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# *Influence of the preliminary sample preparation on the tannins content in the extracts obtained from Mutellina purpurea* Poir

Wpływ przygotowania próbki na zawartość garbników w ekstraktach z *Mutellina purpurea* Poir.

## INTRODUCTION

Tannins (commonly referred to as tannic acid) are water-soluble, polyphenolic compounds naturally occurring in higher plants. The primary definition of this class of secondary metabolites was established by Bate-Smith and Swain. They classified tannins as phenolic compounds with a molar mass between 300 and 3000, showing the usual phenol reactions and precipitating alkaloids and other proteins [1]. However molecules with a molar mass of up to 20000 D have also been described and their molecular structures indicate that they should be classified as tannins. Since those tannins are defined as "macromolecular phenolic substances", they are divided into two major groups: the 'hydrolysable' and 'condensed' tannins [4]. Hydrolysable tannins include glucosides either from gallic acid, i.e. gallotannins, or from ellagic acid, i.e. ellagitannins or complex tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit. Condensed tannins are all oligomeric and polymeric proanthocyanidins composed of flavan-3-ol monomer subunits, such as catechin, epicatechin and their gallates [6]. Tannins play varied biological roles such as protein precipitating agents, biological antioxidants, metal ion chelators, or antymicrobial agents. It is possible because of structural variation among this group of natural products [20].

Antioxidants are important in protecting cellular oxidative damage, including lipid peroxidation and damage of DNA chain. Biological antioxidants are classified into enzymes, inhibitors of radical formation and free radical neutralizing agents [16]. Polyphenoles including tannins neutralise oxygen-free radicals, which are unstable and highly reactive molecules that contain unpaired electrons. Free radical molecules are produced via normal metabolic processes and oxidative stress [6]. Tannins interacting with free radicals are known as protectors of cells against aging, cancer and cardiovascular disease. Many tannin molecules have also been shown to reduce the mutagenic activity of a number of mutagens [5].

The Folin–Ciocalteu (F–C) reaction as a standard method for quantitative determination of phenolic compounds is used. This reaction is based on oxidation in alkaline solution of phenols by the yellow molybdotungstophosphoric heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue [14]. It is a determination of total phenolics, which are reducing agents, and the content of tannins and other compounds such as flavonoids, phenolic acids or anthocyanes. Determination of total tannins is partly a chemical reaction and partly a physical interaction. Tannins are measured as the reduction in phenolics that occurs when a binding agent (polyvinyl polypyrrolidone) is added to the extract [17].

*Mutellina purpurea* (Poir.) Thell. is a perennial herb growing on Alpine pastures, among mountain pines or on the glades. It is the herb typical of the Carpathian Mountains and of the Polish Tatra Mountains [2]. *M. purpurea* grows up to 50cm and like other *Apiaceae* plants produces umbels with seeds ripening at summer [12]. In alternative medicine, *M. purpurea* is used for substitution of calcium and potassium. Since mineral balance is disturbed during the cancer preventing diet excluding proteins, tea made from *M. mutellina* herb supplements lack of calcium and potassium [3]. Methanolic extracts from *M. purpurea* herb are active against some *Staphylococcus* and *Pseudomonas* species [Sieniawska E. et al. article under revision]. The essential oil obtained from roots of different collections of *M. purpuraa* contains ligustylid as one of the major constituents [13]. Ligustilide suppreses reactive oxygen species production and extracellular signal-related kinases. Thus, ligustilide contributes to be an effective agent in preventing cardiovascular diseases and cancer[10].

## MATERIAL AND METHODS

E x p e r i m e n t a l  d e s i g n . Chemical reagents of high purity were purchased from Sigma–Aldrich Chemie GmbH (Munich, Germany) and Merck (Darmstadt, Germany). The plant material was collected in the Botanical Garden of the Medical University in Lublin in June 2010. Plants were dried at room temperature, powdered and extracted.

### EXTRACT PREPARATION OBTAINED BY POLISH PHARMACOPOEIA VIII METHOD [12]

Pulverized herb (1 g) of *M. purpurea* was subjected to 30 min extraction on a water bath under reflux with 150 ml of deionised water. The cooled extract was left for precipitation of a sediment. The extract was filtrated with filter paper and 50 ml of filtrate was discarded. The obtained solution (solution I) was filled with deionised water up to 250 ml.

### EXTRACTION WITH PRELIMINARY SAMPLE PREPARATION

H o m o g e n i z a t i o n . The first portion of pulverized herb (1 g) of *M. purpurea* was subjected to 30 min. homogenization with 150 ml of deionised water in the mechanical homogenizer (Homogenizer type 302, Mechanika Precyzyjna, Poland) with 5000 rpm speed. The obtained blend was extracted for 30 min. on a water bath under reflux as described in the Pharmacopoeia method (H-FPVIII).

M i x i n g . The second portion of pulverized herb (1 g) of *M. purpurea* was subjected to 60 min maceration with 150 ml of deionised water in the orbital shaker (RS10 Control, IKA Werke GmbH,

Germany), speed of shaking 180 $min^{-1}$. Subsequently, the obtained mixture was extracted for 30 min on a water bath under reflux (M-FPVIII).

Ultrasonic. The third portion of pulverized herb (1 g) of *M. purpurea* was subjected to 30 min sonification with 150 ml of deionised water in the ultrasonic water bath (Sonorex Digitral 10P, Bandelin, Germany), extraction temperature was approximately 60ºC, with 100% of sonifcation power. Subsequently, the obtained mixture was extracted for 30 min. on the water bath under reflux (U-FPVIII).

## DETERMINATION OF TOTAL PHENOLICS

5 ml of solution I was taken and filled with deionised water up to 25 ml (solution II) . Subsequently, 2 ml volume of solution II was added to 1 ml of molybdotungstophosphoric heteropolyanion reagent, 10 ml of water and filled up to 25 ml with sodium carbonate. Spectrophotometric measurement was done after 30 min time in the $\lambda$=760 nm ($A_1$) (Cary 50 UV-Vis Spectrophotometer, Varian INC, USA). Kinetics of reaction and maximum absorbance were obtained by using Cary WinUV Software.

## DETERMINATION OF CONDENSED TANNINS (PHENOLICS NOT INTRACTING WITH HIDE POWDER)

10 ml of solution I was taken, 100 mg of *hide powder* was added and the mixture was shaken for 60 min (speed 180 $min^{-1}$). Then the extract was filtrated, 5 ml was taken and filled with deionised water up to 25 ml (solution III). Subsequently, 2 ml volume of solution II was added to 1 ml of molybdotungstophosphoric heteropolyanion reagent, 10 ml of water and filled up to 25 ml with sodium carbonate. Spectrophotometric measurement was done after 30 min. time in the $\lambda$=760 nm ($A_2$).

## REFERENCE SOLUTION SPECTROPHOTOMETRIC MEASUREMENT

50 mg of pirogallol was suspended in deionised water and filled up to 100 ml volume, *ex tempore* (standard solution I). 5 ml of standard solution I was taken and filled with deionised water up to 100 ml (standard solution II). Subsequently, 2 ml volume of solution II was added to 1 ml of molybdotungstophosphoric heteropolyanion reagent, 10 ml of deionised water and filled up to 25 ml with sodium carbonate. Spectrophotometric measurement was done after 30 min. time in the $\lambda$=760 nm ($A_3$). The percentage of tannin content in the plant material on the basis of the following equation was calculated.

$$x_i(\%) = 62{,}5\ (A_1 - A_2)\ m_2 / A_3 \times m_1$$

$x_i$ = tannins in the plant material, $m_1$ = plant material weight [g]; $m_2$ = pirogallol weight [g]

Statistical analysis, according the Pawlaczyk et al. [14] on the basis of Q-Dixona test was conducted. A the confidence level $\alpha$ = 0.05% (P = 95%) it was verified that the results in the particular preliminary extraction method belong to the same test population [14]. Statistical parameters: arithmetic means, standard deviation of the individual value and arithmetic means and relative standard deviation are given in Table 1.

Table 1. Total tannins content in the aerial parts of *M. purpurea* calculated on pirogallol. Tannins amounts coresponding to the preliminary sample preparation and not modified FP VIII method of extraction. Statistical analysis of percent tannins content

| Polish Pharmacopoeia method | | | | |
|---|---|---|---|---|
| n | A1 | A2 | Percentage content | Statistical parameters |
| 1 | 0.1314 | 0.1098 | 0.2368 | n = 10. $t_{\alpha,f}$ ($\alpha$ = 0.05. f = 9) = 2.262<br>$\bar{x}$ = 0.2241%; $S_1$= 5.3173 x $10^{-3}$; $S_1^2$ = 2.8273 x $10^{-5}$;<br>S$\bar{x}$ = 1.603 x $10^{-4}$<br>$\mu$ = 0.2241% ± 0.0038; RSD = 0.0237 |
| 2 | 0.1300 | 0.1100 | 0.2193 | |
| 3 | 0.1301 | 0.1095 | 0.2259 | |
| 4 | 0.1300 | 0.1096 | 0.2237 | |
| 5 | 0.1302 | 0.1096 | 0.2259 | |
| 6 | 0.1301 | 0.1096 | 0.2248 | |
| 7 | 0.1301 | 0.1097 | 0.2237 | |
| 8 | 0.1303 | 0.1099 | 0.2237 | |
| 9 | 0.1302 | 0.1104 | 0.2171 | |
| 10 | 0.1299 | 0.1098 | 0.2204 | |
| H-FPVIII | | | | |
| 1 | 0.0987 | 0.0864 | 0.1349 | $\bar{x}$ = 0.1297%; $S_1$= 3.9847 x $10^{-3}$; $S_1^2$ = 1.5878 x $10^{-5}$;<br>S$\bar{x}$ = 1.260 x $10^{-3}$<br>$\mu$ = 0.1297% ± 0.0028; RSD = 0.0307 |
| 2 | 0.0984 | 0.0866 | 0.1294 | |
| 3 | 0.0983 | 0.0867 | 0.1272 | |
| 4 | 0.0983 | 0.0863 | 0.1316 | |
| 5 | 0.0987 | 0.0865 | 0.1338 | |
| 6 | 0.0990 | 0.0874 | 0.1272 | |
| 7 | 0.0980 | 0.0867 | 0.1239 | |
| 8 | 0.0981 | 0.0863 | 0.1294 | |
| 9 | 0.0978 | 0.0864 | 0.1250 | |
| 10 | 0.0988 | 0.0865 | 0.1349 | |
| M-FPVIII | | | | |
| 1 | 0.1010 | 0.0795 | 0.2357 | $\bar{x}$ = 0.2387%; $S_1$= 8.2901 x $10^{-3}$; $S_1^2$ = 6.8727 x $10^{-5}$;<br>S$\bar{x}$ = 2.6215 x $10^{-3}$<br>$\mu$ = 0.2387% ± 0.0059; RSD = 0.0347 |
| 2 | 0.1007 | 0.0799 | 0.2281 | |
| 3 | 0.1012 | 0.0797 | 0.2357 | |
| 4 | 0.1017 | 0.0796 | 0.2423 | |
| 5 | 0.1022 | 0.0794 | 0.2500 | |
| 6 | 0.1019 | 0.0795 | 0.2456 | |
| 7 | 0.1023 | 0.0798 | 0.2467 | |
| 8 | 0.1022 | 0.0799 | 0.2445 | |
| 9 | 0.1015 | 0.0803 | 0.2325 | |
| 10 | 0.1007 | 0.0801 | 0.2259 | |
| U-FPVIII | | | | |
| 1 | 0.1339 | 0.1075 | 0.2895 | $\bar{x}$ = 0.3016%; $S_1$= 7.6735 x $10^{-3}$; $S_1^2$ = 5.8882 x $10^{-5}$;<br>S$\bar{x}$ = 2.4266 x $10^{-3}$<br>$\mu$ = 0.3016% ± 0.0055; RSD = 0.0254 |
| 2 | 0.1344 | 0.1069 | 0.3015 | |
| 3 | 0.136 | 0.1069 | 0.3191 | |
| 4 | 0.1344 | 0.1067 | 0.3037 | |
| 5 | 0.1345 | 0.1066 | 0.3059 | |
| 6 | 0.1342 | 0.1066 | 0.3026 | |
| 7 | 0.1340 | 0.1066 | 0.3004 | |
| 8 | 0.1338 | 0.1064 | 0.3004 | |
| 9 | 0.1337 | 0.1067 | 0.2961 | |
| 10 | 0.1339 | 0.1068 | 0.2971 | |

A1 – absorbance of total phenolics; A2 – absorbance of phenolics not intracting with *hide powder*; A3 = 0.285

## RESULTS AND DISCUSSION

On the basis of UV spectrum of *M. purpurea* extract with proper reagents (molybdotungstophosphoric heteropolyanion reagent, deionised water, sodium carbonate), the maximum of absorbance for this complex was estimated as λ=760 nm (Fig. 1). In this wave length, the kinetics of the spectrophotometrical reaction was determined. It is shown in Fig. 2 that the reaction became stable after 25 min. The result concerning the tannins content in the aerial parts of *M. purpurea* in the Table 1 was shown.

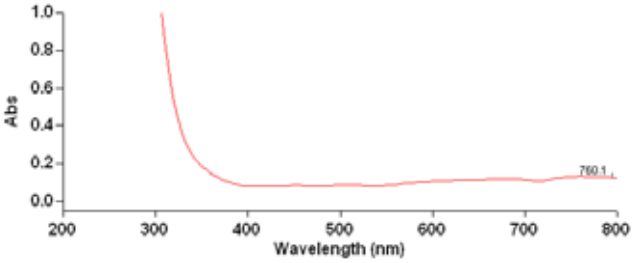


Fig. 1. UV spectrum for the *M. purpurea* extract with added reagents (molybdotungstophosphoric heteropolyanion reagent, deionised water, sodium carbonate)

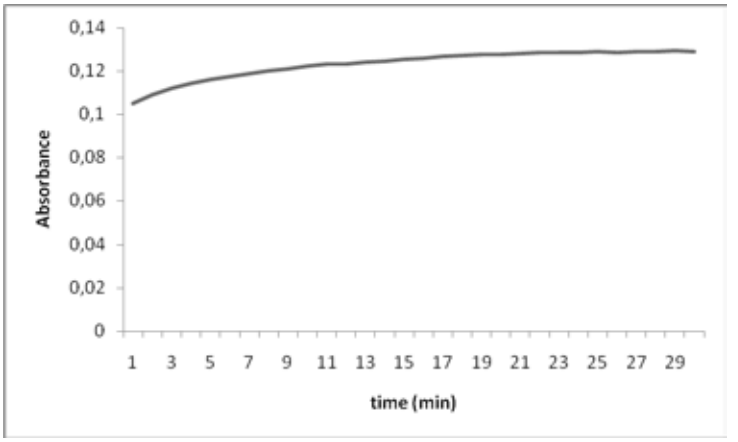


Fig. 2. Kinetics of tannins estimation reaction in *M. purpurea*, λ = 760

As results from Table 1, the highest percent content of tannins in the investigated plant material was achieved applying preliminary ultrasound assisted extraction followed by classical extraction the FP VIII 0.30%. Somewhat lower tannins content was after Polish Pharmacopoeia VIII method for isolation of tannins, 0.22%. The lowest stated amount of tannins in *M. purpurea* was after previous

homogenisation of plant material, 0.13%. The percent amount after preliminary maceration was slightly higher than for FP VIII method, 0.24%.

According to Polish Pharmacopoeia VIII, standard tannins extraction procedure involves the boiling of plant material with hot water [15]. This method reduces the number of possible solvent interaction with water. Recently, many authors have applied different extraction methods to find the most efficient one [7–9, 19]. These researches focus on comparing individual extraction techniques. On the contrary, the present authors' present pre-extraction procedures coupled with proper extraction, because preliminary sample preparation gives a possibility to enhance solvent penetration to plant tissue. This is the first time when the influence of sample preparation on tannins content is investigated.

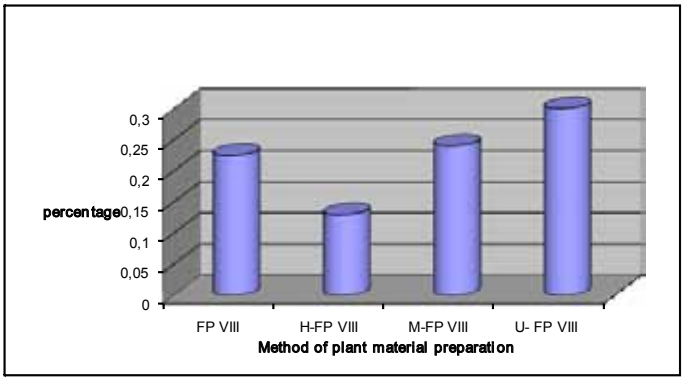


Fig. 3. Influence of the preliminary sample preparation on tannins content in the *M. purpurea.* FP VIII- Polish Pharmacopoeia VIII extraction procedure, H- FP VIII – homogenisation + FP VIII, M-FP VIII – maceration + FP VIII, U-FP VIII – ultrasound assisted extraction + FP VIII

Among the investigated methods of preextraction, compared with standard tannins extraction FPVIII, only U-FPVIII gave an increase in the amount of the extracted tannins (Fig. 3). Recently, ultrasound assisted extraction (UAE) has been used quite often on the phytochemical field however, it gives no information about using ultrasounds in the tannins extraction. It is difficult to apply UAE methods for tannins extraction because even modern ultrasound water baths have a maximum temperature of approximately 80°C. However ultrasounds joined with the following classical extraction (U-FPVIII ) give good results (Table 1). Unexpectedly, using homogenisation (H-FPVIII) as a sample preparation technique, the lowest quantity of tannins was obtained (Tab. 1). This result can be caused by drastic, mechanical mincing of plant tissue effecting tannin compounds hydrolysis in the water medium. Two of the compared methods of preliminary sample preparation involve a kinetic energy increase. In the U-FPVIII and H-FPVIII solvent-substance system higher temperature should enhance the extraction process. However, concerning homogenisation, the temperature increase in the solvent-substance system was from ambient temperature to 55°C, which could cause decrease in the tannins content. On the contrary, ultrasound energy is strong, but does not rip the plant structures, only improves solvent penetration into the cells and the isolation of biologically active compounds. One hour maceration with constant shaking did not affect extraction improvement (Table 1). This

suggests that the FPVIII extraction method is sufficient for tannins isolation from the plant material. It is the first scientific report about tannins content in the *M. purpurea*. The tannins quantity in this herb is comparable to the tannins content in Black YUNNAN Golden Leaf Tea, Green Oryginal „Celmar" Tea and Black BROOKE BOND Tea [18].

## CONCLUSIONS

In the presented work the joined techniques of preliminary sample preparation and extraction were applied. The authors indicate that preliminary ultrasound assisted extraction increases the amount of tannins extracted from *M. purpurea* compared to classical methods described by the Polish Pharmacopoeia VIII. On this plant material it was revealed that one hour of preliminary maceration did not influence the efficiency of tannins extraction, whereas mechanical homogenization reduced the tannin content by approximately 50%. On the basis of this research, it seems that preliminary ultrasound assisted extraction joined with classical extraction FPVIII is a simple way to improve tannins isolation from plant material. It is the first time the tannins content in the aerial parts of *Mutellina purpurea* was assigned.

## REFERENCES

1. Bate-Smith, E. C., Swain, T.: Flavonoid compounds. In H. S. Mason & A. M. Florkin (Eds.), Comparative biochemistry. Academic Press, New York 1962.
2. Bojnanský V., Fargašová A.: Atlas of Seeds and Fruits of Central and East-European Flora, The Carpathian Mountains Region. Springer Verl., Neu-Isenburg 2007.
3. Breuss R. Krebs - Leukämie und andere scheinbar unheilbare Krankheiten mit natürlichen Mitteln heilbar. Mebus 1990.
4. Chung K.T., Wong T.Y. et al.: Tannins and human health, Crit. Rev. Food Sci. Nutr., 38, 421, 1998.
5. Griffiths D.W. in: Toxic Substances in Crop Plants, ed. D'Mello J. P. F., Duffus D. M. and Duffus J. H., The Royal Society of Chemistry, Cambridge 1991.
6. Khanbabaee K., van Ree T: Tannins: Classification and Definition, Nat. Prod. Rep., 18, 641, 2001.
7. Khennouf S., Amira S. et al.: Effect of Some Phenolic Compounds and *Quercus* Tannins on Lipid Peroxidation. World Appl. Sci. J., 8, 1144, 2010.
8. Komaszewska E., Mucha K., Baj T. et al.: Comparison of classic and modern methods of furocoumarins extraction from *Angelica officinalis* Hoffm. and *Pastinaca sativa* l. fruits, Annales UMCS, sect. DDD, 22, 93, 2009.
9. Kozyra M., Głowniak K.: Influence of the extraction mode and type of eluents on the isolation of coumarins from plant material. Herba Pol., 52, 71, 2006.
10. Lu Q., Qiu TQ., Yang H.: Ligustilide inhibits vascular smooth muscle cells proliferation. Eur. J. Pharm., 542, 136, 2006.
11. Makkar H.P.S.: Quantification of Tannins in Tree and Shrub Foliage. A Laboratory Manual, Harinder Makkar. Kluwer Academic Publishers, Dordrecht 2003.
12. Mirek Z., Piękoś-Mirkowa H.: Kwiaty Tatr. Przewodnik kieszonkowy. Multico Oficyna Wyd., Warszawa 2003.

13. Passreiter C. M., Akhtar Y., Isman M.B.: Insecticidal Activity of the Essential Oil of *Ligusticum mutellina* Roots. Z. Naturforsch., 60C, 411, 2005.
14. Pawlaczyk J., Zając M. [ed.]: Walidacja metod analizy chemicznej. AM, Poznań 2005.
15. Polish Pharmacopoeia VIII. URPL,WMiPB, Warszawa 2008.
16. Simic, M.G., Jovanovic, S.V. In: Ho, C.T., Osawa, T., Huand, M.T., Roren R.T. (Eds.), Food Phytochemicals for Cancer Prevention II. Teas, Spices and Herbs. American Chemical Society: Washington DC 1994.
17. Singleton V.L., Rossi J.A.: Colorimetry of total phenolics with phosphomolybdic– phosphotungstic acid reagents, Am. J. Enol. Vitic., 16, 144, 1965.
18. Stańczyk A.: Garbniki katecholowe różnych gatunków herbat. Bromat. Chem. Toksykol., 12, 95, 2008.
19. Waksmundzka-Hajnos M., Petruczynik A., Dragan A. et al.: Influence of the extraction mode on the yield of some furanocoumarins from *Pastinaca sativa* fruits, J. Chrom. B, 800, 181, 2004.
20. web site: http://www.users.muohio.edu/hagermae/tannin.pdf, access on 27.09.2010.

## SUMMARY

There is no information in the current literature about scientific research describing tannins in the *Mutellina purpurea* Therefore, this work presents investigation of total tannins in the aerial parts of *M. purpurea*. Determination of tannins was done according to the method described in the Polish Pharmacopoeia VIII, with the molybdotungstophosphoric heteropolyanion reagent. The authors applied the preliminary sample preparation procedure before the classic extraction. Maceration of plant material, homogenization and ultrasonication were tested. The best result with the preextraction in the ultrasounds was obtained (0.30%).

*Keywords: Mutellina purpurea*, tannins, extraction, homogenization, maceration, ultrasonic

## STRESZCZENIE

W danych literatury brak jest doniesień dotyczących zawartości związków garbnikowych w *Mutellina purpurea*, dlatego też autorzy pracy przeprowadzili analizę sumy garbników w nadziemnych częściach tej rośliny. Oznaczenie prowadzono metodą farmakopealną (FP VIII) z odczynnikiem fosforomolibdenowolframowym. W pracy zbadano także wpływ przygotowania próbki na zawartość oznaczanej grupy związków. Przed klasycznym procesem ekstrakcyjnym (FP VIII) substancja roślinna była poddana homogenizacji, maceracji lub działaniu ultradźwięków. Z uzyskanych danych wynika, iż po wstępnej ekstrakcji ultradźwiękowej udało się uzyskać najwyższą zawartość związków garbnikowych w badanej substancji roślinnej (0,30%). Wpływ pozostałych metod wstępnej ekstrakcji był nieznaczny.

*Słowa kluczowe: Mutellina purpurea*, garbniki, maceracja, homogenizacja, ultradźwięki