Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(5): 2733-2737
Received: 16-07-2018
Accepted: 18-08-2018

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Determination of biologically active substances with antioxidant potential in different extracts of *Fragaria vesca* L. leaves and flowers

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Abstract
Fragaria vesca L. (wild strawberry) belongs to the Rosaceae family. The aerial part from wild strawberry was applied in traditional medicine. *Fragaria vesca* are a rich source of biologically active substances like tannins, proanthocyanidins, flavonoids and phenolic acids. The aim of this study was to compare the value of phytochemical compounds and antioxidant activities in infusion, decoction glycerine extracts and tinctures obtained from the F. vesca leaves and flowers. The extracts were analyzed regarding their secondary metabolites content (total polyphenols, total tannins, and total proanthocyanidins) and antioxidant activities (DPPH and FRAP methods). The analysis of 50% glycerin extracts under ultrasound irradiation shown the highest level of total polyphenols (150.2 mg GAE/g dw), total tannins (65.8 mg TAE/g dw) total proanthocyanidins (193.3 mg LE/ 100g dw) and antioxidant activities – radical scavenging activity (DPPH – 1255.2 m mol TE/ g dw) and metal reducing ability (FRAP – 1129.2 mmol TE/ g dw). The results showed that the 50% glycerin extracts from leaves and flowers of *Fragaria vesca* are appropriate additives for the preparation of emulsion for natural cosmetic products with improved biological activity. The most suitable solvent for tincture preparation was 50% ethanol. With this solvent, the biologically active substances were best extracted (total polyphenols – 10847.3 mg GAE/ L). The influence of the storage period at room temperature on the amount of biologically active surfactants in the obtained tinctures was also monitored. The optimal shelf life for tinctures has been determinate at 12 months.

Keywords: *Fragaria vesca* L., tinctures, glycerin extracts, antioxidant activity

Introduction
Fragaria vesca L. (wild strawberry) is epigeal perennial herb belongs to the Rosaceae family. Wild strawberry is a plant that grows in subtropical zones of the Northern hemisphere, and it is native to Europe and temperate regions of Asia. The leaves and roots from wild strawberry are herbal materials used in traditional medicine. *Fragaria vesca* are rich source of biologically active substances like tannins, procyanidins, anthocyanidins, flavonoids, phenolic acids organic acid, vitamins, micro- and macroelements (Ca, Mg, K, Fe, Cu, Mn, Zn) (Mudnic *et al*., 2009, Buricova *et al*., 2011, Liberal *et al*., 2014, Liberal *et al*., 2015, Dias *et al*., 2015a,b) [2, 4, 5, 10, 11, 15]. The leaves are gently astringent and are used in gastrointestinal and skin diseases, sclerosis, in traditional medicinal phytotherapy (Landjev, 2010) [9]. The several studies of wild strawberry have been reported that this plant possessed antioxidant (Ivanov *et al*., 2015) [7], anti-inflammatory (Liberal *et al*., 2014) [11], vasodilatory, cardiovascular (Mudnic *et al*., 2009) [15], anti-thrombotic (Naemura *et al*., 2005) [16], antibacterial (Borah *et al*., 2012) [1] activities.
Fragaria vesca leaves and flowers a potential source of bioactive compounds with high antioxidant potential. Therefore, the aim of this work is to disclose the bioactivity of different extracts (infusions, decoctions, ultrasound assisted extracts) obtained from *Fragaria vesca* L. leaves and/or flowers on and to characterize the total phenolic content, total tannins content and total proanthocyanidins content and investigated their antioxidant potential. Furthermore, it has been investigated the change in the amount of phytochemical compounds and their antioxidant potential in tinctures obtained from the wild strawberry leaves in a period of 18 months.

Materials and methods
1. Plant material
Aerial parts (leaves and flowers) by several random chosen plants of *F. vesca* L., were collected from their natural habitats nearby "Zdravec" hut Rhodope Mountains (Balkan Peninsula) (Coordinates: 41°59′42″N 24°40′57″E) in Jun 2016. The samples were dried at ambient temperature (25°C) for 7 days, and finely ground by homogenizer (MKM 6000.

BOSCH, Slovenia). The powder (standard size of particles 0.63 mm) was used for directly for different extraction.

2. Extraction procedure

Two aqueous extracts (infusion and decoction) were prepared according to Pistón *et al*., 2014 [19]. Briefly, for decoction preparation, the dried leaves and flowers (1 g) were added to 50 mL of hot ultrapure water, than heated, kept in boiled water for 15 min and after that the mixture was removed from the heat, stood for 20 min and filtered through filter paper (Whatman® 1). Infusion was prepared by adding 50 mL of ultrapure hot water at 95°C to 1 g of dried leaves and the mixture was left to stand for 15 min to be also filtered using filter paper. 1 gram of dry samples were placed in a plastic tube and 50 mL solvents 50 % glycerol or 50 mL water were added. Ultrasound-assisted extraction was performed in ultrasonic bath SIEL UST 5.7-150 (Gabrovo, Bulgaria) with frequency 35 kHz and power 240 W at temperature 40 °C for 15 min. The tinctures were prepared by maceration for 14 days with 40%, 50%, 60% and 70% (v/v) ethanol at room temperature and storage in a dark place. The material/solvent ratio was as 1:10. Solids leaves were separated by filtration and tinctures were stored at room temperature (18 - 23°C) for 18 mounts. Each extracts were analyzed for polyphenol content and antioxidant activity.

3. Determination of total polyphenolic compounds

The total phenolic contents were measured using a Folin-Ciocalteu assay. Folin-Ciocalteu reagent (1mL) (Sigma) diluted five times was mixed with 0.2 mL of sample and 0.8 mL 7.5 % Na_2CO_3. The reaction time was 20 min at room temperature in darkness. After reaction time, the absorption of sample was recorded at 765 nm using a spectrophotometer (Camspec M107, UK) against blank sample, developed the same way but without extract. Total phenols content was expressed as mg of gallic acid equivalent (GAE) per g of dry matter (DW) according to calibration curve; build in range of 0.02 - 0.10 mg gallic acid used as a standard.

4. Total tannins assay

Phenolic Browning Assay: A spectrophotometric assay of the rate of browning of low-molecular-weight phenolic compounds was adapted to measure the browning of tannins. The samples 0.3 mL (diluted 1:2 with 70% ethanol) was dissolved in 7.7 mL pH 10 buffer (5 mM Na_2CO_3: 5 mM $NaHCO_3$ in ratio 6:4). Absorbance was measured at 415 nm, beginning at 15 sec after the addition of the sample. Subsequent measurements were made with a kinetic protocol every 60 sec over a period of 8 min. The initial, linear rate of browning (Abs/min) was measured within the first 6 min of the reaction. A pentagalloyl glucose (tannic acid) standard was run on each day to confirm that measurements were consistent through time. Plots of browning rate vs. sample concentration (mg) were made for each sample to confirm that rate was a linear function of concentration for each compound. The slopes of these plots were used as the browning rates (normalized for sample concentration as Abs/min/mg phenolics) (Moilanen, 2015, Lincheva *et al*., 2017) [12, 14].

5. Total proanthocyanidins assay

Acid butanol was used for assaying proanthocyanidins, according to Porter *et al*. (1986) [20]. Six milliliters of the acid butanol reagent (950 mL of n-butanol with 50 mL concentrated HCl), 0.5 mL aliquot of the sample, and 0.1 mL of the iron reagent (2 % ferric ammonium sulphate in 2 mol/L HCl) were added to 10 mL screw cap tube and then vortexed. The tube was capped loosely and put in a boiling water bath for 15 min. The absorbance of formed colored complex was read at 550 nm. Condensed tannins were expressed as mg leucocyanidin equivalent per 100 g dw (Hagerman, 2011) [6].

6. Antioxidant activity

DPPH radical scavenging activity: Investigated extract (150 µL) were mixed with 2850 µL freshly prepared DPPH solution (0.1 mM in methanol). The mixtures were incubated for 15 min at 37 °C in darkness and the reduction of absorbance at 517 nm was measured by spectrophotometer. A standard curve was created with Trolox in concentration between 0.005 and 1.0 mM. The results are expressed in mM Trolox® equivalents (TE) per g dry weight (dw).

Ferric reducing antioxidant power (FRAP) assay: The assay was performed according to method, as follow: the FRAP reagent was freshly prepared before analyzes by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka) in 40 mM HCl (Merck) and 1 part 20 mM $FeCl_3.6H_2O$ (Merck) in d. H_2O. The reaction was started by mixing 3.0 mL FRAP reagent with 0.1 mL of investigated extract. Blank sample, prepared with methanol instead of extract was developed as well. The reaction time was 10 min at 37 °C in darkness and the absorbance at 593 nm of sample against blank was recorded. Antioxidant activity was expressed as mM TE/g dw by using calibration curve, build in range of 0.05- 0.5 mM Trolox (Fluka) dissolved in methanol (Merck).

Result and discussion

There is an increasing interest for using of bioactive phytochemicals from natural origin. The plant extracts obtained from different part had different effects for human health (Petkova *et al*., 2017) [18]. Extraction efficiency depends on large number of parameters - extraction method, solvents, temperatures and extraction times. In this reason it is very important to find optimal extraction parameters for obtaining extracts with the highest content of biologically active compounds (Cvetanovic *et al*., 2015, Mašković *et al*., 2016, Lincheva *et al*., 2017) [3, 12, 13].

The extracts from aerial part of wild strawberry contained mainly phenolic compounds (Liberal *et al*., 2014, Liberal *et al*., 2015, Ivanov *et al*., 2015) [7, 10, 11]. The amount of total phenolic contents varied with the used the different solvents and extraction methods (Table 1). The highest values for total phenols were found in 50% glycerin extracts of *F. vesca* leaves and flowers after ultrasound assisted extraction – 150.2 ± 1.8 mg GAE/g dw and 135.2 ± 1.4 mg GAE/g for flowers and leaves, respectively. The results were significantly higher than the previously reported data by Ivanov *et al*., (2015)[7]. Extraction methods strongly influence of polypenolols content (Table 1). Polyphenols content in decoction extracts was 16 % higher than infusions for both aerial parts leaves and flowers. Ultrasounds irradiation did not influence on extraction of polyphenols. But, the addition of glycerin to the system increases the amount of extracted polyphenols in both leaves and flowers – about 30% (from 100 to 130 mg GAE/ g dw) (Table 1). The capacity of glycerol to increase polyphenol extraction efficiency has been investigated previously (Vasantha Rupasinghe *et al*., 2011) [21]. The effects are due to its lower polarity, this might be ascribed to its lower dielectric constant (ε= 42.5). Addition of glycerol to water could favorably change the dielectric constant of water (ε= 80.1),

rendering it a reduced polarity for more efficient polyphenol extraction (Shehata *et al*., 2015) [22]. In addition, in comparison the obtained results for polyphenols content were about over 3 times higher than those reported before 37-39 mg GAE/ g dw by Ivanov *et al*. (2015) [7]. That is very important, because glycerin extracts ware used in cosmetics (Panda, 2015) [17].

Table 1: Total polyphenol content, total tannins content, total proanthocyanidins content and antioxidant ability of different extracts obtained from *Fragaria vesca* L. leaves and flowers.

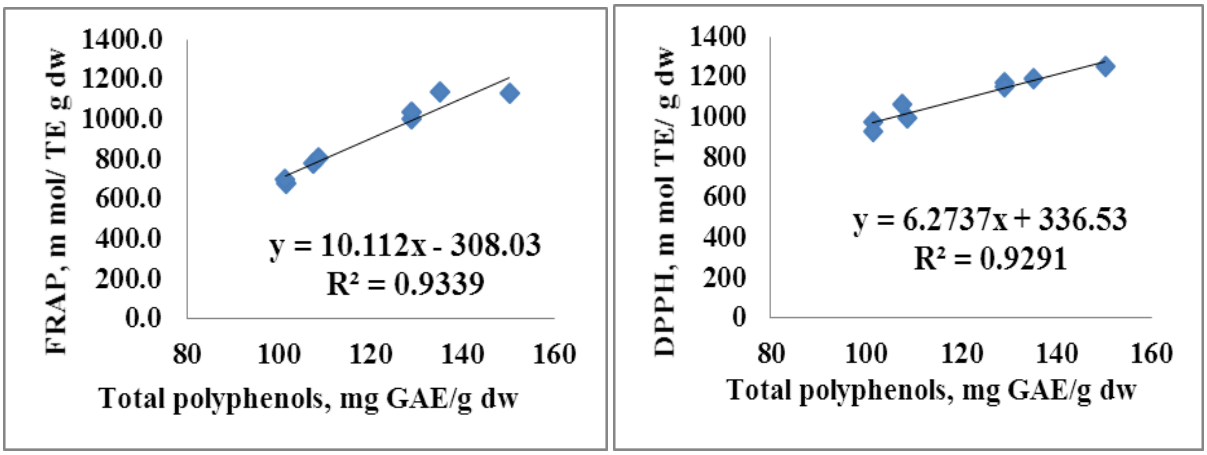
Extraction	Aerial part	Total polyphenol content, mg GAE/ g dw	Total tannins content, mg TEA/g dw	Total proanthocyanidins content, mg LE/ 100g dw	Antioxidant ability	
					DPPH, mmol TE/g dw	FRAP, mmol TE/g dw
Infusion	Leaves	107.5±1.2	46.3±1.2	116.2±1.5	1066.2±14.0	782.2±11.5
	Flowers	108.7±1.0	45.5±1.1	124.8±1.1	995.7±12.1	804.0±9.2
Decoction	Leaves	129.0±1.1	53.3±1.0	134.6±1.2	1171.6±10.4	1002.9±8.5
	Flowers	129.0±1.2	54.1±1.1	142.4±1.0	1152.0±9.5	1035.8±10.5
USE water	Leaves	101.5±1.0	41.6±1.0	111.5±1.5	974.6±8.5	678.2±11.5
	Flowers	101.4±1.0	39.4±1.0	125.6±1.1	928.1±8.8	701.0±10.3
USE 50% glycerin	Leaves	150.2±1.1	60.8±1.2	188.2±1.2	1255.2±10.3	1129.1±11.6
	Flowers	135.2±1.2	65.8±1.2	193.3±1.2	1188.3±12.3	1137.6±11.5

USE – ultrasound-assisted extraction

Water and hydro-alcoholic extracts from *F. vesca* leaves were rich sours of ellagitannins and gallotannins (pedunculagin, castalagin, vescalagin, sanguiin H, agrimoniin derivatives) flavonoids (quercetin-glucosides, kampferol-glucosides) and proantocyanidins ((epi) catechin-(epi) catechin, (epi) catechin-(epi) catechin-(epi) afzelechin and (epi) catechin-(epi) afzelechin derivatives, procyanidine B1 and procyanidine B2) (Mudnic *et al*., 2009, Liberal *et al*, 2014, Liberal *et al* 2015)[10, 11, 15]. In this reason, the extraction efficiency of these components has been investigated (Table 1). The highest concentration of tannins was obtain with 50% glycerin under ultrasound assisted extraction (65.8 ± 0.5 mg TAE/ g dw). Decoction was bather extraction technique than infusion (16% higher total tannins content). With solvent 50% glycerin was extracted 30% more tannins from wild strawberry leaves and flowers than water under ultrasound irradiation (Table 1). A similar occurrence was observed in the total polyphenols content (Table 1). In all investigated extracts tannins content was about 50% from total polyphenols content. Total proantocyanidines in different extracts were presented from 111.5 mg LE/100g dw (flowers, USE) to 193.3 mg LE/ 100g dw (leaves, 50% glycerin USE). Leaves from *F. vesca* were a rich source for proanthocyanidins than flowers (Table 1). The obtained results for total proanthocyadinins in accordant with previously described by Ivanov *et al*. (2014) [8].

In present study has been decided to evaluate antioxidant activities of different extracts of *F. vesca* leaves and flowers by application of two methods, based on mixed hydrogen atom transfer (HAT) mechanisms (DPPH method) and a method, based only on and single electron transfer SET mechanism (FRAP method). To evaluate antioxidant activities of investigated extracts, their abilities to scavenge DPPH radicals, as well as their power to reduce ferric (Fe^{3+}) ions (FRAP method) were investigated (Table 1). The result shown that all obtained extracts possessed higher radical scavenging ability than metal reducing activity. The highest antioxidant potential was established extract obtained from aerial part of wild strawberry with 50 % glycerin under ultrasound irradiation (1255.2 m mol TE/ g dw). In addition, the results in comparison with results for antioxidant activities obtained by Ivanov *et al*. (2015) [7] they were about over 3 times higher.

The results showed the high correlation between total polyphenolics content in investigated extracts and their antioxidant activities $R^2 = 0.9339$ and $R^2 = 0.9291$ for FRAP and DPPH methods, respectively. Correlation between total proanthocyanidins and antioxidants activity was lower than total polyphenols and total tannins - $R^2 = 0.7776$ and $R^2 = 0.6247$ for FRAP and DPPH methods, respectively. These results suggest that antioxidant activities of *F. vesca* leaves and flowers extracts were obtained mainly from hydrolysable tannins as evidenced by the high coefficients of determination - $R^2 = 0.9396$ and $R^2 = 0.8518$ for FRAP and DPPH methods (Figure 1). The high correlation with antioxidant capacity efficiency possessed that the measurement of total phenolics may be the better assay to use for the later stages in a wild strawberry qualitative analysis in an herbal natural pharmaceutics.



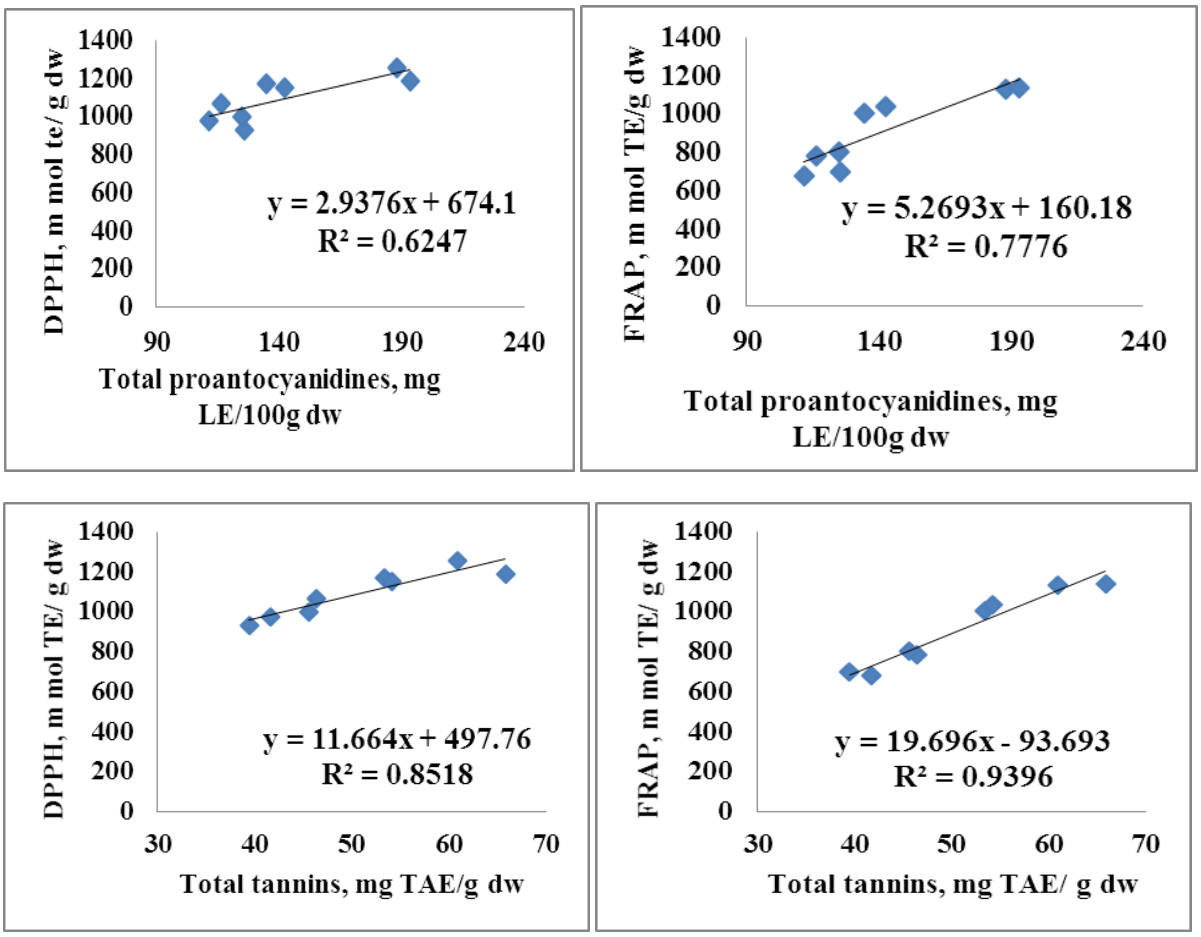


Fig 1: Correlation between antioxidant activity and total polyphenols, tannins and proanthocianidins content.

Wild strawberry tinctures from leaves with 40, 50, 60 and 70% ethanol was prepared. After 14 days of maceration, the amount of extrapolated polyphenols was determined. The 50% ethanol extract obtained the largest amount of total polyphenols – 10847.3±40.1 mg GAE/ L, and at least 70% ethanol extract 8756.8±39.4 mg GAE/ L (Figure 2). Relevant changes in relation to the start value in the fresh tinctures are summarized in Figure 2. After the preparation of tinctures, samples were re-analyzed after 1, 12 and 18 months and compared regarding relative and absolute changes. Figure 2 illustrates the change of the total polyphenols content of tinctures. The reduction of total polyphenol content for 1 month was about 24-25% for all tinctures (Figure 2), but change over 18 months was about 32-33%. In all tinctures did not indicate divergent stability over time according to the solvent.

The effect of the storage period on the amount of total proanthocyanidins over the 18 months period was monitored (Figure 3). Again, the 50% ethanol extract obtained the largest amount of total proanthocyanidins – 196.4 mg LE/ L, but at least was 40 % ethanol extract 159.6±2.4 mg LE/ L (Figure 7). The reduction of total proanthocyanidins content for 1 month for all tinctures were different (Figure 3), tinctures obtain with 50% and 70% decrease amount of proanthocyanidins about 16%, but change of tinctures obtain with 40% ethanol and 60% ethanol were lower 9% and 5%, respectively. For the 18 months period amount of total proanthocyanidins decreased about 44%-45%. In addition obtain results were in accordant with previously reported by Mudnic *et al*. (2009) [15] for polyphenolic content of aqueous extracts from *F. vesca* leaves.

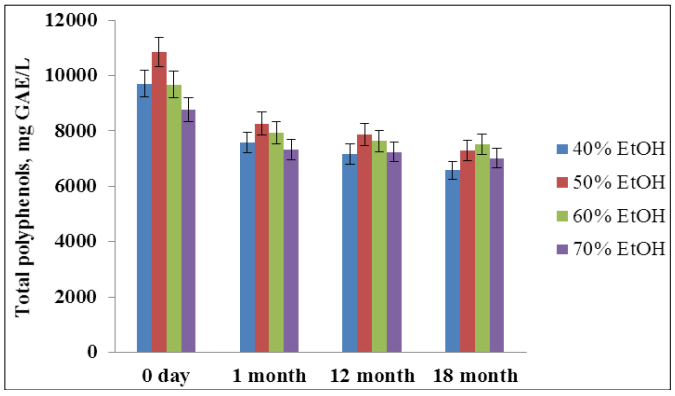


Fig 2: Change of total polyphenols content of tinctures obtained from *Fragaria vesca* L. leaves with 40%-70% ethanol during storage at room temperature.

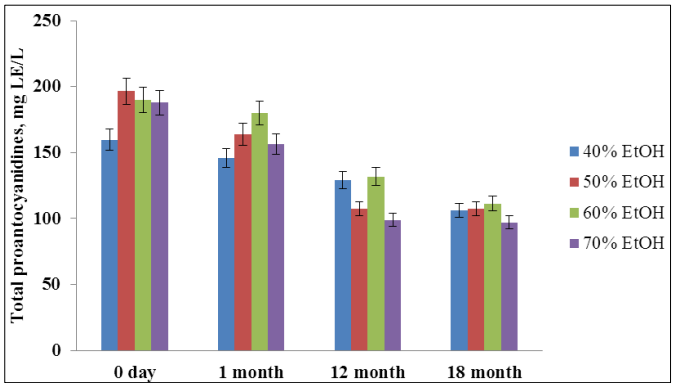


Fig 3: Change of total proanthocyanidins content of tinctures obtained from *Fragaria vesca* L. leaves with 40%-70% ethanol during storage at room temperature

Relevant changes in relation to the start value in the fresh tinctures are summarized in Figure 8. Similar of obtained result for total polypheniols content, antioxidant activity decrease 30% in investigation period (18 mounts) from 50651.2 m mol TE/L to 41071.3 m mol TE/L (50% ethanol), the most stability tincture decrease only 20% antioxidant potential – 60% ethanol (from 55701.0 m mol TE/L to 44437.8 m mol TE/L) (Figure 4).

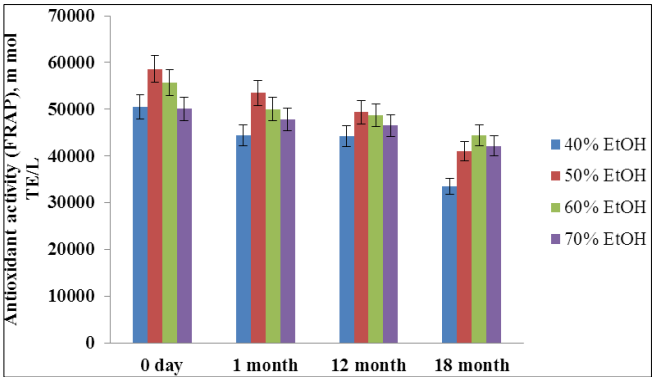


Fig 4: Change of antioxidant activity of tinctures obtained from *Fragaria vesca* L. leaves with 40%-70% ethanol during storage at room temperature

Conclusion

The present work has shown the huge potential of leaves and flowers of *F. vesca* and their glycerin extractions and tinctures, to have a promising and huge health benefits effect with potential application in medicinal cosmetics and pharmaceutical industry. On the basis of the obtained results, it has been shown that the storage period of tinctures obtained from the wild strawberry leaves should be maximum 18 months, as a recommended shelf life of 12 months.

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