METABOLOMIC PROFILING AND BIOLOGICAL ACTIVITY STUDIES OF *PRUNUS SPINOSA* L. BRANCHES IN INLAMMATION-RELATED DISEASES

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INTRODUCTION

***Prunus spinosa* L. (blackthorn)** is a small tree, from the rose family (*Rosaceae*), which young branches are used in traditional medicine as **antihypertensives, vasoprotectives and anti-inflammatory agents in skin diseases** [1]. *P. spinosa* branches **is a rich source of polyphenol fraction**, especially **A-type proanthocyanidins, with broad regulatory effects on oxidation-reduction and inflammatory processes** in living cells including vascular inflammation [2,3]. While *P. spinosa* branches hold promise as source for production of modern phytotherapeutics, their practical application requires resolving issues, like effective extraction method, understanding biological mechanisms, identifying structures of active markers, and ensuring quality control.

AIM OF THE PROJECT

The first phase of the study was aimed at **preparative extraction**, preceded by **optimisation of extraction** process assessing the effect of solvent and method on extraction yield and total polyphenol and total proanthocyanidin content of the extracts, in order to obtain an **extract rich in polyphenols and proanthocyanidins**, and detailed **phytochemical profiling** of the obtained extract. In addition, the second objective of this phase of the study was preliminary ***in vitro* screening** to assess the extract's effect on key factors in the etiology of oxidative-inflammatory disorders.

OPTIMISATION OF EXTRACTION

❶ **Plant material:** branches of *P. spinosa* collected from the natural habitat in spring 2023 in Rzeczyca 51°44'N, 18°50'E (following ethnobotanical guidance) and properly prepared (drying, removing buds, grinding).

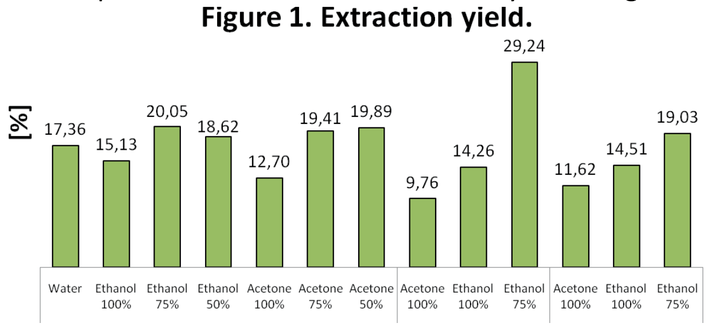
❷ **Reflux extraction:** boiling of plant material and solvent at boiling point of solvent for 30 min., filtration, repeating steps 3 times**;**

Ultrasonic extraction: heating of plant material and solvent on an ultrasonic cleaner for 15 min at 40°C, filtration, repeating steps 3 times;

Maceration: maceration of plant material and solvent at room temp. for ca. 24h and shaking, filtration after 3, 6, 9 days, process took 9 days.

All extracts are evaporated to dry on rotary evaporator, weighed, extraction yield was calculated and quantitative evaluation of obtained extracts was performed. Results of extraction yield in Figure 1.

Figure 1. Extraction yield.



❸ **Quantitative studies**: total polyphenolic content (TPC) were determined spectrophotometrically acc. the Folin-Ciocalteu (F-C) method and total content of proanthocyanidins (total tanin content, TTC1, TTC2), were determined acc. vanillin and butanol/HCl methods respectively. Methodology of these assays in Figure 2, results in Figure 3.

Figure 2. Methodology of spectrophotometric assays for TPC, TTC1 and TTC2 analysis.

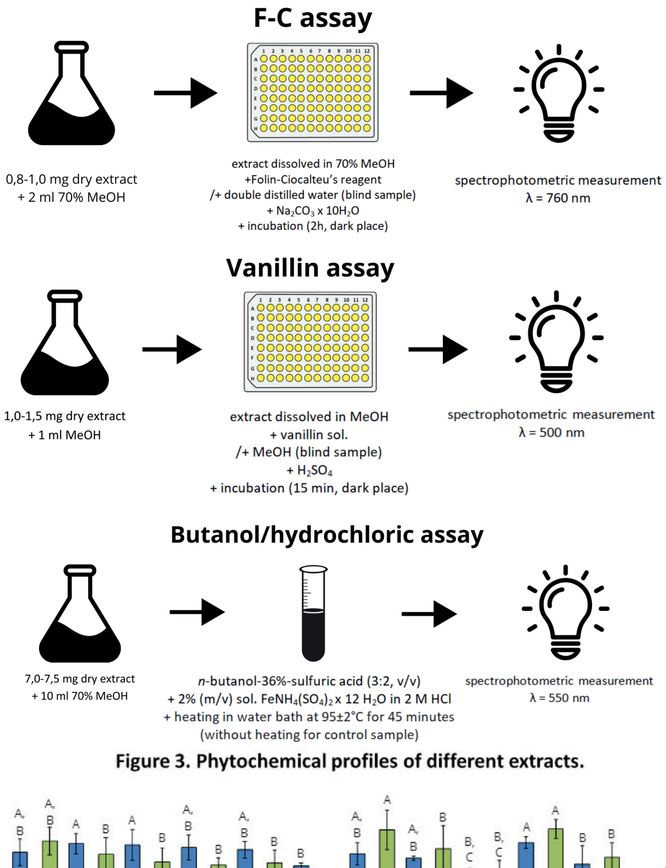
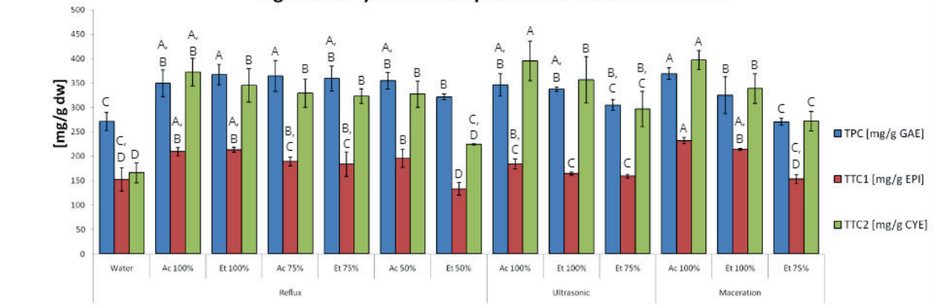


Figure 3. Phytochemical profiles of different extracts.



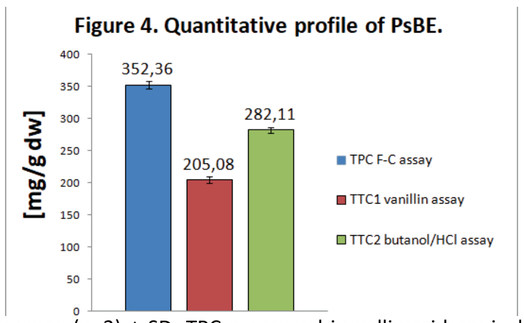
Values are means (n=3) ± SD. For each parameter different letters A-D indicate significant differences (p<0,05). TPC expressed in gallic acid equivalents GAE (mg/g dw); TTC1 expressed in epicatechin equivalents EPI (mg/g dw), TTC2 expressed in cyanidin equivalents CYE (mg/g dw).
Ac - acetone, Et - ethanol, dw- dry weight.

PROFILING OF THE EXTRACT

Considering the optimisation results and other important factors in developing pharmaceutical formulations, **ethanol-water solvent (3:1, *v/v*) and reflux extraction were chosen to prepare *P. spinosa* branches extract (PsBE).**

❹ **Quantitative chemical profiling of PsBE:** determined by spectrophotometric assays of TPC, TTC1, and TTC2 (methodology in Figure 2, results in Figure 4).

Figure 4. Quantitative profile of PsBE.

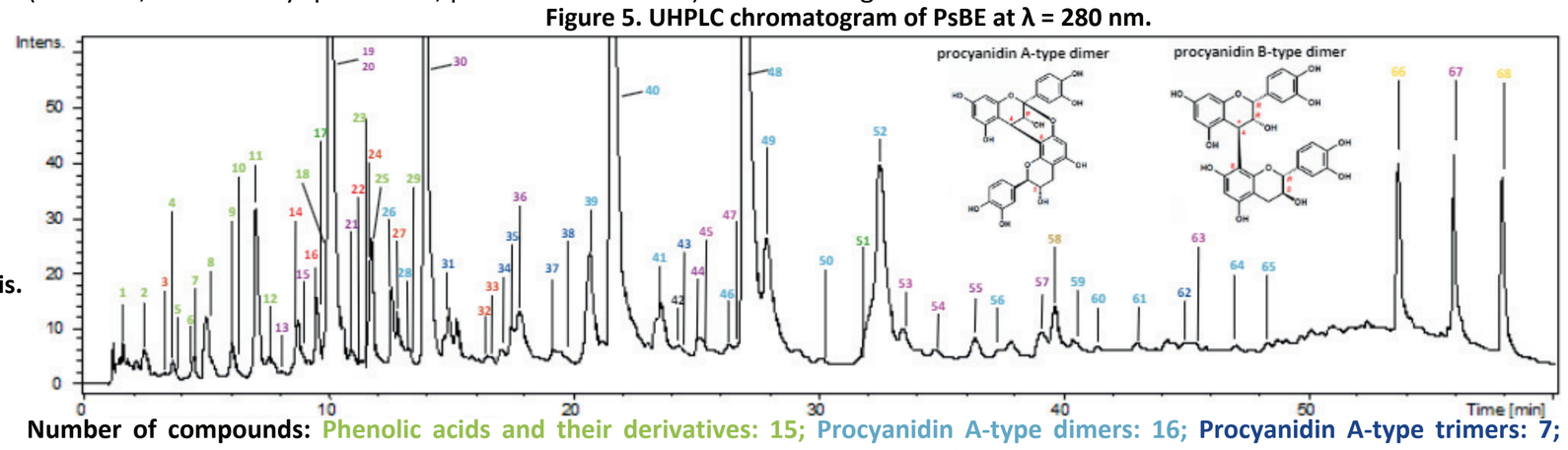


Values are means (n=3) ± SD. TPC expressed in gallic acid equivalents GAE (mg/g dw); TTC1 expressed in epicatechin equivalents EPI (mg/g dw), TTC2 expressed in cyanidin equivalents CYE (mg/g dw); dw - dry weight.

❺ **Fractionation:** performed by CC chromatography (Sephadex-LH20, eluent: methanol) for fractionation of the part of crude extract into low-molecular compounds and high-molecular polymeric compounds.

❻ **Qualitative profiling**: performed by the UHPLC-PDA-MS/MS method acc. Magiera et al [4]. The analytes were structurally characterised based on their chromatographic and spectral properties with the literature data or reference standards (catechin, 3-*O*-caffeoylquinic acid, protocatechuic acid). Results in Figure 5.

Figure 5. UHPLC chromatogram of PsBE at λ = 280 nm.



Number of compounds: Phenolic acids and their derivatives: 15; Procyanidin A-type dimers: 16; Procyanidin A-type trimers: 7; Procyanidin B-type dimers: 3; Procyanidin B-type trimers: 5; Procyanidin A/B trimer: 1; Quercetin derivatives: 6; Kaempferol derivatives: 2; Flavan-3-ol derivatives: 10; Other flavonoids and their derivatives: 2; Unidentified: 1.

1 - Quinic acid; **2** - Vanillic acid-dihexoside derivative; **3**- Procyanidin B-type trimer; **4** - Protocatechuic acid hexoside; **5** - Vanillic acid hexoside; **6** - Protocatechuic acid; **7** - Protocatechuic acid hexoside; **8** - Syringic acid hexoside; **9** – Protocatechuic acid hexoside derivative; **10** - 3-*O*-Caffeoylquinic acid (neochlorogenic acid); **11** - Caffeic acid hexoside; **12** - Hydroxybenzoic acid hexoside; **13** - (Epi)gal hexoside; **14** - Procyanidin B-type dimer; **15** - (Epi)cat hexoside; **16** - Procyanidin B-type dimer; **17** - Taxifolin hexoside; **18** - Quinic acid derivative; **19** – Catechin; **20** – Catechin dimeric ion; **21** - (Epi)cat hexoside; **22** - Procyanidin B-type dimer; **23** - Caffeic acid derivative; **24** - Procyanidin B-type trimer; **25** - Ferulic acid hexoside; **26** - Procyanidin A-type dimer; **27** - Procyanidin B-type trimer; **28** - Procyanidin dimer [(Epi)afz–(Epi)cat]; **29** - Ferulic acid derivative; **30** – (Epi)cat derivative; **31** - Procyanidin A-type trimer; **32** - Procyanidin B-type trimer; **33** - Procyanidin B-type trimer; **34** - Procyanidin A-type trimer; **35** - Procyanidin A-type trimer [(Epi)gal–(Epi)gal–(Epi)gal]; **36** - (Epi)afz; **37** - Procyanidin A-type trimer; **38** - Procyanidin A-type trimer; **39** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)gal)]; **40** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)gal)]; **41** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)gal)]; **42** - Procyanidin A/B-type trimer [(Epi)afz–(Epi)cat–(Epi)cat]; **43** - Procyanidin A-type trimer; **44** - Procyanidin derivative; **45** - Quercetin hexoside-rhamoside; **46** – Procyanidin A-type dimer [(Epi)afz–A–(Epi)gal)]; **47** - Quercetin hexoside; **48** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)gal)]; **49** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)cat]; **50** - Procyanidin A-type dimer [(Epi)gal–A–(Epi)cat]; **51** - Naringenin hexoside; **52** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)cat]; **53** - Quercetin pentoside; **54** - Quercetin rhamnoside; **55** - (Epi)afz derivative; **56** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)afz]; **57** - (Epi)afz derivative; **58** – Unidentified; **59** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)gal)]; **60** – Procyanidin A-type dimer [(Epi)afz–A–(Epi)cat]; **61** - Procyanidin A-type dimer [(Epi)gal–A–(Epi)cat]; **62** - Procyanidin A-type trimer; **63** - Quercetin acetyl-hexoside-rhamoside; **64** – Procyanidin A-type dimer [(Epi)gal–A–(Epi)cat]; **65** - Procyanidin A-type dimer [(Epi)afz–A–(Epi)afz]; **66** - Kaempferol derivative; **67** - Quercetin deriviative; **68** - Kaempferol derivative.

*(Epi)gal - (Epi)gallocatechin; (Epi)cat - (Epi)catechin; (Epi)afz - (Epi)afzelechin.

❼ **Evaluation of antioxidant and antidiabetic activity:** scavenging efficiency of PsBE towards various ROS (ang. *reactive oxygen species*) such as superoxide anion and hydrogen peroxide were tested *in vitro* by spectrophotometric assays; potential antidiabetic effects verified by spectrophotometric glycolytic enzyme inhibition tests (α-glucosidase). Figure 6,7,8.

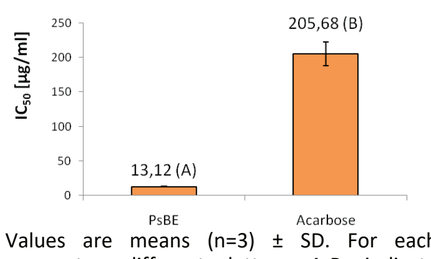
Antioxidant activity assays: evaluated by spectrophotometric methods; results expressed as half-maximal scavenging concentration (SC50) values; Positive control: Trolox.

$O_2^{\bullet-}$: determination of the level of formazan formed by the reduction of nitrotetrazolium.

H_2O_2: determination of quinoimine amount formed as a product of the reaction of phenol, 4-aminoantipyrine, and hydrogen peroxide under the influence of horseradish peroxidase.

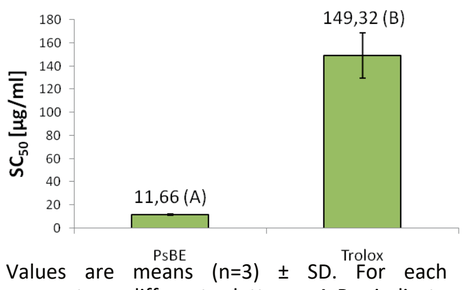
Antidiabetic activity assay: the inhibitory activity of α-glucosidase evaluation; enzyme activity measured by the *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) hydrolysis; results expressed as IC50 (half-maximal inhibitory concentration); Positive control: Acarbose.

Figure 6. Inhibition of α-glucosidase.



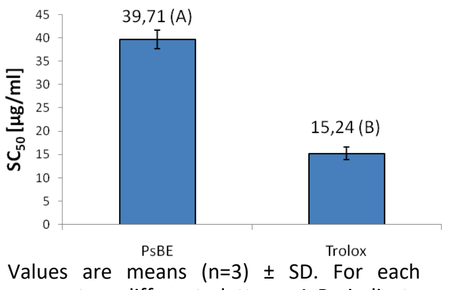
Values are means (n=3) ± SD. For each parameter different letters A-B indicate significant differences (p<0,05).

Figure 7. Scavenging efficiency of $O_2^{\bullet-}$.



Values are means (n=3) ± SD. For each parameter different letters A-B indicate significant differences (p<0,05).

Figure 8. Scavenging efficiency of H_2O_2.



Values are means (n=3) ± SD. For each parameter different letters A-B indicate significant differences (p<0,05).

CONCLUSIONS

- The quantitative and qualitative analysis confirmed the hypothesis that **blackthorn branches ethanol-water (3:1, *v/v*)** extract chosen on the basis of optimisation results is **rich source of polyphenols, especially proanthocyanidins.**
- The qualitative profiling by the UHPLC-PDA-MS/MS method resulted in full or partial **identification of about 68 phenolic constituents (proanthocyanidins, phenolic acids, flavonols)** in the extract. Among detected compounds can be distinguished: phenolic acids and derivatives (15), procyanidin A-type dimers (16), procyanidin A-type trimers (7), procyanidin B-type dimers (3), procyanidin B-type trimers (5), procyanidin A/B trimer (1), quercetin derivatives (6), kaempferol derivatives (2), flavan-3-ol derivatives (10), other flavonoids (2). **The chemical analysis of blackthorn branches extract in this project is highly detailed**, bringing significant novelty to *P. spinosa* branches phytochemistry.
- Fractionation led to obtaining 13 fractions which were separated and grouped into two: **low-molecular and high-molecular compounds**. The compositional analysis of the high-molecular ones is underway.
- The extract showed greater scavenging activity of superoxide anion than Trolox (ca. 13 times lower SC50 value for extract) and stronger inhibitory activity against α-glucosidase (the extract's IC50 value was ca. 16 times lower than for acarbose) thereby stronger inhibitory activity against the tested enzyme than acarbose. **Results of *in vitro* tests highlight the extract's substantial potential to affect pathological mechnism of oxidative-inflammatory disorders** (i.e. vascular complications of diabetes) which shows that it is a good candidate for further extended studies in cellular models.

References:

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3. Rauf A.; Imran M.; (...); Rasul Suleria HA; Biomed Pharmacother., 2019, 116:108999.
4. Magiera A.; Czerwińska M.E.; (...); Olszewska M.A.; Molecules, 2022, 27, 1691.

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