Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 7, 2015

Original Article

BIOLOGICAL ACTIVITY OF BULGARIAN *FOLIA BETULAE* DRY EXTRACT

DIMITAR PENKOV[1]*, STELA DIMITROVA[2], VELICHKA ANDONOVA[1], EMILIA MILIEVA[3], MARIANA MURDJEVA[4], IRINA STANIMIROVA[4], MARIAN DRAGANOV[5], MARGARITA KASSAROVA[1]

[1]Department of Pharmaceutical Sciences, Faculty of Pharmacy, [2]Department of Chemistry and Biochemistry, Faculty of Pharmacy, [3]Department of Physics and Biophysics, Faculty of Pharmacy, [4]Department of Microbiology and Immunology, Faculty of Pharmacy, [5]Department of Biology, Faculty of Medicine, Medical University of Plovdiv, 4000 Plovdiv, Bulgaria
Email: dimitar_penkov@abv.bg

Received: 23 Apr 2015 Revised and Accepted: 21 May 2015

ABSTRACT

Objective: The aim of this study was to investigate the biological activity of dry *Folia Betulae* (FB) extract.

Methods: Extracts from birch leaves were obtained by different technological methods–maceration and percolation, extraction with different concentrations of ethanol, changes in temperature regimen. The influence of the technological factors on the content of the biologically active substances (BAS) was examined. A phytochemical characterization of the extracts and their standardization were made, according to important groups of BAS–flavonoids (rutin, quercetin) and terpenes (betulin and betulinic acid), by means of HPLC methods for detection and quantitative determination. A model extract, with optimal content of BAS was chosen for subsequent *in vitro* investigation of its biological activity. Antimicrobial activity was studied via *in vitro* tests using bacterial isolates–*Staphylococcus aureus*, *Escherichia coli and Candida albicans*. The physiological activity was investigated by using *in vitro* test with smooth muscle strips. The antiproliferative activity of FB extract on eukaryotic cells was examined on cell cultures *in vitro*. Two cell cultures were used: the mouse lymphoma cell line *L5178Y* and the serum-free *McCoy-Plovdiv* cells.

Results: The dry extract from FB has a dose–dependent antibacterial effect. The bactericidal effect on *Staphylococcus aureus* is stronger than the one on *Escherichia coli*. Results prove that adding the extract leads to stimulating effect on muscle contractility. It demonstrates biological activity, expressed as changes in cell morphology, proliferation and vitality as well as initiation of apoptosis.

Conclusion: The results obtained largely overlap with literature data and explain some of the applications of this plant in traditional medicine.

Keywords: *Folia Betulae* extract, HPLC identification, Biological activity.

INTRODUCTION

Humans use various plants in their daily life as a source of food, spices, cosmetics and medicines. It has been known for a long time that the leaves of birch (*Betula pendula*, Roth) *(Folia Betulae, (FB)* contain a wide range of biologically active substances–flavonoids, tannins, terpenes, glycosides and essential oils. Anti-allergic, anti-platelet, anti-inflammatory [1], antitumour [2-4], antioxidant [4] and antimicrobial [1, 5-7] activities were found for the flavonoids. Antibacterial [8], antiviral, antifungal [9, 10] and antioxidative [11] activities have been established for the terpenes. Studies were focused on the antitumour activity of dammarane triterpenoids (against *Ehrich ascite* carcinoma) [12, 13], isolated from birch leaves. An antiproliferative effect on different tumour cell cultures was found for the betulinic acid [14]. The dammarane triterpenoids show an antitumour effect, manifested in changing the permeability and the micro-viscosity of the tumour cell membranes [15].

The aim of this study was to investigate the biological activity of dry *Folia Betulae* (FB) extract. For the first time in Bulgaria the antimicrobial, antiproliferative and physiological activity of total dry extract from Birch leaves was examined.

MATERIALS AND METHODS

Plant material

Plant material (*Betulae folium*) was collected from an environmentally clean region in Bulgaria–Rhodope Mountains, in the most appropriate time between June and August. It meets the requirements of the European Pharmacopoeia 7. The leaves were grinded to a size of approximately 0.5 mm.

Plant extracts

Different liquid extracts were prepared, using the method of percolation (USP 24, 1151, Process P), maceration (USP 24, 1151, Process M) and maceration at increased temperature–50 °C. Thick extracts were obtained after removal of the solvent with rotary evaporator (Rotavapor R II, BUCHI). Dry extracts were prepared by the method of spray drying from two of the most promising thick extracts (respectively Dry 1–obtained from D60 and Dry 2–obtained from M96) by a Mini Spray Dryer B-290 BUCHI. The obtained extracts with their abbreviations, method and preparation conditions are shown in table 1.

Table 1: *Folia Betulae* extracts–technological procedure's details

	Indication of the extract	Type of the extract	Method of obtaining	Used extractant	Working temperature
1.	P96	Thick	Percolation	Ethanol 96 %	25 °C
2.	P60	Thick	Percolation	Ethanol 60 %	25 °C
3.	M96	Thick	Maceration	Ethanol 96 %	25 °C
4.	D60	Thick	Maceration	Ethanol 60 %	Heating to 50 °C
5.	Dry 1	Dry	Spray dryer; Obtained from D60, thick.		
6.	Dry 2	Dry	Spray dryer; Obtained from M96, thick.		

HPLC quantitative determination of the biologically active substances: A phytochemical characterization (the presence of rutin, quercetin, betulinic acid and betulin) of the obtained model extracts was performed via HPLC method for simultaneously quantitative determination. It was developed and based on literature data [16-18] and adapted to the available equipment. HPLC system Varian Prostar with a column Microsorb-MV C18 (150 mm × 4.6 mm; 5 µm) and PDA detector were used. A mobile phase A (H_2O, pH = 3): B

(CH_3CN) (Labscan) was used, in a gradient mode from 90(A):10(B) to 10(A):90(B) and a flow rate 1 ml/min. Rutin and quercetin were detected at 368 nm, betulinic acid and betulin–at 210 nm. Identification of the substances was performed by the times of retention compared to this of pure substances. The quantitative determination was made by the method of the external standard. Results are presented as mean±SD values, provided from 3 independent measurements.

Antimicrobial activity *in vitro*

The *in vitro* study of the antimicrobial activity was performed on bacterial isolates *Staphylococcus aureus (S. aureus)*, *Escherichia coli (E. coli)* and *Candida albicans (C. albicans).* FB Dry 2 extracts, dissolved in 1 % DMSO (Fluka) and distilled water, was added to 24 hours 5 ml glucose broth cultures of *S. aureus*, *E. coli* and *C. albicans* (0.5 Mc Farland densities). The final concentrations of the extract in the media were 1, 2, 4, 10, 20, 24, 40, 50 and 100 mg/ml. The treated cultures were incubated for 24 and 48 hours. Untreated microbial cultures were used as controls. The effect of treatment was assessed by microbial count of the colony-forming units (CFU) in 5% blood agar (for *E. coli* and *S. aureus*) or in Candida Chromagar (for *C. albicans*). Results are presented as mean±SD values, provided from 3 independent measurements.

Antiproliferative activity *in vitro*

Two cell cultures were used: mouse lymphoma cell line *L5178Y* and serum-free *McCoy-Plovdiv* cells. FB Dry 2 extract was dissolved with 10% DMSO and sterilized by filtration. Final concentrations were as follows: 125, 250, 500, 750, 1000, 1500, 2000 µg/ml.

- Mouse lymphoma cells *L5178Y* were seeded with an initial concentration of $2x10^5$ cell/ml in 24-well plates. The cells were incubated with different concentrations of FB dry 2 extract for 2, 4 and 24 hours. The cell number and vitality of lymphomas were determined 24 hours after treatment by haemocytometer using Trypan blue solution and dye exclusion method;
- Serum-free *McCoy-Plovdiv* cells were plated in 96-well plates (200 µl per well suspension with cell number of 2 $x10^5$ cell/ml). After 24 hours they were treated with the extract in selected concentrations for 2, 4 and 24 hours.

Results are presented as mean±SD values, provided from 3 independent measurements.

Physiological activity *in vitro*

Isometric force measurements were performed on the smooth muscle samples. The preparations were taken from a rat stomach, separated in a circulatory direction, without mucosa, and were placed in a modified Krebs solution (in mmol/l): 120 NaCl, 5.9 KCl, 15.4 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11.5 glucose. The solution was aerated (with 95 % O_2 and 5% CO_2) and temperature-controlled (36±1) °C to create optimal conditions for the manifestation of spontaneous contractile activity. One gram of preload was applied to the tissues; equilibrium period was 1 hour.

The evoked contractile activity was triggered with KCl in mmol/l: 5, 50 and 100 (approximately 5 %, 40 % and 80 % depolarization of the resting membrane potential). The initial transient state (Phase 1) and a subsequent sustained state (Phase 2) were investigated in the presence of 0.1 mg/ml and 0.3 mg/ml of the FB Dry 2 extract. Registration of the isometric tension was performed using strain-gauge-measuring bridge and multichannel polygraph Linseis. All data were expressed as a percentage of maximal 50 mmol/l KCl contractions. Results are presented as (mean±SEM) values of at least five independent measurements.

RESULTS AND DISCUSSION

Fig. 1 presents the chromatogram of a standard mixture of rutin, quercetin, betulin and betulinic acid (Fig.1a), and a chromatogram of one of the model extracts (D60 from table 1)–Fig.1b) as an illustration of the results presented on table 2.

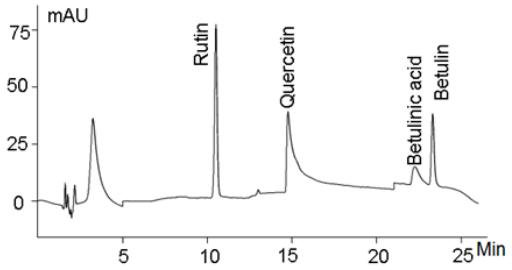

Fig. 1 a): HPLC chromatogram of a standard mixture of rutin, quercetin, betulinic acid and betulin

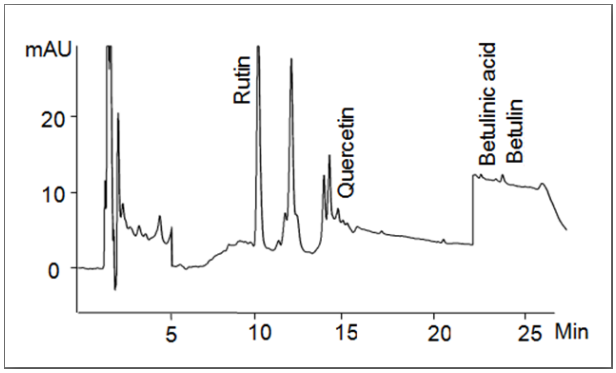

Fig. 1 b): HPLC chromatogram of D60

Table 2: Content of biologically active substances in the FB model extracts

	Extract	Rutin (µg/ml)	Quercetin (µg/ml)	Betulinic acid (µg/ml)	Betulin (µg/ml)
1.	P60	54.4±3.1	46.0±3.5	57.6±3.4	33.6±1.5
2.	P96	59.1±3.0	33.4±5.5	91.2±5.3	74.3±3.4
3.	M96	68.0±5.1	93.0±4.4	84.0±5.0	78.0±3.1
4.	D60	79.6±5.4	45.6±2.6	70.6±3.2	61.4±2.2
5.	Dry 1(from D60)	151.2±9.2	51.2±2.9	36.4±1.9	35.6±2.0
6.	Dry 2(from M96)	148.8±8.9	64.0±4.9	75.6±3.9	39.2±2.2

Values are mean±SD (n=3)

Data from table 2 show that the application of different technological approaches and various extractants determines the difference in the concentration of biological active substances. For example, rutin is extracted to a greater extend at higher temperature and lower concentration of ethanol (D60), which could be possibly explained by its glycoside structure. The non-polar structure of betulin and betulinic acid is probably the reason for their better extraction with high-concentrated ethanol (M96).

M96 extracts, in the form of dry extract–Dry 2, was examined for its biological activity (antimicrobial, antiproliferative and physiological), due to its phytochemical characteristics.

In vitro study of antimicrobial activity of FB Dry 2 extract showed the bactericidal effect in *S. aureus* broth cultures, treated for 24 and 48 hours with 10, 20, 40 and 100 mg/ml extract. The concentration of 1 mg/ml FB Dry 2 extract caused the bacteriostatic effect on *S. aureus* (CFU 10^3–10^4) whereas untreated cultures showed CFU>10^5. FB extract at concentrations of 1, 10 and 20 mg/ml did not influence *E. coli* growth (CFU>10^5 after incubation with the extract) but 40 и 100 mg/ml showed a bacteriostatic effect. The concentrations 1, 2, 4, 10, 24, 40, 50 mg/ml did not change culture growth of *C. albicans* in comparison with non-treated cultures (CFU 10^5). In summary, the Gram positive *S. aureus* is more susceptible to the bacteriostatic effect of FB Dry 2 extract used in lower concentrations in comparison with the Gram negative *E. coli*. However, the prokaryote fungus–*C. albicans* cannot be affected by all applied concentrations of the extract.

Other studies have demonstrated the antimicrobial activity of essential oils from FB on *Legionella pneumophilla, Fusarium, Microsporum gypseum* and *Rhizopus spp*. [19, 20]. Further investigations are necessary to elucidate the different susceptibility of Gram-positive, Gram-negative bacteria and fungi to the antimicrobial effect of FB extract.

In vitro study of antiproliferative activity of FB Dry 2 extract showed that the extract caused irreversible morphological changes in mouse lymphoma *L5178Y* cells several hours after the initial treatment (fig. 2a). Reduction of cell numbers was reported after 2, 4 and 24 hours exposure to the extract (fig. 2b). Concentration of 1000 μg/ml FB Dry 2 extract lead to single living cells for only 2 hours of exposure, while after 4 and 24 hours all cells were dead. Decrease of the extract concentrations increased the number of surviving cells. At a concentration of 125 μg/ml (for 4 and 24 hours of treatment) surviving cells were over 90 % and for 2 hours after treatment there were no dead cells.

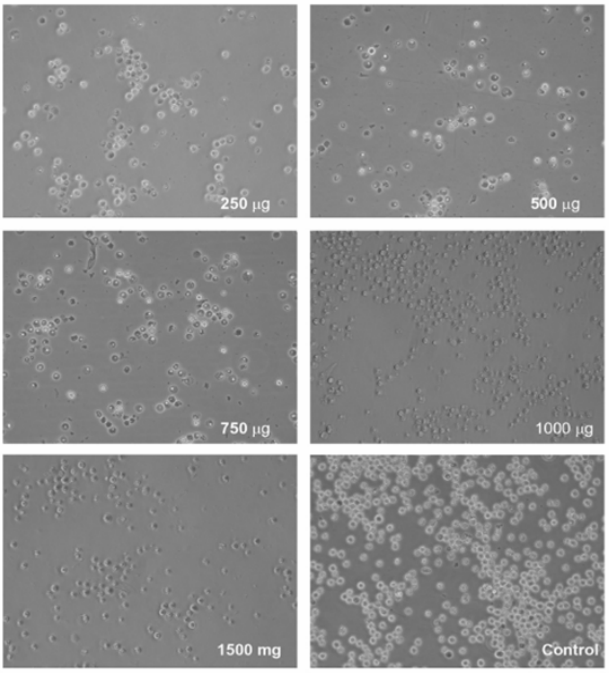

Fig. 2a): Microphotography of *L5178Y* cells treated with FB Dry 2 extract for 6 hours with concentrations: 250, 500, 750, 1000, 1500 μg/ml (x 200)

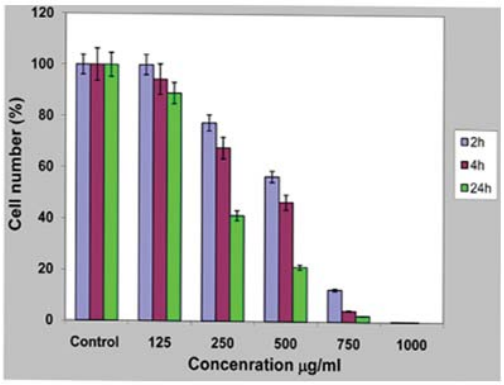

Fig. 2b): Cell numbers in cultures of mouse lymphoma cells treated with FB Dry 2 extract for 2, 4 and 24 hours with concentrations: 125, 250, 500, 750, 1000, μg/ml

Changes in cell monolayer cultures of *McCoy-Plovdiv* cells were observed at 250 µg/ml FB Dry 2 extract concentration. Cells acquired round shape, which was even more pronounced at concentrations of 500 and 750 µg/ml (fig. 3a).

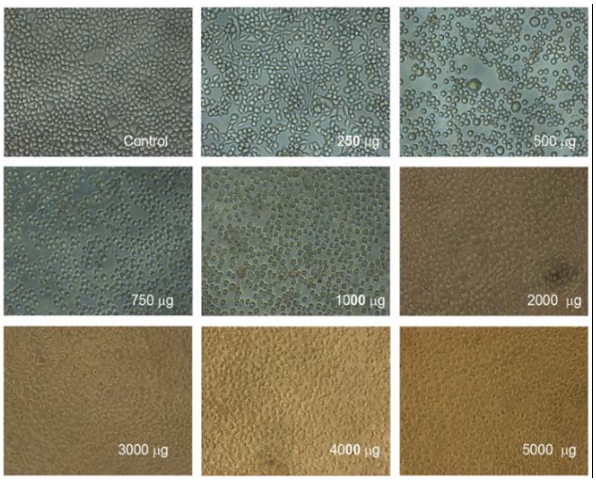

Fig. 3 a): Microphotography of *McCoy-Plovdiv* cells treated with FB Dry 2 extract for 24 hours with concentrations: 250, 500, 750, 1000, 2000, 3000, 4000, 5000 µg/ml (x 200)

However, at concentrations higher than 1000 µg/ml, cells were not round. They remained firmly bound to the substrate, but with strongly altered morphology and vitality (fig. 3b).

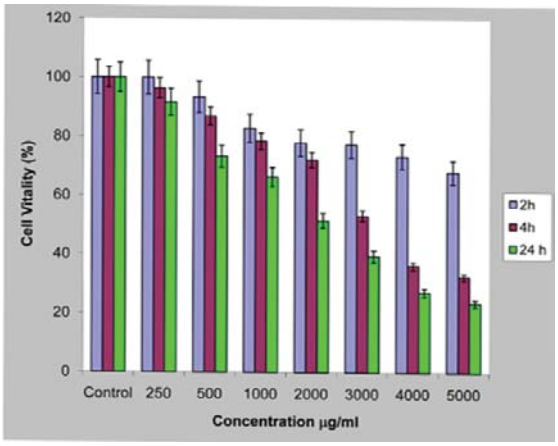

Fig. 3 b): Reduction of cell vitality in cultures of *McCoy-Plovdiv* cells after treatment with FB Dry 2 extract for 2, 4 and 24 hours with concentrations: 250, 500, 750, 1000, 2000, 3000, 4000 and 5000 µg/ml

It has been demonstrated that the cell damage was associated with apoptotic program that was triggered by FB extract components. In the mouse lymphoma cells, it was found that concentration of 2000 µg/ml FB Dry 2 extract led to accumulation of Annexin protein on the cell membrane even for 2 hours treatment.

Annexin binds to the phospholipid phosphatidylserine translocated outside of the cell membrane. This was an early indication of apoptosis occurred. That was also proved on human tumour cell lines–the hepatocellular carcinoma HEp G-2 cells and the adenocarcinoma PC3 cells (results not presented). There are many data in the literature showing that compounds such as betulin and betulin acid, inducing apoptosis and possessing antitumour and antiproliferative activity, have been isolated from the bark of birch and many other plants [8, 12, 19-21].

This gives us grounds to assume that apoptosis observed, was probably due to these compounds, thoroughly present in the extract. The results show higher sensitivity of tumour cells to extract exposure compared with *McCoy-Plovdiv* cells. These results are consistent with the literature [21].

In vitro, study of physiological activity of rat's corpus stomach sample was illustrated on fig. 4. Spontaneous contractile activity was shown with: tonic (TT) and phasic (PhT) isomeric force tension as well as two phases of KCl-depolarisation solution.

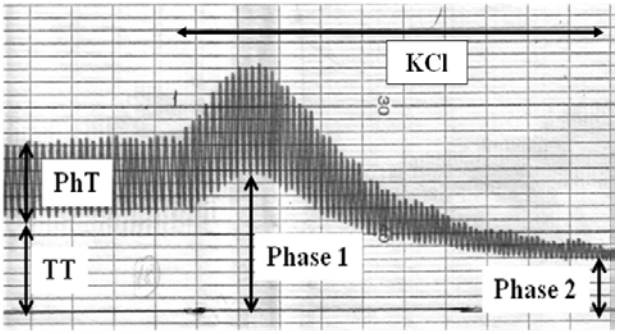

Fig. 4: Mechanogram of smooth muscle samples showing tonic tension (TT), phasic tension amplitude (PhT), an initial transient state–Phase 1 (Ph1) and a subsequent sustained state–Phase 2 (Ph2) of KCl-depolarisation solution (KCl-contracture)

Introduction of the FB Dry 2 extract into the smooth muscle bath (at concentration from 0.1 to 0.9 mg/ml) have a biphasic effect on the smooth muscle isometric tension. Following increase of the concentration, FB extract potentiates isometric force generation. At higher concentrations the extract inhibits the spontaneous muscle activity (fig. 5).

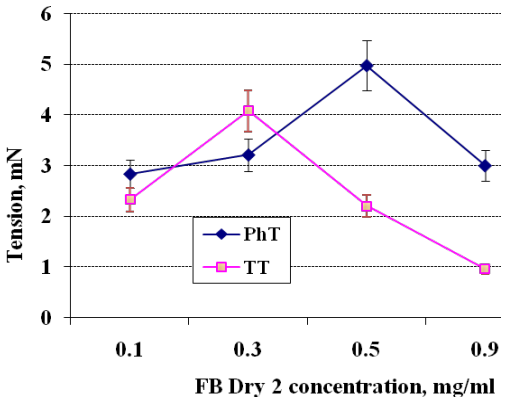

Fig. 5: Dose-response curve of FB Dry 2 extract. Average values of phasic (PhT) and tonic (TT) tension are presented as mean±SEM of 10 (PhT) and 5 (TT) separate samples

Stimulation effect of FB Dry 2 extract concentrations of 0.1 mg/ml and 0.3 mg/ml were investigated by evoked KCl-contracture. The initial transient state (Phase 1) and a subsequent sustained state (Phase 2) are illustrated in the presence of 0.1 mg/ml and 0.3 mg/ml of the extract (on fig. 6).

Adding FB extract into solution with smooth muscle strips leads to stimulating effect on the spontaneous contractility. The intensity of the effect is proportional to the concentration of the filtrate in doses up to 0.3 ÷ 0.5 mg/ml. The higher doses lead to reduction of the contractility. The biphasic profile of the dose–effect curve supports the assumption for the depolarizing effect and Ca^{2+}-influx through

the voltage-operated calcium channels (VOCCs) at low concentrations of FB extract. At higher concentration, the increased quantity of intracellular Ca^{2+}probably activates the K^+-efflux through the Ca^{2+}-dependent K^+-channels, hyperpolarizes the membrane and leads to smooth muscle relaxation [22]. KCl-induced response is a standard tool-set as a calcium-sensitizing stimulus [23].

Applied after priorly treated with FB extract muscle strips, Phase 1 and Phase 2 are changed significantly at 100 mmol/l KCl-contracture (that corresponded to 80 % depolarization of the membrane).

This suggests that FB extract changes the complex Ca^{2+}-signaling system than simply activation of VOCCs.

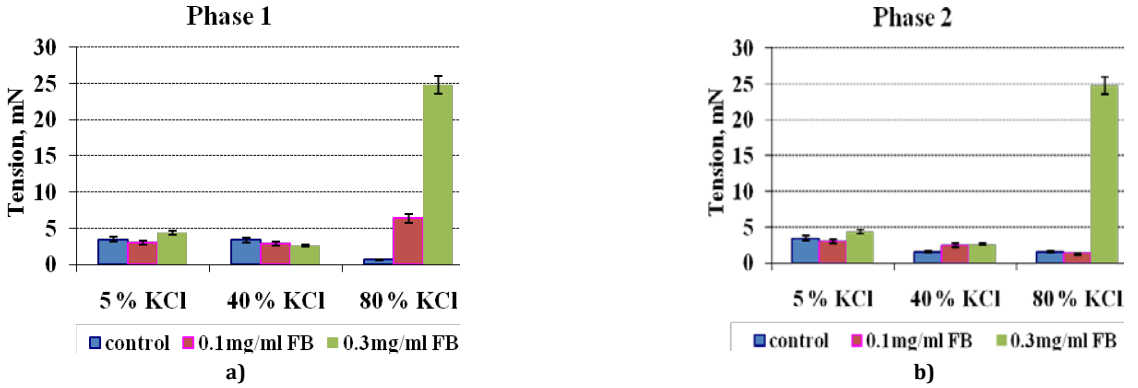

Fig. 6: Effect of 0.1 mg/ml and 0.3 mg/ml FB Dry 2 extract on the size of the KCl-contracture Phase 1 (a) and Phase 2 (b). Values are mean±SEM (4 rats; 8 strips)

In general, the total extract from FB demonstrates biological activity, which is expressed as changes in cell morphology, proliferation and vitality as well as in initiation of apoptosis in *L5178Y* cells and *McCoy-Plovdiv* cell lines. Dried FB extract possesses dose-dependent antibacterial activity *in vitro* on *S. aureus* and *E. coli* cultures, being more prominent in *S. aureus*. The *in vitro* treatment of *C. albicans* did not produce antimicrobial effect. A possible explanation for that may be associated with various mechanisms of maintaining Ca^{2+}-homeostasis (*C. albicans*) and the difference in intracellular Ca^{2+}-stores (*E. coli*). The physiological activity is probably due to the breach of the Ca^{2+}homeostasis on the membrane transport level and ryanodine-and caffeine-sensitive intracellular stores.

CONCLUSION

Our study showed that the application of different technological approaches and extractant defines the difference in the concentration of biological active substances of *Folia Betulae* extracts. The results obtained largely overlap with literature data and explain some of the applications of this plant in traditional medicine. These promising results give grounds to continue with further investigation and clarification of the antitumour activity of the extract.

ACKNOWLEDGEMENT

This work was supported by Research Project HO–8/2009, funded by Medical University of Plovdiv, Bulgaria.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Klinger W, Hirschelmann R, Süss J. Birch sap and birch leaves extract: screening for antimicrobial, phagocytosis-influencing, antiphlogistic and antipyretic activity. Pharmazie 1989; 44(8):558-60.
2. Kandaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT, *et al.* The antitumor activities of flavonoids. *In Vivo* 2005;19:895-909.
3. Drag M, Surowiak P, Drag-Zalesinska M, Dietel M, Lage H, Oleksyszyn J. Comparision of the cytotoxic effects of birch bark extract, Betulin and Betulinic acid towards human gastric carcinoma and pancreatic carcinoma drug-sensitive and drug-resistant cell lines. Molecules 2009;14(4):1639-51.
4. Ju EM, Lee SE, Hwang HJ, Kim JH. Antioxidant and anticancer activity of extract from Betula platyphylla var. japonica. Life Sci 2004;74:1013-26.
5. Smullen J, Koutsou G, Foster H, Zumbe A, Storey D. The antibacterial activity of plant extracts containing polyphenols against Streptococcus mutans. Caries Res 2007;41(5):342-9.
6. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26(5):343-56.
7. Pepeljnjak S, Kalodera Z, Zovko M. Antimicrobial activity of flavonoids from Pelargonium radula (Cav.) L'Herit. Acta Pharm 2005;55(4):431-5.
8. Akiyama H, Fujii K, Yamasaki O, Oono T, Iwatsuki K. Antibacterial action of several tannins against Staphylococcus aureus. J Antimicrob Chemother 2001;48(4):487-91.
9. Webster D, Taschereau P, Belland RJ, SC Rennie RP. Antifungal activity of medicinal plant extracts; preliminary screening studies. J Ethnopharmacol 2008;115:140-6.
10. Qi-he C, Jing L, Hai-feng Z, Guo-qing H, Ming-liang F. The betulinic acid production from betulin through biotransformation by fungi. Enzyme Microb Technol 2009;45(3):175–80.
11. Grassman J. Terpenoids as plant antioxidants. Vitam Horm 2005;72:505-35
12. Prokof'eva NG, Anisimov MM, Kiseleva MI, Rebachuk NM, Pokhilo ND. Cytotoxic activity of dammarane triterpenoids from birch leaves. Izv Akad Nauk Ser Biol 2002;6:645-9.
13. Setzer WN, Setzer MC. Plant-derived triterpenoids as potential antineoplastic agents. Mini Rev Med Chem 2003;3(6):540-56.
14. Rzeski W, Stepulak A, Szymański M, Sifringer M, Kaczor J, Wejksza K, *et al.* Betulinic acid decreases expression of bcl-2 and cyclin D1, inhibits proliferation, migration and induces apoptosis in cancer cells. Naunyn Schmiedebergs Arch Pharmacol 2006;374(1):11-20.
15. Bishayee A, Ahmed S, Brankov N, Perloff M. Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. Front Biosci 2011;16:980–96.
16. Zhao G, Yan W, Cao D. Simultaneous determination of betulin and betulinic acid in white birch bark using RP-HPLC. J Pharm Biomed Anal 2007;43(3):959–62.
17. Abreu I, Porto A, Marsaioli A, Mazzafera P. Distribution of bioactive substances from Hypericum brasiliense during plant growth. Plant Sci 2004;167(4):949–54.
18. Zu Y, Li C, Fu Y, Zhao C. Simultaneous determination of catechin, rutin, quercetin kaempferol and isorhamnetin in the extract of sea buckthorn (Hippophae rhamnoides L.) leaves by RP-HPLC with DAD. J Pharm Biomed Anal 2006;41(3):714-9.
19. Demirci F, Demirci B, Baser KHC, Güven K. The composition and antifungal bioassay of the essential oils of different Betula species growing in Turkey. Chem Nat Compd 2000;36(2):159-65.

20. Shimizu I, Isshiki Y, Nomura H, Sakuda K, Sakuma K, Kondo S. The antibacterial activity of fragrance ingredients against Legionella pneumophila. Biol Pharm Bull 2009;32(6):1114-7.
21. Demikhova OV, Balakshin VV, Presnova GA, Bocharova IV, Lepekha LN, Chernousova LN, *et al.* Antimycobacterial activity of a dry birch bark extract on a model of experimental pulmonary tuberculosis. Probl Tuberk Bolezn Legk 2006;(1):55-7.
22. Berridge MJ. Cell Signalling Pathways. In: Cell Signalling Biology, Module 2; 2012.
23. http://www.auburn.edu/academic/classes/biol/6190/CellSignalingBiology/csb002. pdf
24. Ratz PH, Berg KM, Urban NH, Miner AS. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. Am J Physiol Cell Physiol 2005;288(4):C769-83.