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Achillea millefolium L. s.l. herb extract: Antioxidant activity and effect on the rat heart mitochondrial functions

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ABSTRACT

An extract of *Achillea millefolium* herb (YE) was investigated for antioxidant activity using chemical and biological assays. Qualitative and quantitative analysis of some major phenolics was carried out by HPLC. An on-line HPLC-DPPH assay showed that YE possesses significant antiradical activity which is due to the presence of active components amongst phenolic compounds. Furthermore, direct effects of YE and a mixture of its identified phenolic compounds (MPC) on isolated rat heart mitochondrial function were investigated. We found that YE in concentration-dependent manner induce a decrease in State 3 respiration rate without any changes in the integrity of inner mitochondrial membrane. Thus, pyruvate oxidation was affected only by the highest used YE concentrations; meanwhile succinate oxidation was reduced even at lower YE concentrations. MPC had no effect on mitochondrial State 3 respiration rate. Fluorimetric measurements demonstrated that YE at concentrations that had no effect on the State 3 respiration rate significantly decreased H_2O_2 production in mitochondria.

1. Introduction

The genus *Achillea* L. (yarrow) comprises over 100 perennial herb species indigenous to the Northern Hemisphere (Si, Zhang, Shi, & Kiyota, 2006). In Lithuania *Achillea millefolium* L. s.l., the best-known and most widespread species of yarrow, is listed amongst the most commonly used plant species in both folk and conventional medicine (Radušienė & Gudaitytė, 2005). The diversity and complexity of the phytochemical composition of yarrow species may explain their polyvalent pharmacological activity. The raw material of *A. millefolium* L. s.l. contains terpenes, alkaloids and bases, tannins, coumarins, saponins, sterols, vitamins, amino and fatty acids (Blumenthal, Goldberg, & Brinckmann, 2000; Si et al., 2006). Phenolic compounds, such as flavonoids and phenolcarbonic acids, constitute one of the most important groups of pharmacologically active principles in yarrow. It is suggested that anti-inflammatory (Blumenthal et al., 2000), antimicrobial (Aljancic et al., 1999), choleretic (Benedek, Geisz, Jager, Thalhammer, & Kopp, 2006) and cytotoxic (Trifunović et al., 2006) activities of *Achillea* plants are mainly attributed to the flavonoid and phenolcarbonic acid complex. However, further scientific investigations are still required to clarify its phytochemicals as well as the spectrum of their potential utilisation.

Oxidation reactions and the decomposition of oxidation products are major causes of deterioration of various food products. To prevent these processes, antioxidants are widely used as additives in some foods. Owing to increased safety concerns about synthetic antioxidants and their possible involvement in chronic diseases, research efforts have been directed toward natural antioxidants (Shahidi, 2000). Medicinal plants and their extracts constitute one of the most important targets to search for new sources of natural antioxidants for consideration as components for functional ingredients and nutraceuticals as well as feasible and natural alternatives to synthetic antioxidants in the food industry. Since plant-derived antioxidants are generally considered to be multifunctional and their activity depends on various parameters, any herb or its extract should be thoroughly tested involving several methods of assessing antioxidant activity.

It has been shown that the anti-diabetic (Yazdanparast, Ardestani, & Jamshidi, 2007) and gastroprotective (Potrich et al., 2010) properties of extracts from *Achillea* plants may be

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linked to their antioxidant potential, therefore, it is of high importance to investigate their antioxidant effectiveness. Recent reports indicate that the *Achillea* genus displays a relevant antioxidant activity that is associated or correlated well with its flavonoid and total phenolic contents (Giorgi, Mingozzi, Madeo, Speranza, & Cocucci, 2009; Konyalioglu & Karamenderes, 2005). However, the observed similarities or close correlation between the profiles of the antioxidant capacity and of the total phenolic and flavonoid contents must be interpreted with care, since the latter parameter is usually measured using traditional spectrophotometric assays, which are based on non-specific reaction of phenolic compounds with Folin–Ciocalteu's reagent and complexation of flavonoids with Al(III) (Naczk & Shahidi, 2004).

The relevance of botanicals as antioxidants in biological systems cannot be reliably predicted according to the results shown solely using the assays based on chemical reactions. Thereby they should be tested using *in vitro* biological techniques (MacDonald-Wicks, Wood, & Garg, 2006). Thus, for a more thorough evaluation of the antioxidant potential of the *A. millefolium* herb extract (YE), it is important to investigate its behaviour in both chemical (free radical-scavenging activity) and biological systems (for instance, in isolated mitochondria). Mitochondria are an important intracellular source of reactive oxygen/nitrogen species (ROS/RNS) and, on the other hand, are also a critical target of their damaging effects (Cadenas, 2004). Moreover, oxidative stress-mediated impairments in mitochondrial function are considered to be implicated in a large variety of diseases and patho-physiological processes, such as cardiovascular, neurodegenerative diseases, ageing and carcinogenesis (Gellerich et al., 2004). Assessment of influence of YE on cellular energy metabolism would gain more insights into the mechanism of their action by counteracting detrimental effects of ROS. As mitochondria produce most of the cell ATP required for cell function and play an important role in health and diseases, the characterisation of functional status of mitochondria exposed to various biologically active compounds is of great importance.

Nowadays, international attention has been directed toward bioactive food components such as polyphenols that could have the potential to modulate mitochondrial function. Our previous study showed that flavonoids (quercetin and its derivatives as rutin, hyperoside, quercitrin) even at low concentrations cause concentration-dependent heart mitochondrial uncoupling, while at higher concentrations they reduce mitochondrial State 3 respiration rate (Trumbeckaite et al., 2006). Moreover, we revealed a powerful uncoupling effect of *Ginkgo biloba* leaves extract (Baliutyte, Baniene, Trumbeckaite, Borutaite, & Toleikis, 2010; Trumbeckaite et al., 2007) and slight uncoupling effect of *Crataegus monogyna* fruit extract (Bernatoniene et al., 2009), both rich in flavonoids. Recently, it was shown that *A. millefolium* exhibits hypotensive, cardiosuppressant, and vasodilatory activities, thereby confirming the traditional use of yarrow in cardiovascular disorders (Khan & Gilani, 2010). However, despite the fact that many of cardiovascular diseases are associated with disturbances of the mitochondria, to our knowledge, no study has been carried out on the effects of *A. millefolium*, which also contains phenolic compounds such as flavonoids and phenolcarbonic acids, on heart mitochondrial oxidative phosphorylation.

Therefore, the aims of this study were (1) to evaluate antioxidant activity of *A. millefolium* herb extract (YE) assessed by chemical (on-line HPLC-DPPH) and biological (in relevance to ROS production in rat heart mitochondria) systems and (2) to investigate *in vitro* the direct influence of YE on the respiration of isolated rat heart mitochondria.

2. Materials and methods

2.1. Chemicals

Authentic standards of reference compounds apigenin, apigenin 7-*O*-glucoside, luteolin, luteolin 7-*O*-glucoside, luteolin 3′,7-di-*O*-glucoside, rutin, vicenin-2, and chlorogenic acid were purchased from Fluka (Buchs, Switzerland), Roth (Karlsruhe, Germany), ChromaDex (Santa Ana, CA) and Quality Phytochemicals LLC. Trifluoroacetic acid was obtained from Sigma–Aldrich (Seelze, Germany). Acetonitrile, labelled as HPLC grade, was supplied by Sigma–Aldrich (Buchs, Switzerland). Ethanol 96.3% (v/v) was provided by Stumbras (Kaunas, Lithuania). (R)-6-Methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®) was supplied by Acros Organics (Geel, Belgium), while DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, glutamic acid, malic acid, succinic acid, cytochrome *c* from bovine heart, adenosine 5′-diphosphate (ADP) sodium salt , ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), Tris–HCl, KH_2PO_4, antimycin A, atractyloside were obtained by Sigma–Aldrich Chemie (Steinheim, Germany). Sodium citrate, citric acid, KCl and magnesium chloride were purchased from Roth (Karlsruhe, Germany). Amplex® Red was purchased from Invitrogen (Carlsbad, CA). All other chemicals used were analytical grade and obtained from Sigma–Aldrich Chemie (Steinheim, Germany) and Fluka (Buchs, Switzerland). Ultrapure water was used throughout and was prepared using a Millipore water purification system (Bedford, MA).

2.2. Preparation of YE

The plant material represents tops of *A. millefolium* L. s.l. plants (~10 cm in length) collected at full flowering stage in 2007 from the field collection of the Institute of Botany, Lithuania. The raw material was air dried at room temperature (20–25 °C), in a ventilated lodge, avoiding direct sunlight for two weeks. Dry material was packed into multilayer paper bags and stored in a dark room at ambient temperature. The air-dried aerial parts of *A. millefolium* were milled at room temperature and sieved using a sieve with 355 μm mesh. The hydroalcoholic extract of powdered plant material was prepared by maceration with 40% ethanol at room temperature (1:10, v/v), initially for 48 h and thereafter until exhaustion. The hydroalcoholic extract obtained was filtered and concentrated under vacuum (at 50 °C) and then subjected to freeze drying. Extract was frozen at −30 °C in a glass jar then freeze dried for 48 h using a laboratory freeze dryer with shell freezer FD8512S (Ilshin Lab. Co., Ltd., Gyeonggi-do Korea). Sublimation was carried out at −80 °C condenser temperature and a pressure of 0.007 mbar. A thermocouple PT100 was used to measure the temperature in the centre of the sample during freeze drying. Freeze-dried yarrow powder was packed into a glass jar.

2.3. Quantitative analysis of phenolic compounds by HPLC

HPLC analysis of YE was performed using a liquid chromatographic Waters 2690 Alliance HPLC system (Waters Corporation, Milford, MA) equipped with Waters 2487 dual λ absorbance detector (UV/Vis) and a Waters 996 photodiode array (PDA) detector. Separations were carried out using a 5-μm Ascentis™ RP-Amide analytical column (150 × 4.6 mm) with guard column 5-μm Supelguard™ Ascentis™ RP-Amide (20 × 4.00 mm) (Supelco, Bellefonte, PA). The chromatographic separation was carried out using 0.1% trifluoroacetic acid solution in water as solvent **A** and 0.1% trifluoroacetic acid solution in acetonitrile as solvent **B** with a previously published gradient elution programme (Benetis, Radušienė, Jakštas, Janulis, & Malinauskas, 2008). The elution was monitored

at 360 nm with a UV/Vis detector. This LC method was used at a flow rate of 1.5 mL/min at ambient temperature. The sample injection volume was 10 μL. The confirmation of the identity of chromatographic peaks was achieved by comparison of retention times of samples with those of standard compounds and spectral characteristics of the eluting peaks, scanned with diode-array detector (λ = 200–400 nm), with those of authentic standards. The quantification of identified analytes was achieved by external standardisation with reference compounds. YE was analysed in triplicate and the mean values ± SD are presented.

2.4. On-line detection of radical scavengers by HPLC-DPPH assay

The instrumental and experimental setup for investigation of radical-scavenging activity of YE by the on-line HPLC-DPPH method was based on the setup previously published by our group (Raudonis, Jakštas, Burdulis, Benetis, & Janulis, 2009). A linear binary gradient is formed by chromatograph Beckman programmable solvent module 126 (Fullerton, CA) at constant flow rate of 1.5 mL/min. A Rheodyne 7125 manual injector (Rheodyne, Rohnert Park, CA) with a 20-μL loop was used for injection of samples. The eluting compounds were detected with a UV absorption detector (Beckman System Gold 166 programmable detector module) at 360 nm. From the detector, the mobile phase with analytes is delivered by mixing tee to the reaction coil, where DPPH reagent solution is supplied. HPLC pump Gilson pump 305 (Middleton, WI) was used to supply DPPH solution. The reaction coil was made of PEEK tubing of the following size: 15 m × 0.25 mm i.d., 0.36 mm o.d. Detection of DPPH bleaching was carried out at 520 nm. A Gilson UV/VIS detector 118 connected to a PC was used to monitor DPPH conversion.

Reference antioxidant Trolox was used for quantitative assessment of antioxidant compounds' (which possessed radical-scavenging properties) activity in YE. Direct dependence between the amount of Trolox (μM) and the depth of the peak for bleaching of DPPH (negative peak area) was confirmed by high correlation factor (R^2 = 0.998). Trolox content equivalent (TE) of an active compound is expressed by the amount of Trolox (μM), which under the same experimental conditions shows an appropriate DPPH radical-scavenging activity, evaluated by Trolox calibration curve ($y = 948.8x - 7568$). Trolox equivalent antioxidant capacity (TEAC) was used to evaluate the activity of radical scavengers present in YE. TEAC is the amount of Trolox (μmol) having an antioxidant capacity equivalent to that of one gram of antioxidant active compound present in YE.

2.5. Preparation of rat heart mitochondria

All experimental procedures were performed according to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Licence No. 0006). Hearts of male Wistar rats were excised and rinsed in ice-cold 0.9% KCl solution. Subsequently the tissue was cut into small pieces and homogenised in a buffer (10 mL/g tissue) containing 180 mM KCl, 10 mM Tris/HCl, and 1 mM EGTA (pH 7.7) for 45 s. The homogenate was centrifuged at 750*g* for 5 min and the supernatant obtained was centrifuged at 6800*g* for 10 min. The mitochondrial pellet was resuspended in buffer containing 180 mM KCl, 20 mM Tris/HCl, and 3 mM EGTA (pH 7.35) to approximately 50 mg/mL protein, and stored on ice. The mitochondrial protein concentration was determined by the biuret method using bovine serum albumin as a standard (Gornall, Bardawill, & David, 1949).

2.6. Measurements of mitochondrial respiratory rates

Oxygen uptake rates were recorded at 37 °C by means of the Clark-type electrode system in a solution containing 150 mM KCl, 10 mM Tris/HCl, 5 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.2, with 5 mM pyruvate + 5 mM malate and 15 mM succinate (+2 mM amytal) as substrates. The solubility of oxygen was estimated to be 420 nmol O/mL. The final mitochondrial protein concentration in all experiments was 0.5 mg/ml. The basal (State 2) respiration rate (V_2) was measured with the respiratory substrates, then after the addition of 1 mM ADP State 3 respiration rate (V_3) was recorded. For investigation of effect of YE on mitochondrial function different amounts (2.5, 5, 10 and 15 μL) of yarrow extract (3.3 mg of freeze-dried extract of *A. millefolium* herb was dissolved in 1 mL of 40% ethanol) were added into 1.5 mL of incubation medium during State 2 respiration rate. After addition of 2.5 μL of this extract, final concentrations of phenolic compounds in 1 mL of incubation medium were: 101 ng/mL of chlorogenic acid, 9 ng/mL of vicenin-2, 8 ng/mL of luteolin 3′,7-di-*O*-glucoside, 4 ng/mL of luteolin 7-*O*-glucoside, 17 ng/mL rutin, 10 ng/mL apigenin 7-*O*-glucoside, 53 ng/mL of luteolin and 74 ng/mL of apigenin. The mixture of pure phenolic compounds (MPC) was prepared by dissolving standards in 96% ethanol. Different amounts of MPC (0.1, 0.5, 1, 2, 5 and 10 μL/1.5 mL) were added into the incubation medium during State 2 respiration. After addition of 1 μL of MPC, final concentrations of phenolic compounds in 1 mL of incubation medium were: 82 ng/mL of chlorogenic acid, 76 ng/mL of vicenin-2, 58 ng/mL of luteolin 3′,7-di-*O*-glucoside, 68 ng/mL of luteolin 7-*O*-glucoside, 104 ng/mL rutin, 66 ng/mL apigenin 7-*O*-glucoside, 73 ng/mL of luteolin and 68 ng/mL of apigenin. Amounts of solvent (ethanol) used in the experiments did not have any effect on mitochondrial function. Mitochondrial respiration rates were expressed as nmol O/min/mg protein.

2.7. Measurement of H_2O_2 generation

H_2O_2 generation was estimated fluorimetrically using Ascent Fluoroskan plate reader (Thermo Fisher Scientific, Waltham, MA). Mitochondria (0.25 mg/mL) were incubated in the same medium as for mitochondrial respiration (at 37 °C) supplemented with 6 mM pyruvate + 6 mM malate, 0.016 μg/mL antimycin A, 5 μM Amplex Red and 2 U/mL horseradish peroxidase (excitation at 544 nm, emission at 590 nm). Fluorescence signal was calibrated using known amounts of H_2O_2.

2.8. Statistical analysis

The results are presented as means ± SEM of three separate experiments. The mean of individual experiment was obtained from three repetitive measurements. Statistical analysis was performed using Student's *t* test, and $p < 0.05$ was taken as the level of significance. Statistical analysis was performed using the software package SPSS Version 16.0 for Windows. Graphical presentation of results was performed using the software SigmaPlot for Windows, Version 10.0.

3. Results

3.1. Qualitative and quantitative composition of phenolic compounds in YE

Eight phenolic compounds – chlorogenic acid and seven flavonoids, namely vicenin-2, luteolin 3′,7-di-*O*-glucoside, luteolin 7-*O*-glucoside, rutin, apigenin 7-*O*-glucoside, luteolin, and apigenin – were identified in *A. millefolium* herb extract (YE). Quantitative

analysis revealed that chlorogenic acid (19.3 ± 1.3 mg/g) predominated in the mixture of identified secondary metabolites. Regarding the composition of the flavonoid complex, the pattern of distribution in YE was characterised by the dominance of apigenin (14.2 ± 0.4 mg/g) and luteolin (10.2 ± 0.3 mg/g), whereas their glucosides were determined in considerably lower quantities (2.0 ± 0.1 mg/g of apigenin 7-*O*-glucoside, 0.67 ± 0.01 mg/g of luteolin 7-*O*-glucoside and 1.5 ± 0.1 mg/g luteolin 3′,7-di-*O*-glucoside). Furthermore, vicenin-2 (1.8 ± 0.1 mg/g) and rutin (3.2 ± 0.1 mg/g) were also found to be minor components amongst the identified flavonoids in YE.

3.2. Assessment of the antioxidant activity of YE

Recently, high resolution screening techniques for the rapid identification of antioxidants in complex mixtures following chromatographic separation from the matrix were developed (Niederländer, van Beek, Bartasiute, & Koleva, 2008). The results from on-line HPLC-DPPH assay showed the presence of constituents within a complex of the flavonoid and phenolcarbonic acids in YE, which were capable of scavenging free radicals. According to the lower chromatographic profile (DPPH quenching chromatogram), we found that the main components amongst the identified analytes in the YE, that possessed radical-scavenging properties, were luteolin and chlorogenic acid, accompanied by minor scavengers such as rutin and luteolin 7-*O*-glucoside (Fig.1). The selectivity of the DPPH on-line assay is nicely illustrated by the major UV-active compound (retention time 42.89 min.) that showed no antiradical activity and was identified as apigenin. Quantitative determination of contribution of individual compounds to radical-scavenging properties of YE was achieved by using Trolox calibration curve and results are presented in Table 1. The calculated TEAC values confirmed that luteolin and chlorogenic acid (54.96 and 44.84 μmol/g, respectively) were predominant radical scavengers in YE and they taken together determine approximately one-third of its total antiradical activity. Furthermore, the use of on-line HPLC–DPPH assay in a quantitative manner revealed that unidentified components contributed most (~65%) to the total antioxidant activity of YE (TEAC 308.80 μmol/g).

We also tested whether YE affects the H_2O_2 production in isolated rat heart mitochondria. As shown in Fig. 2, YE at concentration of 2.5 and 5 μL/1.5 mL, clearly suppressed this process: after 14 min incubation YE caused a 45% reduction in the rate of H_2O_2 generation as compared to the rate in the absence of YE.

3.3. Effect of YE on mitochondrial oxidative phosphorylation

In order to investigate the effect of YE on the heart mitochondrial function, mitochondrial respiration rates in various metabolic states (in the presence and the absence of YE) were measured. According to our scheme (Fig. 3), at the beginning we measured the basal (State 2) respiration rate with pyruvate + malate as substrate. Then, YE was added into incubation medium during State 2 respiration of mitochondria. After addition of ADP, the State 3 respiration of isolated mitochondria was recorded. Addition of atractyloside, an inhibitor of ADP/ATP translocator, allowed the measurement of the State 4 respiration rate. Finally, exogenous cytochrome *c* was added in order to check the intactness of mitochondrial outer membrane. The data showed that YE at the concentrations studied (2.5–15 μL/1.5 mL) had no effect on the State 2 respiration rate (V_2) of mitochondria with Complex I-dependent substrate pyruvate + malate (Table 2). Addition of YE up to 10 μL/1.5 mL (total content of the identified phenolics 1105 ng/mL) had no effect on State 3 respiration rate (V_3) either (Fig. 4 and Table 2). However, the further increase in YE concentration (15 μL/1.5 mL) caused a significant decrease in State 3 respiration rate by 53% (from 258 ± 46 to 120 ± 39 nmol O/min/mg; $p < 0.05$). Accordingly, mitochondrial respiratory control index (RCI), i.e. the ratio between States 3 and 2 respiration rates, at lower used amounts of YE (2.5, 5 and 10 μL/1.5 mL) was unchanged, whereas the highest YE concentration induced a significant decrease in RCI from 6.1 (control) to 3.0 (15 μL/1.5 mL of YE).

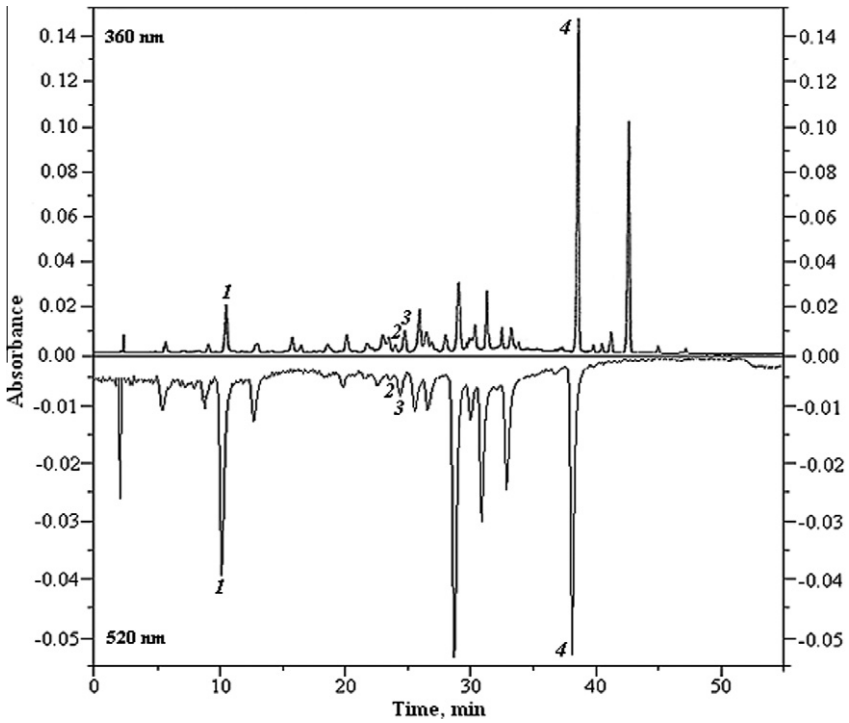

Fig. 1. Chromatographic elution profile (upper trace) and on-line DPPH quenching profile (lower trace) of *Achillea millefolium* herb extract (YE). Numbers refer to identified radical scavengers: (1) chlorogenic acid, (2) luteolin 7-*O*-glucoside, (3) rutin, (4) luteolin.

Table 1
Radical-scavenging activity equivalents of individual compounds present in *Achillea millefolium* herb extract (YE).

No	Retention time (min)	Peak area (in arbitrary units) ($\times 10^3$)	Compounds identity	TE[a] (μM)	TEAC (μmol/g)
-	5.39	24.1	Unknown	33.40 ± 0.76	9.95
-	8.75	16.3	Unknown	25.11 ± 0.65	7.48
1	10.11	135.3	Chlorogenic acid	150.6 ± 5.85	44.84
-	12.67	30.9	Unknown	40.54 ± 1.43	12.07
-	19.81	7.3	Unknown	15.65 ± 0.35	4.66
-	21.46	2.9	Unknown	10.99 ± 0.14	3.27
-	22.46	7.0	Unknown	15.34 ± 0.26	4.57
2	23.52	1.2	Luteolin-7-*O*-glucoside	9.28 ± 0.07	2.76
3	24.35	13.3	Rutin	22.01 ± 0.56	6.56
-	25.51	27.2	Unknown	36.63 ± 1.04	10.91
-	26.56	25.7	Unknown	35.03 ± 1.03	10.43
-	28.69	203.9	Unknown	222.87 ± 10.75	66.37
-	29.96	31.7	Unknown	41.42 ± 1.64	12.33
-	30.86	96.3	Unknown	109.43 ± 4.87	32.59
-	32.87	72.2	Unknown	84.12 ± 3.20	25.05
4	38.09	167.5	Luteolin	184.56 ± 8.30	54.96
			Total	1036.94 ± 47.70	308.80

TE - Trolox content equivalent; TEAC - Trolox equivalent antioxidant capacity.
[a] Each value represents the mean ± SD ($n = 3$).

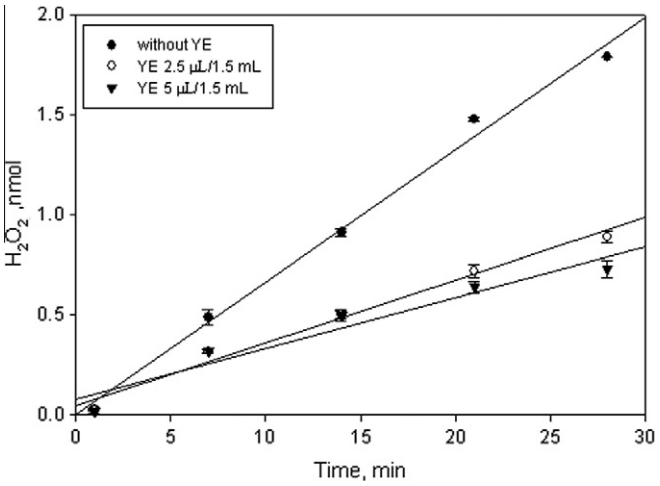

Fig. 2. Effect of *A. millefolium* herb extract (YE) on production of H_2O_2 in isolated rat heart mitochondria. Standard incubation medium was used. $n = 3$ separate measurements with three repeats in each. In all periods of incubation differences were statistically significant ($p < 0.05$).

Other experiments were aimed to check the action of YE on Complex II-dependent substrate succinate oxidation. We found that YE at concentrations up to 2.5 μL/1.5 mL had no effect on State 3 respiration rate (Fig. 4). Higher amounts of YE induced significant inhibition of mitochondrial respiration, V_3 decreased by 27%, 78% and 80% in the presence of 5, 10 and 15 μL/1.5 mL of YE, respectively, followed by the decrease of RCI (by 17%, 72% and 68%, respectively).

In order to clarify the reason for YE-induced diminishing of State 3 respiration rate, we also measured uncoupled respiration rate by adding carbonyl cyanidine *m*-chlorophenylhydrazone (CCCP) instead of ADP. The results presented in Table 3 show that in the presence of the highest used amounts of YE, uncoupled (not associated with ATP synthesis) respiration rate (V_{CCCP}) decreased significantly by 25% and 12% (with both pyruvate + malate and succinate, respectively; $p < 0.05$) as compared to control; however, much less as compared to the inhibitory effect on the State 3 respiration. Data indicate that the highest amounts of YE used in our study may inhibit phosphorylation system and respiratory chain/complexes.

We found that the State 2 (V_2) and State 4 (V_{ATR}) respiration rates were not increased in the presence of YE with substrate pyruvate + malate (Table 2) and succinate (not shown), reflecting that

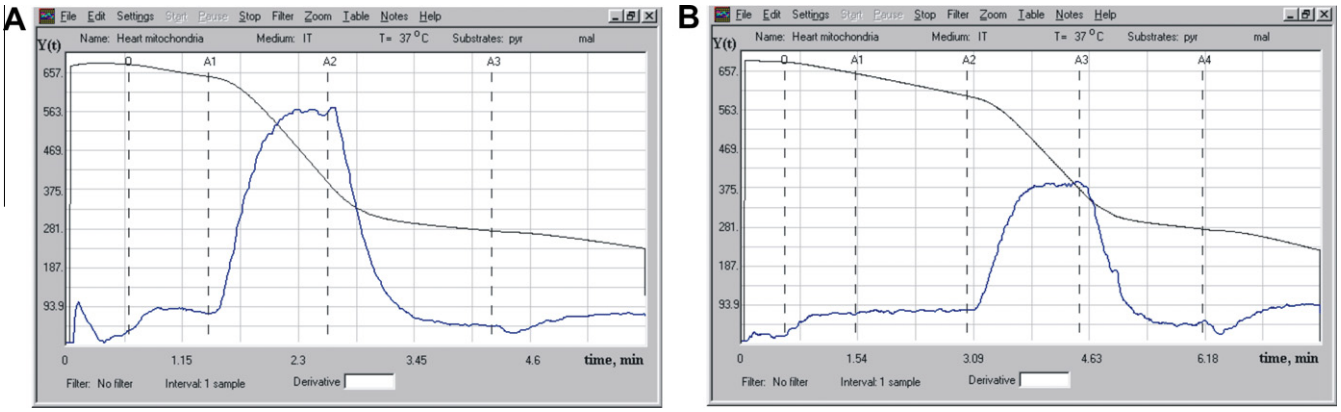

Fig. 3. Original trace of respirometric measurements in rat heart mitochondria. Substrates were 5 mM pyruvate + 5 mM malate. (A) control mitochondria; additions: O, mitochondria (0.5 mg protein/mL); A1, 1 mM ADP; A2, 0.12 mM atractyloside; A3, 32 μM cytochrome *c*. (B) in the presence of 15 μL/1.5 mL of YE. Additions: O, mitochondria, (0.5 mg protein/mL); A1, 15 μL YE; A2, 1 mM ADP; A3, 0.12 mM atractyloside; A4, 32 μM cytochrome *c*.

Table 2
Effects of *A. millefolium* herb extract (YE) on rat heart mitochondrial respiratory parameters when oxidising pyruvate + malate.

	Substrate pyruvate + malate				
	Control	YE (2.5 μL/1.5 mL)	YE (5 μL/1.5 mL)	YE (10 μL/1.5 mL)	YE (15 μL/1.5 mL)
V_2	48 ± 9	48 ± 10	38 ± 6	42 ± 2	43 ± 3
V_2 + YE	-	49 ± 9	39 ± 8	43 ± 6	44 ± 6
V_3	258 ± 46	246 ± 33	237 ± 19	226 ± 41	120 ± 39*
V_{ATR}	40 ± 13	38 ± 7	26 ± 6	31 ± 14	33 ± 5
$V_{ATR + Cyt\ c}$	87 ± 25	96 ± 20	81 ± 38	84 ± 39	93 ± 19
RCI	6.1 ± 1.7	5.9 ± 1.7	6.3 ± 0.6	5.3 ± 0.7	3.0 ± 1.2*

Measurements were performed in the presence of 5 mM pyruvate + 5 mM malate and 15 mM succinate (+2 mM amytal). V_2 – State 2 respiration rate in the presence of 0.5 mg/mL of mitochondria and substrates; V_3 – State 3 respiration rate in the presence of 1 mM ADP; V_{ATR} – State 4 respiration rate in the presence of 0.12 mM atractyloside; $V_{ATR + Cyt\ c}$ - State 4 respiration rate after the addition of 32 μM cytochrome *c*. Mitochondrial respiratory control index (RCI), i.e. the ratio between oxygen uptake rates in States 3 and 2 (RCI = V_3/V_2).
* $p < 0.05$ *vs.* respective control; $n = 3$.

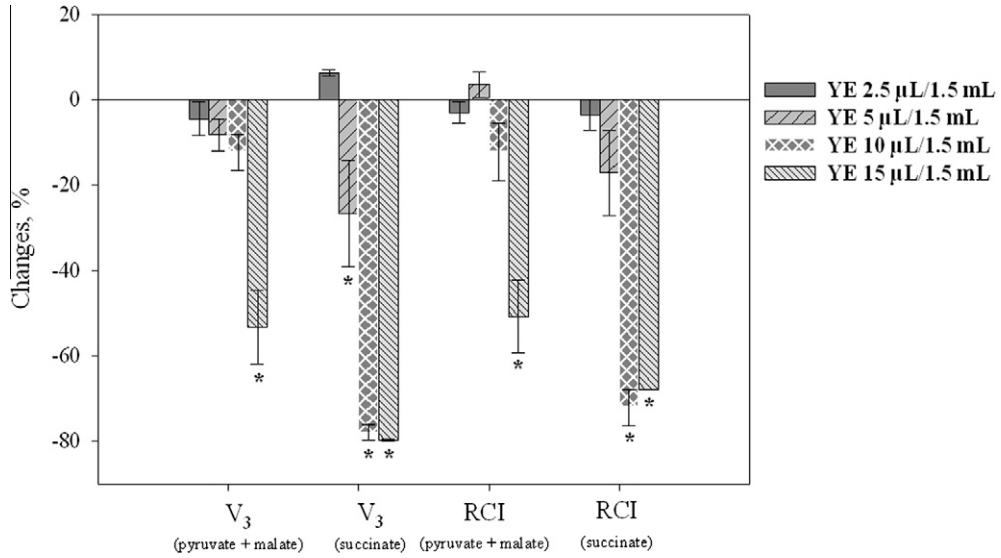

Fig. 4. Effects of *A. millefolium* herb extract (YE) on mitochondrial State 3 respiration rate (V_3) and respiratory control index (RCI). Substrates were 5 mM pyruvate + 5 mM malate or 15 mM succinate (+2 mM amytal). For explanation see Table 2. *$p < 0.05$ *vs.* respective control; $n = 3$.

Table 3
Effects of *A. millefolium* herb extract (YE) on uncoupled from phosphorylation respiration rate of mitochondria energised with pyruvate + malate and succinate.

	Substrate pyruvate + malate		Substrate succinate	
	Control	YE (15 μL/1.5 mL)	Control	YE (15 μL/1.5 mL)
V_{CCCP}	447 ± 13	336 ± 29*	272 ± 3	240 ± 5*
$V_{CCCP + Cyt\ c}$	643 ± 22	426 ± 49*	454 ± 20	370 ± 9*

Measurements were performed in the presence of 5 mM pyruvate + 5 mM malate and 15 mM succinate (+2 mM amytal) as substrates. When measuring uncoupled from phosphorylation respiration rate of mitochondria 0.19 μM of carbonyl cyanidine *m*-chlorophenylhydrazone (CCCP) was added into the incubation medium instead of ADP. V_{CCCP} – respiration rate in the presence of 0.19 μM CCCP; $V_{CCCP+Cyt\ c}$ – uncoupled from phosphorylation respiration rate of mitochondria in the presence of 32 μM cytochrome *c*.
* $p < 0.05$ *vs.* respective control; $n = 3$.

YE had no effect on permeability of mitochondrial inner membrane to protons.

Measurement of Complex IV (cytochrome *c* oxidase) activity in isolated rat heart mitochondria by following *N*,*N*,*N*′,*N*′,-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate oxidation showed no signs of Complex IV inhibition by YE (control, 600 ± 50 nmol O/min/mg; in the presence of 15 μL/1.5 mL of YE, 690 ± 40 nmol O/min/mg; $n = 3$).

Further experiments were designed to elucidate which active compounds of YE might be responsible for the State 3 respiration diminishing effect. As the HPLC analysis showed that the main compounds amongst the identified analytes in YE were chlorogenic acid and flavonoids (vicenin-2, luteolin 3′,7-di-*O*-glucoside, luteolin 7-*O*-glucoside, rutin, apigenin 7-O-glucoside, luteolin and apigenin), we tested the effects of MPC on the mitochondrial oxidative phosphorylation. Lower amounts (0.1–0.5 μL/1.5 mL) of MPC had no effect on the mitochondrial State 2 and State 3 respiration rate (data not shown). It is interesting to note that in contrast to YE, MPC even at higher concentrations used (1–10 μL/1.5 ml) did not induce a decrease in the State 3 respiration rate (Table 4). Moreover, the highest amounts of MPC were found to have the tendency to stimulate slightly the State 2 respiration rate ($V_{2(+MPC)}/V_2$); however this effect was not statistically significant.

4. Discussion

The raw material of yarrow (*A. millefolium*) constitutes one of the oldest and most important medicines widfely used both in folk and conventional Lithuanian medicine (Radušienė & Gudaitytė, 2005). A rich complex of biologically active compounds is responsible for the wide spectrum of pharmacological effects of *A. millefolium* herb. In the past years interest in the antioxidant properties of *A. millefolium* is increasing, therefore, the assessment of radical-scavenging activity of YE is of high importance. The antioxidant properties of different extracts from several *Achillea* species have

Table 4
Effects of mixture of phenolic compounds (MPC) on rat heart mitochondrial respiratory parameters when oxidising pyruvate + malate.

	Substrate pyruvate + malate				
	Control	MPC (1 μL/1.5 ml)	MPC (2 μL/1.5 ml)	MPC (5 μL/1.5 ml)	MPC (10 μL/1.5 ml)
V_2	56 ± 9	54 ± 7	7 ± 8	64 ± 8	69 ± 8
V_2 + MPC	–	62 ± 6	62 ± 7	73 ± 8	89 ± 8
$V_{2(+MPC)}/V_2$	-	1.15 ± 0.14	1.10 ± 0.01	1.14 ± 0.12	1.28 ± 0.21
V_3	358 ± 41	376 ± 38	358 ± 34	395 ± 39	349 ± 31
V_{ATR}	61 ± 10	60 ± 9	57 ± 8	60 ± 9	74 ± 10
RCI	6.4 ± 0.9	7.0 ± 0.7	6.3 ± 0.6	6.2 ± 0.6	5.0 ± 0.5

Measurements were performed in the presence of 5 mM pyruvate + 5 mM malate. For explanation see Table 2; $n = 3$.

been studied using chemical assays based on the ability to scavenge stable free DPPH radical (Ardestani & Yazdanparast, 2007; Giorgi et al., 2009). The results from these model oxidation systems have shown that yarrow extracts possess components with radical-scavenging activity. However, due to batch-like character, DPPH bleaching methods are convenient for a preliminary screening of antioxidants in plant extracts, but are unsuitable for determination of contribution of individual compound(s) to the overall effect (Niederländer et al., 2008).

Therefore, in the present study the antioxidant activity of YE, in relation to free radical scavenging, was investigated by the on-line HPLC-DPPH assay. This coupled approach combines a chromatographic separation of the flavonoid and phenolcarbonic acid complex in YE with a DPPH scavenging assay and enables a rapid pinpointing of individual radical-scavenging compounds, as well as determination of their contribution to antiradical activity of YE. The results showed, that chlorogenic acid and flavone luteolin were found to possess significant radical-scavenging activity amongst the identified analytes in the YE. Since other two identified scavengers (luteolin 7-*O*-glucoside and flavonol rutin) were present in low amounts they contributed less to the total antioxidant activity of YE. The obtained results correspond with the generally accepted assumption, that the presence of the *ortho*-arrangement of two hydroxyl groups (catechol group) in the aromatic B-ring and 2,3-double bond in conjugation with 4-oxo function in the C-ring are structural features essential for the antiradical activity of flavonoids (Pietta, 2000). These pharmacophores are present in the structure of rutin and luteolin; meanwhile in apigenin the catechol group is lacking, this may explain why it has no activity. Chlorogenic acid is also known to possess significant ROS scavenging properties (Firuzi et al., 2003). However, structure elucidation of the active unknowns remains to be established in order to obtain a more comprehensive picture of the radical-scavenging profile of YE. In addition, we applied another method to detect free radicals in biological systems and measured fluorimetrically the mitochondrial H_2O_2 generation in the presence of YE. By using this approach we found a significant reduction of H_2O_2 generation caused by YE, thus confirming the antioxidant activity of YE.

Since YE may have direct effect on mitochondria, in this study we also investigated the effects of YE on the mitochondrial oxidative phosphorylation *in vitro*. We found that extract of *A. millefolium* herb in concentration-dependent manner induces the decrease in State 3 respiration rate in rat heart mitochondria without any changes in the integrity of inner mitochondrial membrane. Our results demonstrate that oxidation of Complex II-dependent substrate succinate oxidation was more affected by YE, as compared to pyruvate + malate. Pyruvate oxidation was affected only by the highest used YE concentrations (total content of the identified phenolics 1658 ng/mL); meanwhile succinate oxidation was reduced even at lower YE concentrations (total content of the identified phenolics 553 ng/mL). Because of a more pronounced decrease in State 3 respiration rate (V_3) than in uncoupled (V_{CCCP}) respiration rate (−53% and −25% with pyruvate + malate; −80% and −12% with succinate, respectively), one may conclude that the inhibition of pyruvate + malate and succinate oxidation caused by YE may be mainly due to the inhibition of phosphorylation system components, such as F_1F_0ATPase and/or ADP/ATP translocator and only partially due to diminished activities of mitochondrial respiratory chain complexes. Concerning F_1F_0ATPase (Complex V) it was shown that some polyphenols inhibit this enzyme (Gledhill, Montgomery, Leslie, & Walker, 2007). Authors discuss that one of the possible ways where inhibition of mitochondrial F_1F_0ATPase by polyphenols might be beneficial is the induction of apoptosis *via* pathways that depend on mitochondria (Gledhill et al., 2007).

The concentrations of phenolic compounds in *A. millefolium* may substantially vary dependently not only on the genotype, but also on environmental conditions, the stage of plant development, the ratio of plant parts (flower/leaf/stem) analysed, drying and storage conditions. Therefore, the solutions of some pure phenolic compounds (MPC) were investigated in a wide range of concentrations from the approximate concentrations of constituent levels found in YE to even higher concentrations. It is noteworthy that in contrast to yarrow extract, MPC investigated in our study had no effect on mitochondrial State 3 respiration. It is well known that very often an original plant extract possesses different effects than single isolated 'active' constituents. This might be explained by the fact that many plant constituents (primarily phenolic compounds) exert polyvalent effects. Thus, our previous study regarding the pure phenolic compounds revealed, that chlorogenic acid at concentration 30 ng/mL (Bernatoniene et al., 2009) and up to 300 ng/mL (our unpublished results) had no effect on the State 3 respiration rate. Flavonol rutin at concentrations up to 44.4 ng/mL also had no effect on the State 3 respiration rate (Bernatoniene et al., 2009; Trumbeckaite et al., 2006), but starting from 60 and 115 ng/mL caused the decrease of the State 3 respiration rate by 18% and 51%, respectively (Bernatoniene et al., 2009; Trumbeckaite et al., 2006). Thus, the possibility exists that inhibitory effect on mitochondrial State 3 respiration rate by higher concentrations of YE could be partially caused by rutin present in *A. millefolium* extract. Taken together, our findings showed that even at low concentrations (ng/mL) YE exerts direct effects on mitochondrial function *in vitro*.

Since flavonoids are weak acids of hydrophobic character (van Dijk, Driessen, & Recourt, 2000), they act similarly to classical uncouplers of oxidative phosphorylation, and can induce the increase in State 2 respiration rate, as we revealed in our previous study (Trumbeckaite et al., 2006). However, in the present study we did not find any uncoupling effect with YE in contrast to our studies where uncoupling effects of *G. biloba* (Baliutyte et al., 2010; Trumbeckaite et al., 2007) and *C. monogyna* fruit extract (Bernatoniene et al., 2009) were detected. However, it was concluded, that the stimulating (uncoupling) effect on the State 2 respiration exerted by the abovementioned plant extracts cannot be explained on the sole basis of phenolic compounds composition, suggesting that the effect of YE on mitochondria can also be

ascribed to antagonistic/synergistic effects of various phytocompounds present in YE. This is in consistency with the synergistic approach of phytotherapy that not single active constituents, but the extract as a multiple component mixture causes the desired therapeutic effect. Our data from previous studies revealed, that pure compounds such as chlorogenic acid at the concentrations 30 ng/mL (Bernatoniene et al., 2009) and 300 ng/mL (our unpublished results) as well as rutin (up to 1.14 ng/mL, Trumbeckaite et al., 2006) had no effect on the mitochondrial State 2 respiration rate. However, rutin at the concentrations of 4.56, 26, 44 and above 60 ng/mL stimulated State 2 respiration by 15%, 70%, 110% and 20%, respectively (Bernatoniene et al., 2009; Trumbeckaite et al., 2006, 2007). It seems that the observed effect of MPC and some individual compounds on the State 2 respiration rate may partially disappear by investigating crude extract of *A. millefolium*, due to a complex mixture of various substances with different biological and chemical properties.

Our current results indicate that phenolic compounds seem to contribute only in a minor extent to the YE-induced decrease in State 3 respiration rate in rat heart mitochondria. The possibility exists therefore that YE-induced suppression of pyruvate + malate and succinate oxidation in mitochondria might be, at least to some extent, due to sesquiterpene lactones. This class of substances is a very prominent feature not only for the genus *Achillea* but for the whole family *Asteraceae*. Moreover, it has been reported that sesquiterpenes from several genera of the *Asteraceae* family markedly inhibited State 3 respiration in mouse hepatic mitochondria (Narasimham, Kim, & Safe, 1989).

The inhibitory effects of YE on mitochondrial respiration add new insights into the benefits of *A. millefolium* as an antimicrobial agent. Interestingly, the mechanism of energy transduction is similar in both mitochondria and bacteria, therefore one may assume that YE might inhibit bacteria respiration and energy production. Thus, inhibition of ATP synthesis in bacteria may also contribute to the antimicrobial activity of *A. millefolium*, since bacteria use energy concentrated in the bond of ATP for the growth and reproduction (Tortora, Funke, & Case, 2008). On the other hand, depending on concentration, polyphenols exert not only antioxidant but also pro-oxidant properties (Galati, Sabzevari, Wilson, & O'Brien, 2002). Moreover, it is believed that pro-oxidant behaviour of flavonoids is involved in the inhibition of mitochondrial respiration (Hodnick, Duval, & Pardini, 1994).

As mitochondria are specialised for energy production and regulation of apoptosis, the possibility exists that slight inhibition in State 3 respiration rate leading to increase in the generation of reactive oxygen species could be beneficial for the cell in mediating its protection, since ROS play an important role in many redox-sensitive signalling pathways (Halestrap, Clarke, & Khaliulin, 2007; Kabir et al., 2005). Moreover, the role of *A. millefolium* as anticancer agent should be also discussed. Flavonoids centaureidin, casticin, apigenin and luteolin, and the sesquiterpenes paulitin and isopaulitin were obtained as antitumour constituents of *A. millefolium* s.l. against HeLa and MCF-7 cells, whereas artemetin, psilostachyin C, desacetylmatricarin and sintenin were proved to be inactive against tested tumour cells in an antiproliferative assay (Csupor-Löffler et al., 2009). Since mitochondria participate in the regulation of intracellular signalling, the effects of *A. millefolium* extract on mitochondrial oxidative phosphorylation found in our study could be one possible mechanism for YE cytostatic activity.

Phenolic compounds (flavonoids and phenolcarbonic acids) constitute a very large and diverse group of secondary metabolites that are formed in plants from the ubiquitous amino acid phenylalanine *via* the highly-branched phenylpropanoid pathway (Pietta, 2000; Shahidi, 2000). Flavonoids are generally considered as non-nutritive compounds and these bioactive dietary components might contribute significantly to the health benefits of diets rich in fruits and vegetables through additive and synergistic effects. Furthermore, it is thought that most of the biological effects (particularly in which the prevention of oxidative stress is beneficial) exerted by phenolics may be related, partially at least, to their antioxidant activity. It is estimated that flavonoids account for approximately two-thirds of the polyphenol intake, while phenolcarbonic acids account for one-third (Tapiero, Tew, Ba, & Mathé, 2002). Moreover, it is known, that polyphenols after intake undergo various metabolic conversions, but the literature data on different metabolites and their effects on mitochondria are very limited. Because of the hydrophobic nature of polyphenols their accumulation in blood and membranes, including those of the mitochondria, might vary after intake and depend on blood, tissue and species differences. Therefore, the interpretation of results is complicated and further studies should be performed to determine whether the direct effects of YE on mitochondria observed in *in vitro* studies might also occur *in vivo*.

5. Conclusions

The screening of antioxidant properties of *A. millefolium* herb extract by on-line HPLC-DPPH assay revealed that it possesses significant antiradical activity, which is due to the presence of radical-scavenging components within a complex of phenolic compounds. In addition, fluorimetric measurements demonstrated that YE significantly decreased H_2O_2 production in isolated mitochondria. These results taken together with phytochemical data, showing that YE contains considerable amounts of phenolics, demonstrate that *A. millefolium*, or phenolic constituents therefrom with antiradical activity, could be considered as a potential source of natural health-promoting antioxidants for medicinal and food applications. YE in concentration-dependent manner induces the decrease in State 3 respiration rate in rat heart mitochondria without any changes in the integrity of the inner mitochondrial membrane. The diminished capacity of mitochondria to synthesise ATP caused by higher concentrations of YE might be mainly due to the inhibition of F_1F_0ATPase and/or ADP/ATP translocator. Hence, all these findings in *in vitro* experiments give first insights into the molecular mechanism(s) of *A. millefolium* action on cellular energy metabolism. However, further experiments are needed to test *A. millefolium* extract in different food systems, as well as for a more comprehensive evaluation of the complex effects of YE as a multiple component mixture on mitochondrial function.

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