


EREM 72/4 Journal of Environmental Research, Engineering and Management Vol. 72 / No. 4 / 2016 pp. 17-27 DOI 10.5755/j01.erem.72.4.16494 © Kaunas University of Technology	Isolation of Proteins which Interact with Phospholipase A₂ (PLA) from Human Serum after Myocardial Infarction	
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Isolation of Proteins which Interact with Phospholipase A₂ (PLA) from Human Serum after Myocardial Infarction

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The aim of the work was to isolate proteins which interact with phospholipase A₂ (PLA₂) from human serum after myocardial infarction. During this study, the effect of flavonoid inhibitors from the extract of *Bidens Tripartita* was examined. First, the paper describes phytochemical characterisation of compounds found in the plant *Bidens tripartita*. Plant material was harvested at different vegetation stages, and extracts of each were studied for presence of flavonoids by methods such as spectrophotometry and high-performance liquid chromatography (HPLC). Six different flavonoids were identified in extracts. The largest amount of phenolic compounds (mainly rutin and quercetin) was found in the intensive growth vegetation stage, and antioxidant activity corresponds with this result. The conducted analysis shows the dependence of phytochemical composition on the vegetation stage when the plant was collected. These results support the use of bur marigold extracts in pharmaceutical or food industry as a potential source of natural inhibitors of PLA₂. Biochemistry analysis using the pull-down method shows that 7 proteins which bind sPLA₂ were found in healthy blood serum and after myocardial infarction. The biggest fraction was albumins. According to the variant of the sample, different proteins are bound to PLA₂. The data of the pull-down analysis correspond with phytochemical analysis, i.e., they support the presence of natural inhibitors of PLA₂ in *Bidens tripartita* extract.

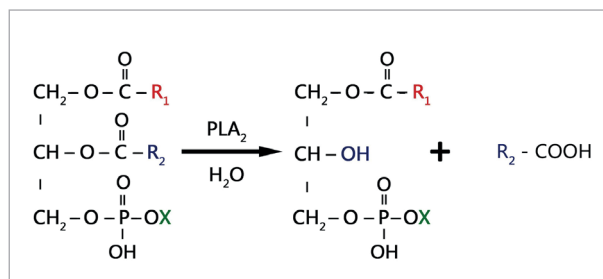
Keywords: phospholipase A₂, PLA₂ inhibitors, protein-protein interactions, flavonoids, *Bidens tripartita*.

Introduction

Secreted phospholipases A₂ (sPLA₂, E.C. 3.1.1.4) are enzymes which catalyse the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids, forming free fatty acids and lysophospholipids. These 13–18 kDa enzymes are structurally very similar (Ho et al., 2001), but they display an impressive variety of different physiological and pathological activities (Dennnis, 1994). Most of sPLA₂ have been identified in mammals: they are found in components of pancreatic juices, liver cells, synovial fluids and in many different mammalian tissues. In normal conditions, sPLA₂ regulate the turnover of free fatty acids in membrane phospholipids, affecting membrane stability and fluidity. Phospholipase activity is also responsible for the generation of intracellular messengers, including arachidonic acid metabolites (Hanasaki and Arita, 1999), shown in Figure 1.

Fig. 1

A specific reaction catalysed by phospholipase A₂ at the sn-2 position of the glycerol backbone; X - any of a number of polar head groups; R₁, R₂ - fatty acid chain (alkyl or alkenyl) groups

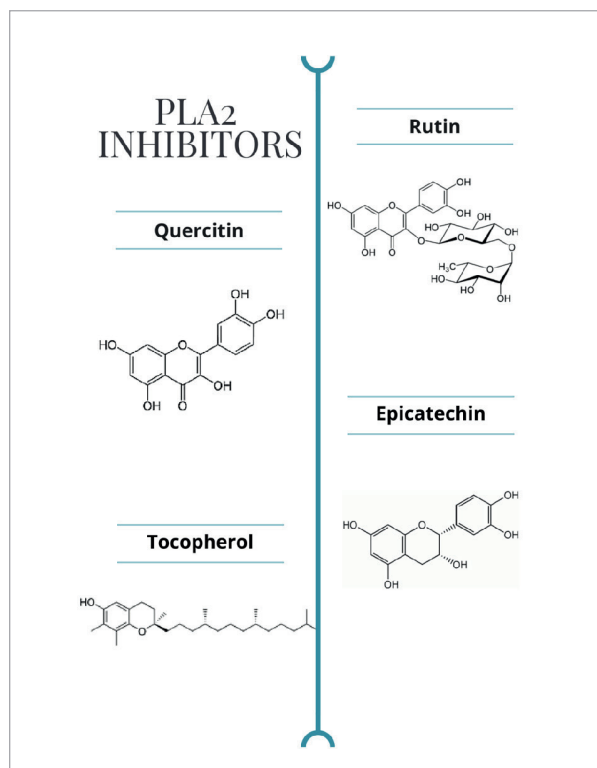


Interestingly, *Viperidae* snake venom contains sPLA₂ enzymes which induce effects like neurotoxicity and myotoxicity (Kini, 1997). Besides, certain snake venom phospholipase A₂ have been identified as specific, non-competitive blood coagulation inhibitors that bind to human activated blood coagulation factor X (FXa) (Kini, 1997). Secreted PLA₂s can also interact with other proteins, for example, calmodulin, antibodies anti-PLA₂ and protein kinase C (PKC) (Kovacic et al., 2009, Faure et al., 2011). On the other hand, those enzymes can interact with non-protein targets – natural phospholipase inhibitors (PLI), flavonoids and polyphenols (e.g., rutin and quercetin) (Wolniak et al., 2007, Lindahl and Tagesson, 1997) (Figure 2).

Flavonoids are a large group of plant secondary metabolites; they are polyphenolic compounds and have a common chemical structure (15-carbon skeleton, which consists of 2 phenyl rings and heterocyclic ring) (Kołodziejczyk, 2012). Flavonoids may be further divided into 6 subclasses. These chemicals are widely distributed in plants and fulfil many functions (Williams et al., 2003). For example, antioxidant activity of flavonoids and their ability to scavenge free radicals cause special preventing effects: they can prevent cardiovascular diseases, cancer or neurodegenerative diseases. Scientists are interested in potential health benefits of flavonoids and look for some plants which contain a lot of polyphenolic compounds to use them for creation of new drugs or treatments (Beecher, 2003). In addition to the content of flavonoids, researchers are also interested in radical scavenging activity of plants. A great number of aromatic, medicinal and other plants contain chemical compounds exhibiting antioxidant properties (Miliaus-

Fig. 2

Chemical structure of flavonoid inhibitors of PLA₂



kas et al., 2004). One of those kinds of plants is *Bidens tripartita* L. *Bidens tripartita* L. (family *Asteraceae*) is an annual plant, 30–100 cm in height with yellow flowers (known as threelobe beggarticks, water agrimony and burr marigold) (Pozharitskaya et al., 2010) and is widely distributed throughout Europe (Tutin, 1996, Szafer et al., 1988). We can also find this plant in North America, North Africa and Asia (mainly China and India).

Some subspecies of *Bidens* have been used in folk medicine, such as *Bidens tripartita*, *Bidens pilosa*, *Bidens frondosa*, etc. (Song, 1999). In this review, I focus directly on *Bidens tripartita* L. Burr marigold has a lot of precious therapeutic value, for example, antioxidant, antibacterial, antifungal or anti-inflammatory properties (Wolniak et al., 2007, Tomczykowa et al., 2008). The herb *Bidens tripartita* has been used in folk medicine as a diuretic and anti-inflammatory agent. It is also used in the treatment of fevers, skin diseases, bladder and kidney troubles, and as a stimulant of the immunological system (Ożarowski, 1993, Strzelecka and Kowalski, 2003, Evans, 1996). The plant was usually combined with a carminative herb such as ginger to treat digestive tract ailments (Chevallier, 1996). The object of investigation is harvested as it comes into flower and is dried for later use (Bown, 1995). Burr marigold is seldom used as a medicine nowadays, but knowledge of its use in folk (Chinese or other) medicine can give a great opportunity to create new therapies and drugs, to use it in the better way. Phytochemical studies on burr marigold herb have shown the presence of flavones, flavanones, chalcones and auronones (Serbin et al., 1975, Olejniczak et al., 2002), coumarins, small amounts of vitamin C, carotenoids and a volatile oil (Tomczykowa et al., 2005). In several papers (Tomczykowa et al., 2005, Kaškonienė et al., 2011), the characterisation of volatile compounds was included, and p-cymene, β -ocimene, phellandrene and β -elemene are the most common of them. What is most interesting, a lot of flavonoids found in the plant extract play a role as natural inhibitors of phospholipases A_2 (Wolniak et al., 2007, Lindahl and Tagesson, 1997). It has been found that rutin (Ljungman et al., 1996), quercetin (Lindahl and Tagesson, 1993) and tocopherol (Pentland et al., 1992) are able to inhibit PLA_2 enzymatic activity. It is not known if they can also inhibit phospholipases protein-protein interactions. All of the mentioned papers are based on investigation into snake venom PLA_2 and its interactions. There is a lack

of works about human phospholipase A_2 , which makes this paper unique. Furthermore, experimental work was carried out using blood serum of both healthy patients and those after myocardial infarction. Blood serum is considered as the gold standard and it remains the required sample matrix for many types of assays (Odoze et al., 2012, Raffick et al., 2010). Serum is easy to collect and more stable than blood plasma. Another reason for using blood serum after myocardial infarction was the fact that this disease is one of the most common causes of death in high- or middle-income countries. The World Health Organization estimated that 12.2% of worldwide deaths in 2004 came from ischemic heart disease (World Health Organization, 2008). Nowadays, this number is rapidly increasing. According to the report of Nichols et al. (2014), cardiovascular disease, in particular myocardial infarction, causes more deaths in Europe than any other disease, and in many countries it causes more than twice as many deaths as cancer (Nichols et al., 2014). Scientists and doctors try to find an appropriate way to reduce mortality of myocardial infarction. Clarification of the PLA_2 molecular properties, their function and interactions with different proteins and inhibitors may be helpful in investigation of new myocardial infarction disease markers. It is also known (Annurad et al., 2010, Li et al., 2010) that one of cardiovascular disease markers can be lipoprotein-associated phospholipase A_2 (Lp- PLA_2), which is a 45-kDa protein of 441 amino acids also known as a platelet-activating factor acetylhydrolase (Birkner et al., 2011).

Materials and methods

Phytochemical characterisation

Plant material and extraction procedure

Bidens tripartita plants were harvested from the collection of medical plants of Kaunas Botanical Garden at Vytautas Magnus University, Lithuania. The herb was collected during the flowering period in 2015 and dried. To conduct the experiment, 5 different stages of vegetation were used:

- Z_1 (beginning of flowering)
- Z_2 (massive flowering)
- Z_3 (the end of flowering)

- _ A (intensive growth)
- _ B (flower bud development)

The dried, powdered aerial parts of *B. tripartita* (500 µg) were extracted successively with 75% methanol at room temperature per 24 hours. For the largest amount of dry material (Z_3), 3 repetitions of extraction were done (Z_3 1; Z_3 2, Z_3 3).

For the solid phase micro-extraction (SPME) method, 20 mg of each sample in 2 repetitions was prepared.

Determination of total content of flavonoids

The calibration graph was built by mixing a 80-µL reference rutin solution in methanol (the same aliquots as in determination on phenolic compounds) with 1920 µL of stock solution. The absorbance was recorded after 30 minutes at 407 nm.

For the determination of flavonoids in the sample, 80 µL of each extract (in 2 different dilutions – 2 times and 4 times) was used. Methanol was used as a blank sample. The total amount of phenolic compounds was calculated from the regression equation and expressed in rutin equivalents (mg RE/g dry extract). The experiment was carried out in triplicate.

HPLC analysis for determination of flavonoids

The separation of samples was carried out using LiChroCART 125-4 C18 column (5 µm; 4×125 mm, Merck Millipore). Flavonoids were detected at 517 nm and 254 nm, respectively, corresponding to the λ_{max} of analysed compounds in methanol solution. The mobile phase consisted of solvent A (water with 0.5% orthophosphoric acid), solvent B (100% methanol) and solvent C (water, acetonitril and methanol at a ratio 2:1:1). The elution profile is shown in Table 1. All the solutions were filtered by means of a 0.2-µm membrane filter and a paper/organic filter (pure methanol).

The flow rate was 0.75 mL/min. For each sample, 2 repetitions were done. In the HPLC analysis, 16 most common flavonoid standards were used. The calibration graph was determined by rutin solution in 0.1-1.0 aliquots.

Animal material and pull-down assays

The experiment was carried out using blood serum of healthy people and blood serum after myocardial infarction. All animal material was received from the Municipal Specialist Clinic in Toruń, Poland. Particular variant sam-

Table 1

Elution profile used in the HPLC analysis

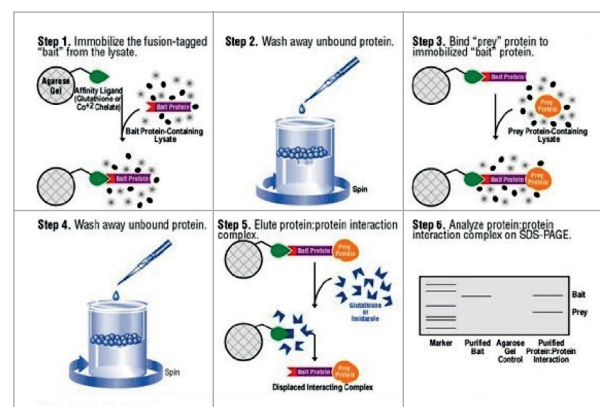
Elution profile		
Time [min]	A % (H ₂ O)	B % (MeOH)
1	2	3
0	99	1
5	75	25
15	56	44
25	55	45
40	1	99
45	1	99
48	99	1
58	99	1

ples (Figure 3) were incubated with shaking on ice for 1 hour. The effect of flavonoid inhibitors was examined by adding extract of *Bidens Tripartita* or 50 mM α -tocopherol and 25 mg/mL rutin to particular samples.

After incubation, each sample was applied to pre-equilibrated nickel iminodiacetic acid (Ni²⁺-IDA) sepharose column. The column was washed with 25 mM Tris-HCl pH 8.0, 300 mM NaCl and 20% glycerol buffer and the protein was eluted with 50 mM EDTA buffer. The eluted protein was concentrated on Microcon® Centrifugal Filters (the sample was centrifuged at 12,000 g at 4°C for 15 min, 5 times). The concentrated fractions obtained after the pull-down assay were applied to electrophoresis SDS-PAGE. Fifteen

Fig. 3

Generalised scheme of the pull-down assay and variants of samples used in the experiment, based on Thermo Scientific Pierce, Protein Interaction Technical Handbook, 2010



SAMPLES:

1. blood serum+PLA2+buffer
2. blood serum+PLA2+CaM+buffer
3. blood serum+PLA2+anti-PLA2+buffer
4. blood serum+buffer (control)

microliter aliquots were analysed by SDS–PAGE on 12% polyacrylamide gels under reducing conditions. Gels for electrophoresis were prepared according to the (Ogita and Markert, 1993). The protein quantity was determined by the Bradford colorimetric assay (Bradford, 1976).

Results and discussion

Phytochemical analysis

Figure 4 shows the total content of flavonoids in *Bidens tripartita* extracts. The concentration of flavonoids

Fig. 4

Total content of flavonoids in different vegetation stages of *Bidens tripartita*

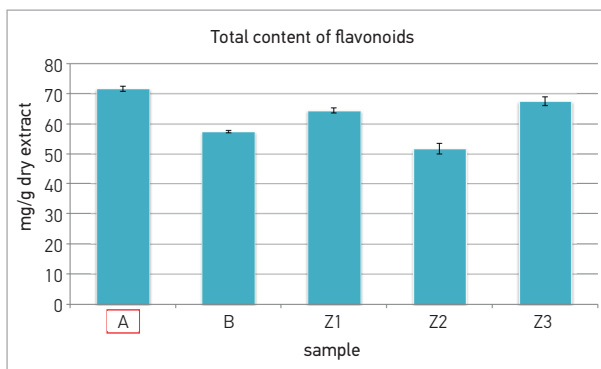
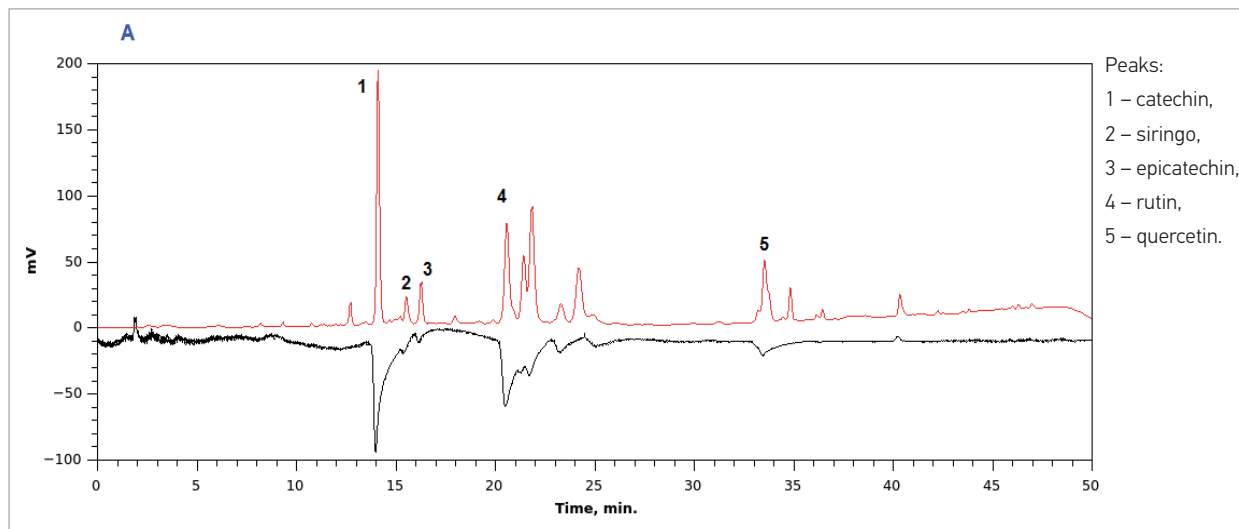


Fig. 5

HPLC chromatogram of *Bidens tripartita* L. in the intensive growth stage



varied between 51.792 and 71.616 mg/g in RE. This amount was determined from regression equation ($y = 0.9767x - 0.036$; $R_2 = 0.973$).

The highest amount of flavonoids was obtained during the intensive growth stage (A) and the lowest was found in the massive flowering stage. The data from the qualitative-quantitative analysis of the extracts made using high performance liquid chromatography (HPLC) are presented in Table 2, and the chromatogram with detector responses at 254 nm is presented in Figure 5.

The components – epicatechin, quercetin, rutin, syringo and catechin – were identified by comparison with the retention time of standards. The quantitative data were calculated from their respective calibration graph of rutin.

Figure 5 shows that the dominant compounds in sample A were catechin and rutin; they constituted 24.28% and 15.02%; the retention time for their standards was 14.23 and 20.807. According to the sources (Pozharitskaya et al., 2010, Tomczykowa et al., 2007), chlorogenic acid, caffeic acid, luteolin, cynaroside and flavanomarein should also be present in extracts. The results may differ depending on extraction method and geographic origin of plants as well – in Pozharitskaya paper The herbs of *B. tripartita* were collected from the plantation of Finland and Leningrad.

Pull-down assays

Figure 6 shows that 7 proteins which bind sPLA₂ were found in healthy blood serum. The biggest fraction were albumins (65–70 kDa) (He et al., 1992). According to the variant of the sample, different proteins were bound to PLA₂. In sample 2 (phospholipase A₂ with calmodulin), we can observe an interaction with unidentified protein of 25–30 kDa. In sample 3 (PLA₂ with anti-PLA₂), we cannot observe the same effect. Figure 7 shows that similar protein fractions were found in blood serum after myocardial infarction. In this case, the biggest fraction was also albumins (65–70 kDa) (He et al., 1992), but

we can observe differences between sample 2 and sample 3: in both of them, protein of 25–30 kDa is presented. According to Gungor et al. (2012), apolipoprotein E (APOE) can play an important role in increasing the risk of coronary heart disease; APOE is a 299 amino acids long, arginine-rich protein at 34.2 kDa weight (Ćwiklińska et al., 2015). Previous studies were related only to the study of the concentration of apoE and Lp-PLA₂ in blood plasma of patients using standard clinical procedures. The pull-down method used in this experiment did not include the examination of Lp-PLA₂; it was used to test human PLA₂. In the serum of patients after myocardial infarction (Figure 7), was observed five common

Fig. 6

Gel electrophoresis for healthy blood serum

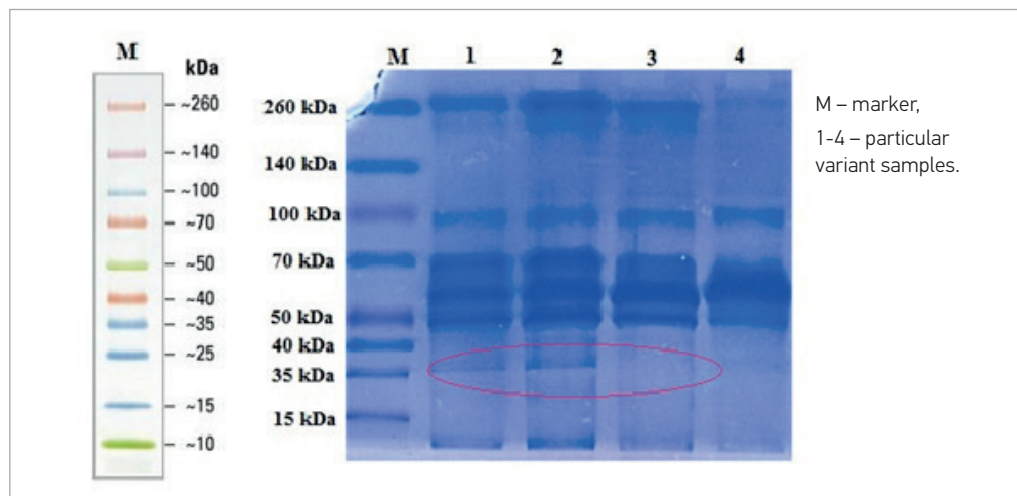
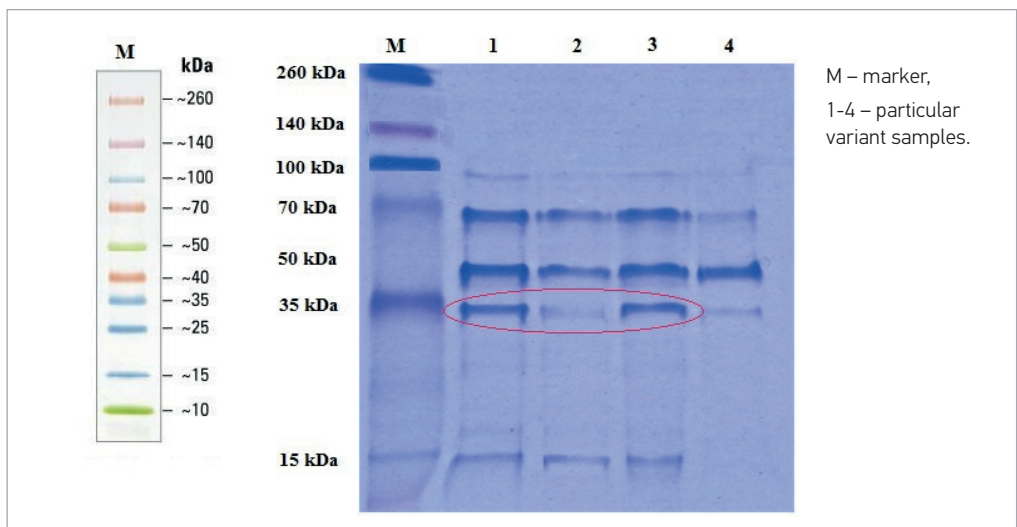


Fig. 7

Gel electrophoresis for blood serum after myocardial infarction



to all attempts protein fractions – according to their molecular weight profile there were albumins. In addition, in sample 3 (interaction studies of phospholipase – specific antibody complex and serum proteins), there are additional bands. It is interesting that they correspond to ApoE (approx. 35 kDa) size. This band is missing in sample 2 (PLA₂-CaM), which suggests that anti-PLA₂ do not interfere with the interaction of the 35 kDa protein fraction with PLA₂, but calmodulin may inhibit the trapping of this fraction from blood by human phospholipase A₂. In order to obtain a full certainty of the origin of the observed band in sample 3, protein identification by MALDI-TOF MS should be performed. Moreover, the

differences in the serum of healthy patients and those after myocardial infarction were observed; it indicates the potential use of the pull-down assay and human PLA₂ as diagnostic for cardiovascular diseases.

Pull-down assays with flavonoids inhibitors of PLA₂

As it was mentioned above, some flavonoids, including rutin (Ljungman et al., 1996) and tocopherol (Pentland et al., 1992) are able to inhibit PLA₂ enzymatic activity. The data of the pull-down analysis using *Bidens tripartita* extract, 50 mM α -tocopherol and 25 mg/mL rutin are presented in Figures 9–11. After incubation with

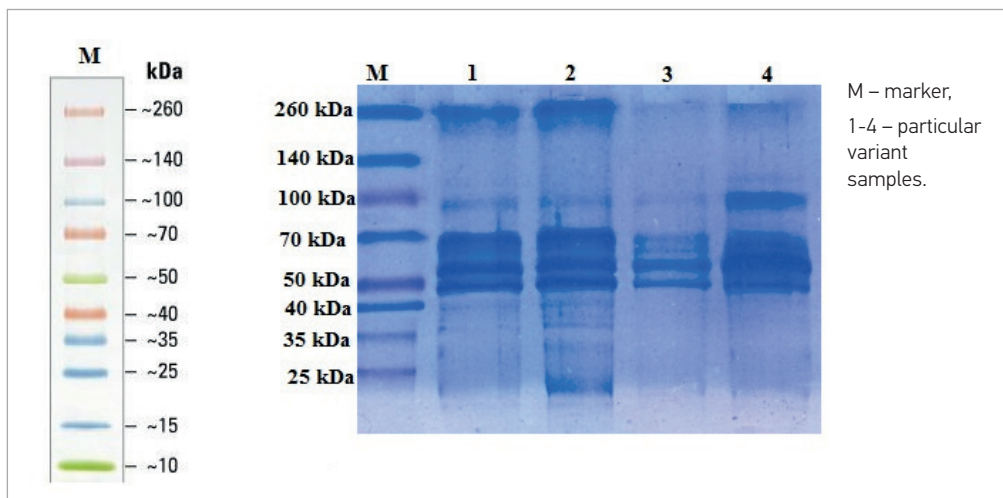


Fig. 9

Gel electrophoresis for healthy blood serum after incubation with 50 mM α -tocopherol

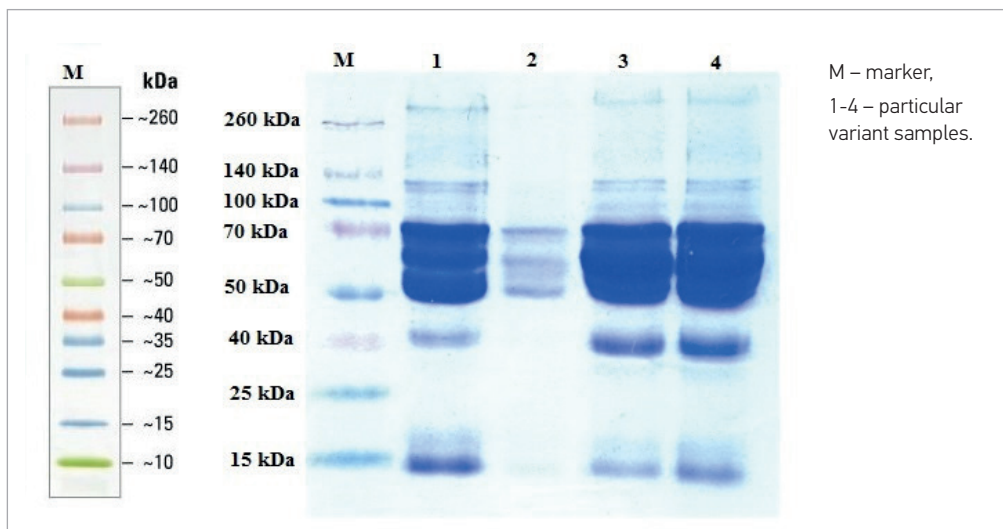
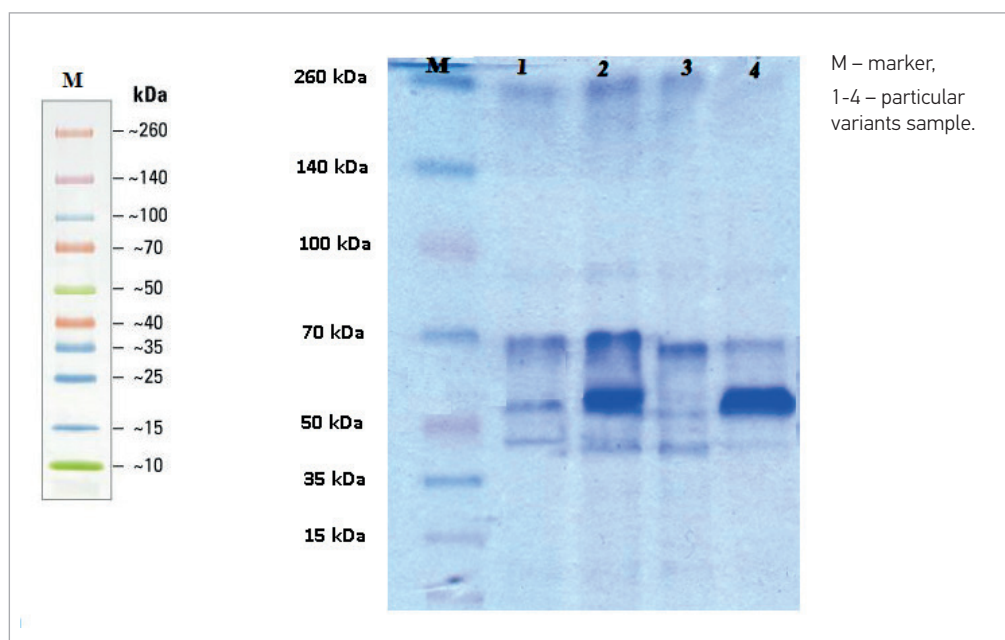


Fig. 10

Gel electrophoresis for healthy blood serum after incubation with 25 mg/mL rutin

Fig. 11
Gel electrophoresis
for healthy blood
serum after
treatment with plant
extract



50 mM α -tocopherol, we can observe a similar effect as in healthy blood serum without inhibitors. In sample 2 (phospholipase A_2 with calmodulin), we can observe an interaction with unidentified protein of 25–30 kDa. In sample 3 (PLA $_2$ with anti-PLA $_2$), we cannot observe the same effect. Figure 11 shows the results of the pull-down assay after treatment with 25 mg/mL rutin. It demonstrates that this kind of flavonoids inhibits an interaction between phospholipase A_2 and calmodulin (sample 2). The last step of the experiment was to investigate if the plant extract of *Bidens tripartita* was a source of PLA $_2$ inhibitors. As shown in Figure 11, the plant extract inhibited efficiently all protein fractions excluding albumins in sample 2 and 4. There is a lack of studies with results of this kind of assays; therefore, it is difficult to discuss them. By analysing both the phytochemistry and pull-down results, we can observe that *Bidens tripartita* is a quite a good source of PLA $_2$ inhibitors. The results of the pull-down assay using the previously described plant extract of *Bidens tripartita* demonstrated that flavonoids inhibited not only the formation of protein complexes between phospholipase A_2 and specific antibody or calmodulin, but also the binding of PLA $_2$ to the serum proteins such as albumin (Figure 11). The specificity of such action may be due to the fact

that the used plant extract contains a mixture of inhibitors in concentrations higher than the other 2 used inhibitors (concentration of flavonoids in the extract was approx. 70 mg/mL, whereas the comparison concentration rutin was 25 mg/mL).

Conclusions

- 1 Blood serum of patients after myocardial infraction and blood serum of healthy people contains various protein fractions which interact with phospholipase A_2 .
- 2 The obtained results of the pull-down assays suggest that 2 places binding proteins can be found in the structure of hPLA $_2$. The first one is able to bind only anti-PLA $_2$ antibodies and the second binding site interacts with calmodulin.
- 3 The HPLC analysis showed presence of 5 flavonoids in *Bidens tripartita* plant during the intensive growth stage. Those substances are inhibitors of secreted phospholipase A_2 (IIA).
- 4 Tocopherol is able to inhibit PLA $_2$ activity, but it does not affect its ability to interact with serum proteins.

- 5 The results demonstrated that rutin efficiently inhibited PLA₂ enzymatic activity and also inhibited an interaction between phospholipase A₂ and calmodulin.

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Addition information

The authors' report was presented at the 10th International Scientific Conference The Vital Nature Sign 2016.

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Baltymų, sąveikaujančių su fosfolipaze A₂ (PLA) esančių žmogaus kraujo serume po miokardo infarkto, išskyrimas

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Šio tyrimo tikslas buvo išskirti baltymus, kurie sąveikauja su fosfolipaze A₂ (PLA₂) ir yra aptinkami žmogaus kraujo serume po miokardo infarkto. Tyrimo metu buvo analizuojamas flavanoidų inhibitoriaus, išskirto iš augalo Triskiautčio Lakišiaus (*lot. Bidens Tripartita*) ekstrakto, poveikis. Pirmiausia, straipsnyje aprašomos augalo Bidens Tripartita ekstrakto išskirtų komponentų, fitocheminės savybės. Augalas buvo auginamas, o skirtingais vegetacijos etapais buvo ruošiami augalo medžiagos ekstraktai, bei naudojant spektrofotometrijos ir skysčių chromatografijos (HPLC) metodus, ekstraktuose tiriama flavonoidai ir jų kiekiai. Ekstraktuose buvo nustatyti šešių skirtingų rūšių flavonoidai. Didžiausias fenolinių junginių kiekis (daugiausia rutinas ir kvercetas) buvo rastas intensyvaus augimo vegetacijos stadijoje, tai patvirtina ir antioksidacinis aktyvumas. Atliktas tyrimas rodo fitocheminės sudėties priklausomybę nuo vegetacijos stadijos, kuomet augalas buvo tiriamas. Šie rezultatai patvirtina medetkų ekstrakto, kaip potencialių natūralių PLA₂ inhibitorių naudojimą farmacijos ar maisto pramonėje. Biocheminė analizė, naudojant išskleidžiamąjį metodą (pull-down method) rodo, kad 7 proteinai, kurie jungiasi sPLA₂ buvo rasti tiek sveiko paciento kraujo serume, tiek po miokardo infarkto. Didžiausia rasta frakcija buvo albuminai. Atsižvelgiant į skirtingus mėginius, skirtingi baltymai buvo surišti su PLA₂. Išskleidžiamosios analizės (pull-down method) duomenys sutampa su fitocheminės analizės duomenimis, t.y. jie patvirtina natūralių PLA₂ inhibitorių buvimą augalo Bidens Tripartita ekstrakto.

Raktiniai žodžiai: fosfolipazė A₂, PLA₂ inhibitoriai, baltymo-baltymo sąveika, flavonoidai, Triskiautis Lakišius (*lot. Bidens Tripartita*).