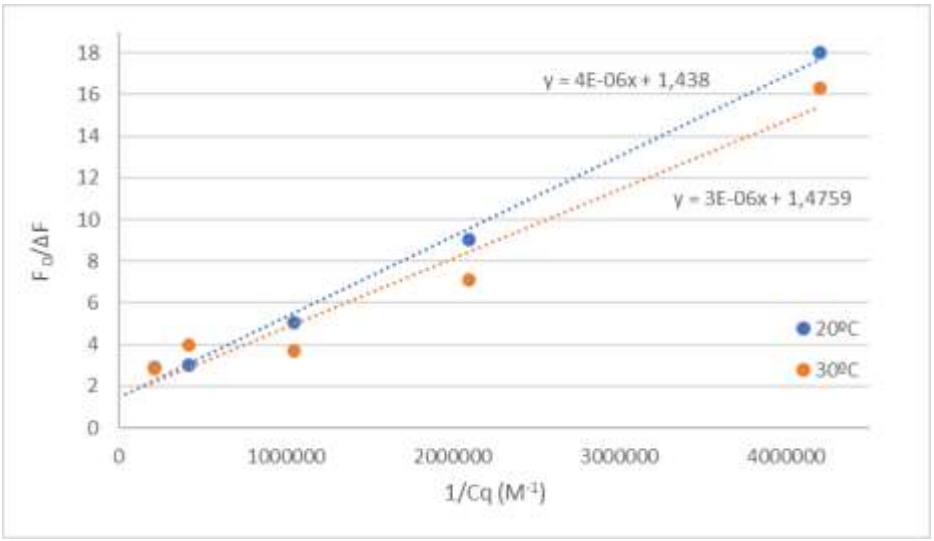

Fig. 3. Modified Stern–Volmer plots for the binding of BSA with the birch extract at 20ºC и 30ºC

Fig. 4 and Fig. 5 show the obtained synchronous fluorescence spectra of 5.25×10^{-7} M human serum albumin (HSA) and its solutions with the obtained extract in the indicated above ratios at $\Delta\lambda = 60$ nm and $\Delta\lambda = 20$ nm at temperatures of 20ºC and 30ºC respectively.

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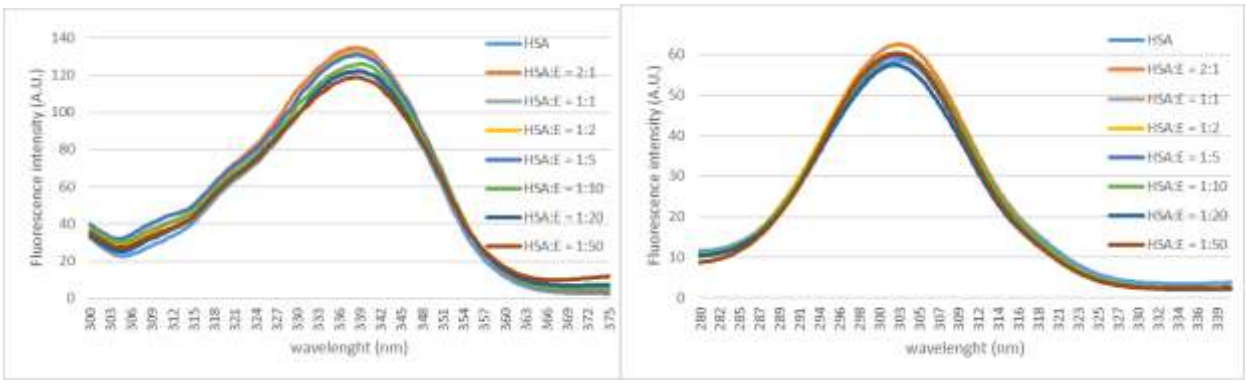
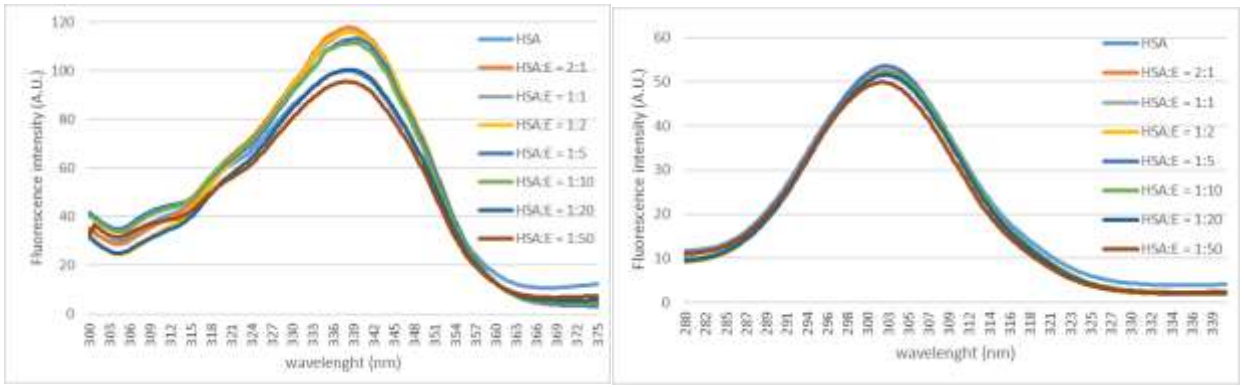


a. b.

Fig. 4. Synchronous fluorescence spectra of 5.25×10^{-7} M HSA and its mixtures with the obtained extract in the indicated ratios at 20ºC: a) Δλ=60 nm; b) Δλ=20 nm



a. b.

Fig. 5. Synchronous fluorescence spectra of 5.25×10^{-7} M HSA and its mixtures with the obtained extract in the indicated ratios at 30ºC: a) Δλ=60 nm; b) Δλ=20 nm

With HSA, a significant decrease in the intensity of the fluorescence spectra and the differences observed at different concentrations were observed. The fluorescence of the tryptophan residues is again significantly more intense than that of the tyrosine residues and changes to a significantly greater extent, from about 119 to 135 and from 95 to 117 fluorescence units at 20ºC and 30ºC, respectively, while the fluorescence of the tyrosine residues is with less intensity and significantly smaller changes – from 57 to 62 and from 50 to 52 fluorescence units at 20ºC and 30ºC, respectively. An increase in the fluorescence intensity at low

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concentrations of the extract used was observed. The fluorescence of tryptophan residues changed about 20%, while the fluorescence changes of tyrosine residues were significantly lower and ranged from 5%.

The quantitative characteristics of the fluorescence quenching as a result of the BSA interacting with the obtained extract was calculated using the Stern–Volmer equation and modified Stern–Volmer plots as shown on Fig.6. To calculate the Stern-Volmer constants, only the range from 1:10 to 1:50 in which the Stern-Volmer equation is valid was considered. According to these results at 340 nm, the calculated values for Kd were 10080 and 16872 at 20ºC and 30ºC respectively. The segment of the linear relation giving the reciprocal value of fa indicates that around 50% of the tryptophan residue's loci were inaccessible during the initial stages of the interaction with the birch extract.

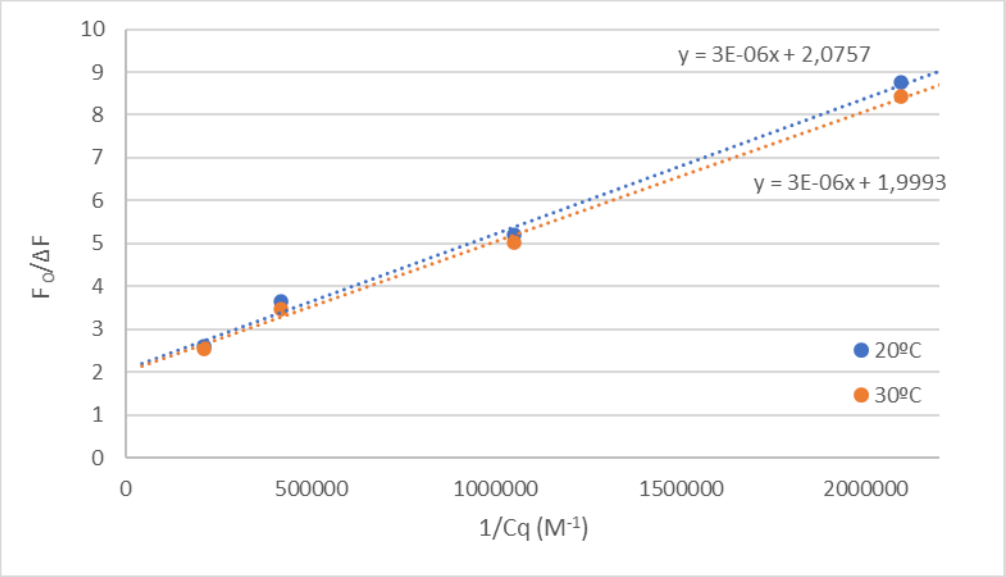

Fig. 6. Modified Stern–Volmer plots for the binding of HSA with the extract at 20ºC and 30ºC

Discussion

The resulting fluorescence spectra show nonlinear quenching of fluorescence at low quencher concentrations. This phenomenon often occurs in fluorescence quenching of proteins in the initial stages of the process, as part of the fluorophores (tryptophan residues) are inaccessible to the quencher under these conditions [26]. Increasing the concentration of the quencher is likely to induce conformational changes, as a result of which, the regions containing tryptophan residues end up in a more hydrophobic local environment, which is more favorable for fluorescence quantum yield. [26, 27]. The decrease in fluorescence intensity using HSA compared to BSA is likely due to the different number of the responsible for the observed emission

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fluorophores. Organisms show a number of differences in their amino acid sequence, despite the fact that serum albumins perform similar functions. A major difference between the two proteins is that BSA has two tryptophan residues (W^{131} and ^{W214}), while HSA has only one (W^{214}). [28]. When the extract was added to HSA, an increase in fluorescence intensity was observed. This effect is likely based entirely on the removal of some static quenching of the W^{214} residue that is present in the native protein [29] or on reduced energy transfer from tryptophan in domain II. The results in recent paper showed that upon guanidine denaturation of acrylodane-labeled HSA, tryptophan fluorescence also increased, due to a decrease in energy transfer from tryptophan to domain II. [31]. The diagrams obtained using the Stern-Volmer equation and modified Stern-Volmer plots allow determining the type of quenching – static, dynamic or combined [26]. The results of the present study indicate that the likely mechanism of fluorescence quenching of BSA and HSA as a result of interactions with the extract is due to a combined quenching procedure and reveals the formation of a complex between them. The calculated quenching constant values in this paper show that the contribution of the two types of interaction is different for BSA and HSA. In fluorescence quenching upon interaction with BSA, the static interaction component predominates, while upon interaction with HSA the contribution of both types of quenching is approximately equal.

In this study, the interactions of a fluorescent protein with triterpene's extract from birch bark were investigated using a fluorescence method. We investigated the interactions of natural product extract with serum albumins, since the binding of active substances to serum albumins affects their presence in the body. A triterpene containing extract forms complexes with both BSA and HSA, resulting in fluorescence quenching by a combined type of quenching.

Acknowledgments

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References

[1] Chudzik, M.; Korzonek-Szlacheta, I.; Król, W. Triterpenes as potentially cytotoxic compounds. *Molecules*, **2015**, *20(1)*, 1610-1625.

[2]. Sun, B.; Wu, L.; Wu, Y.; Zhang, C.; Qin, L.; Hayashi, M. Therapeutic potential of *Centella asiatica* and its triterpenes: A review. *Frontiers in pharmacology*, **2020**, *11*, 568032.

[3]. Darshani, P.; Sen Sarma, S.; Srivastava, A. K.; Baishya, R.; Kumar, D. Anti-viral triterpenes: a review. *Phytochemistry Reviews*, **2022**, 1-82.

Corresponding author: k.kalchev@shu.bg Full Paper
DOI: 10.2478/asn-2022-0019

[4]. Wang, M.; Li, H.; Liu, W.; Cao, H.; Hu, X.; Gao, X.; ... & Li, D. Dammarane-type leads panaxadiol and protopanaxadiol for drug discovery: Biological activity and structural modification. *European Journal of Medicinal Chemistry*, **2020**, *189*, 112087.

[5]. Cheker, R.; Sandur, S.K.; Sharma, D.; Patwardhan, R.S.; Jayakumar, S.; Kohli, V.; Sethi, G.; Aggarwal, B.B.; Sainis, K.B. Potent Anti-Inflammatory Activity of Ursolic Acid, a Triterpenoid Antioxidant, Is Mediated through Suppression of NF-kB, AP-1 and NF-AT. *PLoS ONE*, **2012,** *7(2*): e31318.

[6]. Jiménez, A.; Meckes, M.; Alvarez, V.; Torres, J.; Parra, R. Secondary Metabolites from *Chamaedora tepejilote* (Palmae) are Active against *Mycobacterium tuberculosis*. *Phytother. Res*., **2005**, *19*, 320-322.

[7]. Ma, C.; Nakamura, N.; Miyashiro, H.; Hattori, M.; Shimotohno, K. Inhibitory effects of constituents from *Cynomorium songaricum* and related triterpene derivatives on HIV-1 protease. *Chem Pharm Bull (Tokyo)*, **1999**, *47(2)*, 141-145.

[8]. Wu, H-Y.; Chang, C-I.; Lin, B-W.; Yu, F-L.; Lin, P-Y.; Hsu, J-L.; Yen, C-H.; Liao, M-H.; Shih, W-L. Suppression of Hepatitis B Virus X Protein-Mediated Tumorigenic Effects by Ursolic Acid. *J. Agric. Food Chem.*, **2011**, *59(5)*, 1713-1722.

[9]. Renda, G.; Gökkaya, İ.; Şöhretoğlu, D. Immunomodulatory properties of triterpenes. *Phytochemistry Reviews*, **2021**, 1-27.

[10]. Hisham Shady, N.; Youssif, K.A.; Sayed, A.M.; Belbahri, L.; Oszako, T.; Hassan, H.M.; Abdelmohsen, U.R. Sterols and triterpenes: Antiviral potential supported by in-silico analysis. *Plants*, **2020**, *10(1)*, 41.

[11]. Bano, T.; Kumar, N.; Dudhe, R. Free radical scavenging properties of pyrimidine derivatives. *Organic and Medicinal Chemistry Letters*, **2012**, *2*, 34.

[12]. Mamadalieva, N.Z.; Youssef, F.S.; Hussain, H.; Zengin, G.; Mollica, A.; Al Musayeib, N.M.; ... Wessjohann, L.A. Validation of the antioxidant and enzyme inhibitory potential of selected triterpenes using *in vitro* and *in silico* studies, and the evaluation of their ADMET properties. *Molecules*, **2021**, *26(21)*, 6331.

[13]. Gill, B.S.; Kumar, S. Triterpenes in cancer: significance and their influence. *Molecular biology reports*, **2016**, *43(9)*, 881-896.

[14]. Mitsuda, S.; Yokomichi, T.; Yokoigawa, J.; Kataoka, T. Ursolic acid, a natural pentacyclic triterpenoid, inhibits intracellular trafficking of proteins and induces accumulation of intercellular adhesion molecule-1 linked to high-mannose-type glycans in the endoplasmic reticulum. *FEBS Open Bio*, **2014**, *4*, 229-239.

[15]. Wildner, A.C.; Ferreira, P.L.; Oliveira, S.S.; Gnoatto, S.B.; Bergold, A.M. Variation of ursolic and betulinic acid in five *Malus* × *domestica* clones from Southern Brazil. *Journal of Applied Pharmaceutical Science*, **2018**, *8(9)*, 158-165.

Corresponding author: k.kalchev@shu.bg Full Paper
DOI: 10.2478/asn-2022-0019

[16]. e Silva, M.D.L.; David, J.P.; Silva, L.C.; Santos, R.A.; David, J.M.; Lima, L.S.; ... Fontana, R. Bioactive oleanane, lupane and ursane triterpene acid derivatives. *Molecules*, **2012**, *17(10)*, 12197-12205.

[17]. Soica, C.; Oprean, C.; Borcan, F.; Danciu, C.; Trandafirescu, C.; Coricovac, D.; ... Munteanu, M. The synergistic biologic activity of oleanolic and ursolic acids in complex with hydroxypropyl-γ-cyclodextrin. *Molecules*, **2014**, *19(4)*, 4924-4940.

[18]. Zhu, Z.; Qian, Z.; Yan, Z.; Zhao, C.; Wang, H.; Ying, G. A phase I pharmacokinetic study of ursolic acid nanoliposomes in healthy volunteers and patients with advanced solid tumors. *International journal of nanomedicine*, **2013**, *8*, 129.

[19]. Shao, J.; Fang, Y.; Zhao, R.; Chen, F.; Yang, M.; Jiang, J., ... Jia, L. Evolution from small molecule to nano-drug delivery systems: An emerging approach for cancer therapy of ursolic acid. *Asian Journal of Pharmaceutical Sciences*, **2020**, *15(6)*, 685-700.

[20]. Pisha, E.; Chai, H.; Lee, I.; Chagwedera, T.; Farnsworth, N.; Cordell, G. et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nature Medicine*, **1995**, *1*, 1046-51.

[21]. Galgon, T.; Wohlrab, W.; Drager, B. Betulinic acid induces apoptosis in skin cancer cells and differentiation in normal human keratinocytes. *Experimental Dermatology*. **2005**, *14(10)*, 736-743.

[22]. Soler, F.; Poujade, C.; Evers, M.; Carry, J.; Hènin, Y.; Mayaux, J. et al. Betulinic acid derivatives: A new class of specific inhibitors of human immunodeficiency virus Type 1 entry. *J. Med. Chem*., **1996**, *39*, 1069-83.

[23]. Barreto, A.; Feliciano, G.; Da Costa Nascimento, C.; Luna, C.; Da Motta Lessa, B.; Da Silveira, C. et al. Analysis of the presence of the betulinic acid in the leaves of *Eugenia florida* by using the technique GC/MS, GC/FID and HPLC/DAD: A seasonal and quantitative study. *Column Chromatography*, Martin D. (Ed.). In Tech. **2013**.

[24]. Akihisa, T.; Kojima, N.; Kikuchi, T.; Yasukawa, K.; Tokuda, H.; Masters, H. et al. Anti-inflammatory and chemopreventive effects of triterpene cinnamates and acetates from Shea fat. *J. Oleo. Sci*., **2010**, *59*, 273-280.

[25]. Bonchev, G.D.; Lyubomir, Đ.; Kalchev, K.N. Detecting the presence of antitumor terpenoids in bark of white birch from North-eastern Bulgaria. *Journal of Pharmacognosy and Phytochemistry*, **2017**, *6(2)*, 164-167.

[26] Lakowicz, J.R. (Ed.). Principles of fluorescence spectroscopy. Third edition, Boston, MA: Springer US, **2006**.

[27] Sułkowska, A. Interaction of drugs with bovine and human serum albumin. *Journal of Molecular Structure*, **2002**, *614(1-3)*, 227-232.

Corresponding author: k.kalchev@shu.bg Full Paper
DOI: 10.2478/asn-2022-0019

[28]. Ketrat, S.; Japrung, D.; Pongprayoon, P. Exploring how structural and dynamic properties of bovine and canine serum albumins differ from human serum albumin. *Journal of Molecular Graphics and Modelling*, **2020**, *98*, 107601.

[29]. Gelamo, E.L.; Silva, C.H. T.P.; Imasato, H.; Tabak, M. Interaction of bovine (BSA) and human (HSA) serum albumins with ionic surfactants: spectroscopy and modelling. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, **2002**, *1594(1)*, 84-99.

[30]. Flora, K.; Brennan, J.D.; Baker, G.A.; Doody, M.A.; Bright, F.V. Unfolding of acrylodan-labeled human serum albumin probed by steady-state and time-resolved fluorescence methods. *Biophysical Journal*, **1998**, *75(2)*, 1084-1096.

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