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Improved solubility and increased biological activity of NeoSol™RCL40, a novel Red Clover Isoflavone Aglycones extract preparation

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ABSTRACT

Red clover (*Trifolium pratense* L., Fabaceae; RCL), a perennial plant rich in isoflavones, is a natural alternative for menopausal symptoms, as well as antiaging and antioxidant. Isoflavone preparations usually contain aglycones and β-glycosides. Aglycones, the active moieties, are absorbed slowly and unevenly due to reduced water solubility and biotransformation from β-glycosides. NeoSol™RCL40 is a novel RCL isoflavone aglycones preparation based on active solubilization technologies. In the present study, NeoSol™RCL40 was shown to induce solubilization of isoflavones and to increase estrogenic and antioxidative effects in comparison to a standard RCL extract (RCLE). NeoSol™RCL40 was prepared from RCLE using as host molecules either 2-pyrrolidone, 1-ethenyl homopolymer (PVP), γ-cyclodextrin, or maltodextrin. Solubilisation assays, performed by means of HPLC-UV, showed that solubilization of isoflavone aglycones was highest with RCLE processed with PVP, which was therefore selected for functional assays. In comparison to RCLE, NeoSol™RCL40 containing the same amount of isoflavone aglycones displayed 3.4 times higher estrogenicity in MCF-7 cell, 1.9–2.0 higher antioxidant activity in the DPPH and in the FRAP assay, and was cytoprotective in PC12 cells. As a whole, results support the ability of NeoSol™RCL40 to promote isoflavones solubilization leading to increased biological activity. NeoSol™RCL40 is therefore an interesting novel preparation providing improved availability of active isoflavones aglycones.

1. Background

Red clover (*Trifolium pratense L.*, *Fabaceae*; RCL) is a perennial plant rich in phytoestrogens (mainly biochanin A, daidzein, genistein and formononetin), which has been extensively used in traditional and folk medicine. Scientific evidence currently supports the use of RCL extracts to treat menopausal symptoms [1–3], as antiaging and antioxidant [4,5], for hyperlipidemia, metabolic syndrome and cardiovascular health [6,7], to prevent climateric osteoporosis [8,7], for benign prostatic hypertrophy and possibly even for prostate cancer [9].

Uses of RCL extracts are mainly based on their estrogenic, antioxidant and cytoprotective activities [4,5], which are accounted for by the high content in isoflavone phytoestrogens biochanin A, daidzein, genistein and formononetin [10].

Isoflavone preparations usually contain aglycones and β-glycosides. Isoflavone β-glycosides are highly polar compounds which hardly cross the intestinal epithelium, thus resulting in low bioavailability and weaker biological activities in comparison corresponding aglycones. Aglycones, the active moieties, are however absorbed slowly and unevenly due to reduced water solubility and biotransformation from β-glycosides (reviewed by Vitale et al. [11]). To overcome these potential limitations, hydrolyzed RCL extracts were processed by means of a proprietary active solubilization technology (Asoltech Srl, Italy) called NeoSol™ to obtain NeoSol™RCL40, a novel RCL isoflavone aglycones formulation with optimized solubility [12]. NeoSol™ is based on combination and interaction of the active ingredient with carriers with different characteristics and functions, resulting in increased active compounds availability and improved physiological activity and efficacy [13,14]. Briefly, molecules of botanicals (guest) are inserted into the cavity of carrier (host) creating a «multicomposite» active

Abbreviations: RCL, Red Clover; RCLE, Red Clover Aerial Parts Isoflavone Aglycones Dry Extract; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; TPTZ, 2,4,6 tripyridyl-s-triazine; DMEM, Phenol red-free Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PVP, 2-pyrrolidone, 1-ethenyl homopolymer; CDX, γ-cyclodextrin; MDX, maltodextrin; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; FRAP, Ferric ion Reducing Antioxidant Power; TPTZ, M 2,4,6 tripyridyl-s-triazine; Anx-FITC, Annexin V-FITC; PI, propidium iodide

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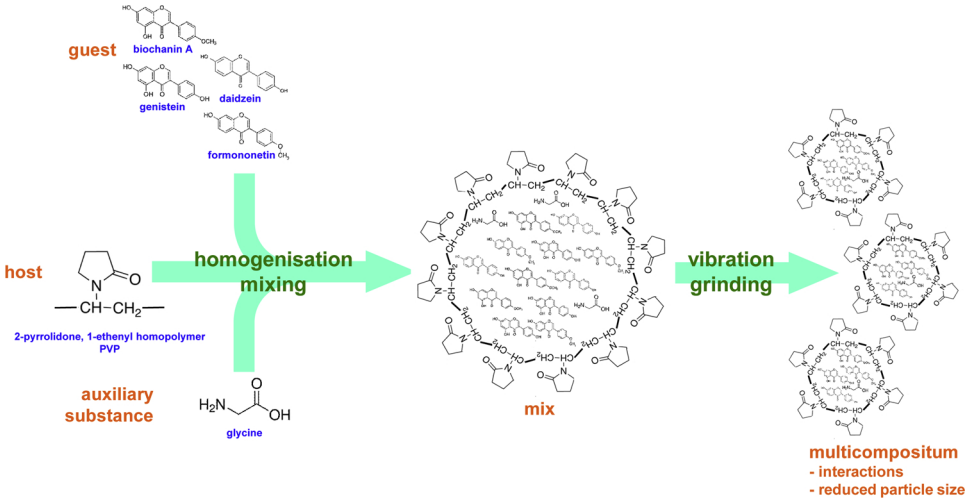

Fig. 1. Schematic representation of NeoSol™ technology applied to RCLE isoflavone aglycones. Briefly, active ingredients (guest) are combined with a carrier (host), eventually together with an auxiliary substance. The ternary composition undergoes mixing and homogenisation, followed by vibration and grinding, resulting in a «multicomposite» active ingredient characterized by increased solubility and rapid release and onset of action.

ingredient characterized by rapid onset of action and a powerful release effect with additional features improving the preexisting characteristics of the active ingredient (Fig. 1).

The present study was undertaken to evaluate NeoSol™RCL40-induced isoflavones solubilization, and estrogenic, antioxidant and cytoprotective effects in comparison to a standard RCL isoflavone aglycone extract (RCLE). To this end, we first tested the solubilisation of isoflavone aglycones from different NeoSol™RCL40 multicomposites in comparison to a standard RCL isoflavone aglycone preparation. The NeoSol™RCL40 with the highest the ratio of solubilized isoflavone aglycones in comparison to the standard RCL preparation was then tested for estrogenicity as well as for antioxidant and cytoprotective activity by use of established *in vitro* models.

2. Material and methods

2.1. General

MCF-7 cells (catalogue number 86012803), PC12 (catalogue number 88022401), ribonuclease A solution (RNAse, catalogue number P4875), propidium iodide (PI, catalogue number P4170), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH, catalogue number D9132), Iron(III) chloride hexahydrate ($FeCl_3$ x 6 H_2O, catalogue number 31232), ethanol (catalogue number 02854), acetic acid (catalogue number A6283), sodium acetate (catalogue number S8625), 2,4,6 tripyridyl-s-triazine (TPTZ, catalogue number 93285) were all from Sigma Milan Italy). Phenol red-free Dulbecco's modified Eagle's medium (DMEM), RPMI1640 medium (code ECM0495 L), fetal bovine serum (FBS, ECS0180 L), 20% charcoal/dextran-treated FBS, Heat-inactivated Donor Horse Serum (ECS0090 L), L-glutamine, penicillin/streptomycin (ECB 3001D), trypsine\EDTA (catalogue number ECB3052D) were from Euroclone (Milan, Italy). FITC Annexin V detection Kit I (catalog number 556547) was from Becton Dickinson, Milan, Italy. Methanol (catalogue number 106009) was from Merck (Darmstadt, Germany).

2.2. Plant material

Red Clover Aerial Parts Isoflavone Aglycones Dry Extract (RCLE) originated from dried aerial parts of *Trifolium pratense* L. (Fam. Fabaceae) grown in Linnea's controlled plantations under Good Agricultural and collection Practice for starting materials of herbal origin (European Medicines Agency, 2006). RCLE is obtained by hydroalcoholic extraction in ethanol 70 ± 10% (Alcosuisse AG, Bern, CH), and contains 36–44% of isoflavones, 90–95% as aglycones, calculated on the dried basis as the sum of daidzein, genistein, formononetin and biochanin A. Daidzein and genistein are always < 1%, and the ratio of 5,7-dihydroxyisoflavones (genistein, biochanin A) vs 7-hydroxyisoflavones (daidzein, formononetin) is in the range 0.1–10, according to the U.S. Pharmacopeia [15].

2.3. Preparation of RCLE solutions

NeoSol™ is a proprietary procedure developed by Asoltech Srl (Italy, https://www.asoltech.it/) with the aim to achieve optimized natural extract solubility based on patented technology [13,14]. NeoSol™ takes advantage of active solubilization technologies based on host-guest chemistry, a technique whereby a guest molecule is inserted within the cavity of a host molecule, thereby modifying the chemical and physical properties of the guest molecule [16–18]. By means of such technology, RCLE isoflavone aglycones (guest) are inserted into host molecules with which they interact through noncovalent bonding.

Host molecules selected for the present study included a ternary composition comprising an active substance, a hydrophilic or hydrophobic carrier and a co-grinding auxiliary substance, chosen among 2-pyrrolidone, 1-ethenyl homopolymer (PVP - CAS n. 9003-39-8), γ-cyclodextrin (CDX - CAS n. 17465-86-0), and maltodextrin (MDX - CAS n. 9050-36-6). These macrocylic molecules have hydrophobic cavities in which guests are embedded, resulting in significantly improved solubility/stability and availability of poorly soluble isoflavone aglycones in physiological environments [19–22]. Steviol gylcosides from *S. rebaudiana* (stevia, rebaudioside A - CAS 58543-16-1) or L-glycine (gly - CAS 56-40-6) were used as auxiliary substances to obtain the final ternary composition (multicomposite).

Briefly, RCLE was processed alone (standard RCLE, Red Clover Extract RCL40, by Linnea SA, CH - CAS 85085-25-2) or added to mixtures of either PVP/gly (w/w: 20/75/5%), CDX/gly (20/70/10), or MDX/stevia (20/70/10), to obtain multicomposite preparations. The procedure included homogenisation for 10 min in a rotating body mixer for powders, and thereafter loading into a vibrational mill, equipped with sintered alumina cylindrical means of grinding, and grinding with a vibrational amplitude comprised between 6 and 10 mm for 2 h. Low temperature indeed prevents possible guest's degradation and the absence of solvents eliminates any problems of contamination, residuals and recovery. The products obtained, with a yield of at least 20% w/w extract (equivalent to 6.8–9.2 mg total isoflavones/100 mg NeoSol RCL40), had a final packing density of 0.65 g/mL.

2.4. HPLC assay of isoflavones

Detection of the four main RCL isoflavone aglycones (biochanin A, daidzein, genistein and formononetin) was performed by means of an HPLC system equipped with an UV detector set at 254 nm, using the

USP 36 method for "powdered red clover extract", modified and validated as previously described [10].

2.5. Estrogenicity in MCF-7 cells

Estrogenicity was tested by use of an established method, based on the proliferation of the estrogen-dependent MCF-7 cells, which express estrogen receptors and exhibit a strong and reproducible proliferative response to 17β-estradiol (E2) as well as to all the compounds which act through estrogen receptors [10,23,24]. Briefly, MCF-7 cells were cultured in phenol red-free DMEM, with added 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin plus 100 µg/mL streptomycin, at 37 °C in 5% CO_2 moist atmosphere. Subconfluent cultures were split 1:3 using trypsine\EDTA and plated in 75 cm^2 flasks. Before treatments, cells were kept for 24 h in 6-well plates containing phenol red-free DMEM with added 20% charcoal/dextran-treated FBS, 2 mM L-glutamine, and 100 U/ml penicillin plus 100 µg/mL streptomycin, and thereafter added with the test solutions and left in culture for additional 24 h. In each experimental session, a well containing cells without any treatment was always included as baseline control, while a well containing cells treated with E2 1×10^{-10} M was included to allow for possible inter-session variability.

MCF-7 cells were finally harvested, washed in phosphate buffered saline (PBS, composition g/l: NaCl 8.4, Na_2HPO_4 1.424, NaH_2PO_4 0.276, pH7.4) at 600 g for 5 min, and fixed/permeabilized with 1.5 ml of 70% ice-cold ethanol for 3.5 h at 4 °C. Pellets were then washed with 3 ml of PBS and resuspended in 50 µl of a 100 µg/ml RNAse solution, and kept at 37 °C for 30 min. Suspensions were then added with 0.6 ml PBS containing 50 µg/ml propidium iodide and incubated in the dark at room temperature for 45 min. Flow cytometric analysis was carried out using a BD FACSCanto II flow cytometer (Becton Dickinson Italy, Milan, Italy) with FACSDiva software (version 6.1.3), and propidium iodide fluorescence (FL3) was collected on a linear scale using a 670-LP filter. At least 25,000 events/sample were acquired and gated on a standard bi-parametric dot plot. Histograms were analysed with FlowJo software (version 8.3.2), and the percentages (%) of cells in G0/G1, S and G2/M phases of the cell cycle were calculated. For the purpose of the present study, the percentage of cells in the S phase of the cell cycle, i.e. the part of the cell cycle in which DNA is replicated, was taken as an index of cell proliferation.

2.6. Antioxidant activity by the DPPH and the FRAP assays

The DPPH assay is based on violet free radical DPPH (1,1-diphenyl-2-picrylhydrazyl radical) reduction to yellow hydrazine DPPHH [25,26]. Briefly, 3.9 ml of 60 µM DPPH methanolic solution and 100 µl of the test solution were allowed to react at room temperature in the dark for 90 min. Absorbance at 514 nm was thereafter measured by means of Nicolet Evolution 100 spectrophotometer (Thermo Fisher Scientific, MA, USA), and its percentage inhibition in the presence of test solutions was assessed and taken as a measure of antioxidant activity.

The Ferric ion Reducing Antioxidant Power (FRAP) assay measures the ferric ion reduction power [27]. Briefly, the test reaction mixture (FRAP reagent) is prepared by mixing 25 ml of acetate buffer (23.125 mL of 0.1 M acetic acid + 1.875 mL of 0.1 M sodium acetate, pH = 3.6), 2.5 ml of 0.02 M $FeCl_3$ x 6 H_2O, and 2.5 ml of 0.01 M 2,4,6 tripyridyl-s-triazine (TPTZ) at 37 °C. Aliquots of 900 µl of FRAP reagent were then prepared and their absorbance was measured at 593 nm by means of Nicolet Evolution 100 spectrophotometer (Thermo Fisher Scientific, MA, USA). Aliquots were then added with 120 µl of the test solutions (30 µl sample + 90 µl water) and the absorbance was monitored over a 4 min period. The difference (delta) between the initial and the final absorbance values was finally calculated referred to a standard curve of 100–2500 mM $FeSO_4$.

2.7. Cytoprotective activity in PC12 cells

PC12 cells were purchased from Sigma (Milan, Italy, catalogue number 88022401) and routinely maintained in RPMI1640 medium supplemented with 5% FBS, 10% heat-inactivated Donor Horse Serum, and 100 U/ml penicillin/streptomycin, at 37 °C in a moist atmosphere of 5% CO_2. Prior to all treatments, cell viability was assessed using Trypan Blue Dye, and only PC12 cells > 95% viable were used in subsequent experiments.

PC12 were cultured in 12-well plates at a concentration of 1×10^6 cells/ml in complete medium and treated with hydrogen peroxide (H_2O_2, 100-300-500 µM) for 24 h to induce apoptosis. Test solutions were added 30 min before H_2O_2, at a final dilution of 1/100 to assess the effect on H_2O_2-induced apoptosis.

Apoptosis was evaluated by flow cytometry, using the Becton Dickinson FITC Annexin V detection Kit I, according to the manufacturer's instructions. Briefly, PC12 cells were harvested, centrifuged at 600 *g* for 5 min at room temperature to remove the culture medium, and washed with 2 ml PBS 1X (composition g/l: NaCl 8.4, Na_2HPO_4 1.424, NaH_2PO_4 0.276, pH7.4). Samples were then resuspended in 400 µL of Annexin V Binding Buffer provided in the kit, and 100 µL of each cell suspension was stained with 5 µL of both Annexin V-FITC (Anx-FITC) and propidium iodide (PI) solution for 15 min at room temperature in the dark. After incubation, 250 µL of binding buffer were added and samples were kept on ice until flow cytometric analysis. Sample acquisition was performed on a BD FACSCanto II Flow Cytometer (Becton Dickinson Italy, Milano, Italy) and data were analyzed using BD FACSDiva software (version 6.1.3). PC12 cells were identified on the basis of their morphological parameters (forward scatter [FSC] and side scatter [SSC]), and a minimum of 20,000 cells/sample was collected in the gate. Gated cells were finally visualized on a bi-parametric dot plot PI vs Anx-FITC and the percentage (%) of Anx^-PI^- (viable) and Anx^+PI^+ (late apoptotic/necrotic) cells was calculated.

2.8. Statistics

Results are shown as means ± SEM of the data in each experimental group, and statistical significance of the differences between groups was assessed by ANOVA followed by the Dunnet or the Bonferroni post test, or the Student's *t* test for paired or unpaired data, as appropriate. Concentration-response relationships were analyzed by nonlinear regression, and sigmoidal concentration-response curves with variable slope were fitted to the data, according to the following function:

$$Y = a + (b - a)/(1 + 10\hat{}((\mathrm{Log}(EC_{50}) - X)*n))$$

In this function, Y is the response, and X corresponds to the dilution of the tested solution. Parameter *a* equals the baseline and *b* is the plateau of the curve (i.e. the maximal response, E_{max}). $\mathrm{Log}(EC_{50})$ is the Log of the concentration value when the response is halfway between baseline and plateau, that is the effective concentration, 50% (i.e. the concentration which elicits 50% of the maximal response). Parameter *n* is the Hill coefficient, which describes the steepness of the curve and

may be related to the cooperativity of the ligand-receptor binding (the higher the value, the more cooperative the binding). The mean values of EC_{50}, E_{max} and Hill coefficient were finally calculated together with 95% confidence interval (C.I.).

Calculations were performed using commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

3. Results

3.1. Solubilisation of isoflavone aglycones from RCLE and from different NeoSol™RCL40 multicomposites

In a preliminary set of experiments, total isoflavone aglycones in solutions obtained from RCLE and from each NeoSol™RCL40 multicomposite (PVP/gly, CDX/gly, and MDX/stevia) were assessed by HPLC-UV spectrophotometry. To this end, 130 mg of RCLE and 650 mg of each multicomposite were placed in 10 mL of ultrapure water (MilliQ). The amount of each multicomposite was calculated taking into account that RCL extract in multicomposites represents 20% of total weight. As a consequence, all the solutions finally contained 130 mg of RCL extract. Solutions were then centrifuged at 200 rpm and 25 °C for 1 h. After 30 min resting, 5 mL were taken from each sample and centrifuged at 5000 rpm for 10 min, 2 mL were filtered on 0.45 μM filters, and 1 mL was finally diluted in ethanol 96% (Alcosuisse AG, Bern, CH) up to a total volume of 50 mL. Final solutions were clear and trasparent in the case of RCLE and of the PVP/gly multicomposite, while they were opalescent with CDX/gly and MDX/stevia multicomposites.

To assess the concentration of individual isoflavone aglycones in solution, 100 mg of RCLE and 500 mg of each NeoSol™RCL40 multicomposite were dissolved in 50 mL water at pH 4.0 by means of tartaric acid/NaOH 0.01 M. After centrifugation at 200 rpm and 25 °C for 1 h and subsequent 30 min resting, supernatants were sampled with a syringe, filtered on 0.45 μM filters and isoflavone aglycones were assayed by means of HPLC-UV. Results show that the amount of solubilized isoflavone aglycones (mean ± SD, n = 3) was 0.50 ± 0.03 mg from RCLE (on average, 125% of total isoflavone aglycones), 2.49 ± 0.40 mg from the PVP/gly (6.22%), 0.70 ± 0.35 mg from the CDX/gly (1.75%), and 1.36 ± 0.43 mg from the MDX/stevia multicomposite (3.40%) (Fig. 2). Thus, the ratio of solubilized isoflavone aglycones in comparison to RCLE, was 5.0 with PVP/gly, 1.4 with CDX/gly, and 2.7 with MDX/stevia.

NeoSol™RCL40 PVP/gly multicomposite was therefore finally selected for functional assays, and the solubilization of individual isoflavone aglycones was further determined in comparison to solutions obtained with RCLE and used as reference control in the assays (Fig. 3). In comparison to solutions from RCLE, solutions from NeoSol™RCL40 PVP/gly multicomposite contained higher concentrations of all isoflavone aglycones (Fig. 3A). The ratio NeoSol™RCL40 PVP/gly multicomposite vs RCLE for individual isoflavone aglycones was 5.6 for biochanin A, 1.7 for daidzein, 2 for genistein, and 4.7 for formononetin (Fig. 3B).

3.2. Functional comparison of NeoSol™RCL40 PVP/Gly and RCLE

3.2.1. Estrogenicity

Both NeoSol™RCL40 and RCLE increased the percentage of MCF-7 cells in the S phase of the cell cycle in a concentration-dependent fashion (Fig. 4). As shown in Table 1, however NeoSol™RCL40 500 (which contains the same amount of isoflavone aglycones as RCLE) displayed higher potency in comparison to RCLE (NeoSol™RCL40 500/RCLE mean EC_{50} ratio = 3.4). The effect of NeoSol™RCL40 was indeed proportional to the title of isoflavone aglycones: indeed, the mean EC_{50} ratio was 1.8 for NeoSol™RCL40 500/250 and 5.1 for NeoSol™RCL40 500/100 (Table 1). The potency of NeoSol™RCL40 100 (which contains just one fifth of isoflavone aglycones in comparison to RCLE) was not significantly different from that of RCLE (NeoSol™RCL40 100/RCLE mean EC_{50} ratio = 0.7). PVP/gly at the concentration contained in NeoSol™RCL40 500 at the lowest tested dilution (1:100) had no effect on the fraction of cells in the S phase of the cell cycle (15.8 ± 0.9% vs 15.7 ± 0.4% in control samples, n = 5, P = 0.763).

3.2.2. Antioxidant activity

In both the DPPH and the FRAP assay NeoSol™RCL40 500 consistently displayed higher antioxidant activity in comparison to RCLE (Fig. 5). In particular, the antioxidant activity ratio of NeoSol™RCL40 500 1:1 vs RCLE 1:1 was 1.9 in the DPPH assay and 2.0 in the FRAP assay. PVP/gly at the concentration contained in NeoSol™RCL40 500 1:1 had no effect either in the DPPH assay (6.2 ± 1.7%, n = 6, P > 0.05 vs control) or in the FRAP assay (15.3 ± 10.6 μM of $FeSO_4$ equivalents, n = 6, P > 0.05 vs control).

3.2.3. Cytoprotection

Flow cytometric analysis showed that H_2O_2 100 μM reduced viable PC12 cells by (mean ± SEM) 17.6 ± 2.5% (P < 0.05 vs control, n = 4) and increased late apoptotic/necrotic cells by 46.2 ± 9.9% (P < 0.05 vs control, n = 4). NeoSol™RCL40 500 1:100 had no effect in control conditions (not shown) however it prevented H_2O_2-induced cytotoxicity, while RCLE 1:100 had no effect (Fig. 6). PVP/gly at the concentration contained in NeoSol™RCL40 500 1:100 had no effect on viable cells (101.1 ± 1.3% of control, n = 3, P = 0.440 vs control) as well as on late apoptotic/necrotic cells (87.3 ± 5.9%, n = 3, P = 0.163 vs control).

4. Discussion

The results of this study show that application of the NeoSol™ technology to RCLE promotes increased solubilization of isoflavone aglycones, providing enhanced functional activity in terms of estrogenicity, antioxidant activity and cytoprotection. NeoSol™ is an innovative and successful result of the application of host-guest chemistry for biomedical and health applications, especially to botanical compounds and complex molecules characterized by uncertain or poor (limited) bioavailability. Molecules of botanicals (guest) are inserted into the cavity of carrier (host) creating a «multicomposite» active ingredient characterized by a rapid onset of action and a powerful release effect with additional features improving the preexisting features of the active ingredient. All this translates into a unique overall therapeutic profile of the multicomposite. Advantages are due to moleculare separation, as single molecules (instead of crystals) of the active ingredient are released within the body, greater solubility, as the hydrophobic parts of the guest are hidden within the host/carrier's cavity, and carrier effect, inasmuch the active ingredient is released rapidly from the multicomposite thanks to the weak bonding effect between host and guest.

This is to our best knowledge the first study documenting the effectiveness of this novel technology, both from the biochemical as well as from the functional point of view. Specifically, we first evaluated the solubilizing effects of different host/carrier and auxiliary substances, namely PVP, CDX and MDX, and glycine and rebaudioside A, respectively. The ternary complex RCLE/PVP/Gly (w/w: 20/75/5 = %) provided the best results in terms of increased solubility of isoflavone

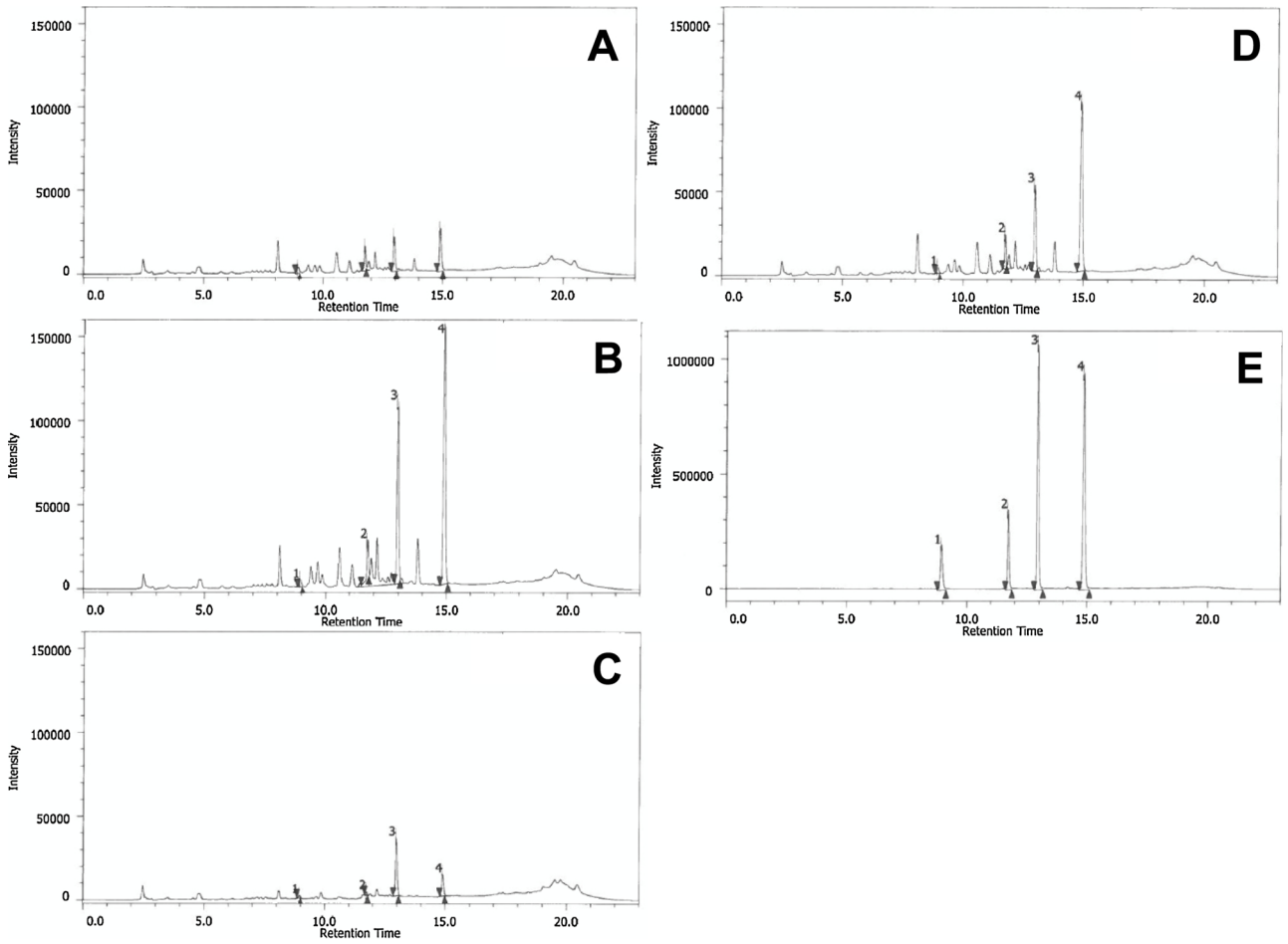

Fig. 2. HPLC-UV analysis of the amount of solubilized isoflavone aglycones from RCLE (A), and from PVP/gly (B), CDX/gly (C), and MDX/stevia multicomposite (D). Chromatograms are from one representative of 3 independent assays. A chromatogram from a standard solution of daidzein (peak 1), genistein (peak 2), biochanin A (peak 3) and formononetin (peak 4) is shown for comparison (E).

aglycones. Indeed, with PVP/gly the ratio of solubilized isoflavone aglycones in comparison to RCLE was 5.0, while it was just 1.4 with CDX/gly, and 2.7 with MDX/stevia.

Both PVP and CDX were previously used to increase the solubility of individual isoflavone aglycones. PVP may inhibit crystallization in both solution [28] and amorphous solid dispersions [29], and a spray-dried ipriflavone with PVP was developed to increase oral absorption of the synthetic isoflavone ipriflavone [30]. On the other side, various studies tested CDX with natural isoflavones. For instance, genistein water solubility and availability has been reported to increase by 4 times with β-CDX and by even 9 times with γ-CDX [31]. Complexation of genistein with γ-CDX also resulted in increased transport of these inclusion complexes across Caco-2 cell monolayers, an established model of human small intestinal mucosa which may predict the absorption of orally administered compounds [32]. Interestingly, in the same study γ-CDX did not result in any increase of Caco-2 transport of daidzein, which was on the contrary enhanced by β-CDX [32], suggesting that the host/carrier effect may vary across different guest isoflavones.

Indeed, a distinct enhanced solubilization for different isoflavones was evident also in the case of NeoSol™RCL40 PVP/gly: while solubility of biochanin A and formononetin increased about 5-fold, solubility of daidzein and genistein increased only 1.7-2-fold. Different solubilization of individual isoflavone aglycones might possibly explain why the potency ratio of the estrogenic activity between RCLE and NeoSol™RCL40 500 (which contains the same amount of isoflavone aglycones as RCLE) is 3.4 ad not 5, and between RCLE and NeoSol™RCL40 100 (which contains just one fifth of isoflavone aglycones in comparison to RCLE) is 0.7 and not 1. In previous studies [10], we showed that genistein displayed the highest potency while formononetin was the less potent and biochanin A and daidzein were nearly equipotent. In the present study, the limited solubility increases of daidzein and in particular of genistein, in comparison to the less potent formononetin likely explains why the relative potency of NeoSol™RCL40 vs RCLE containing equivalent amounts of isoflavone aglycones was less than 5.

Results of functional experiments performed in the present study indicate that NeoSol™RCL40 retains the whole pharmacological profile of RCL extracts, including estrogenicity, antioxidant and cytoprotective activities [4,5]. In particular, estrogenicity assessed in the MCF-7 cell model is in line with previous findings (Spagnuolo et al., 2014), while cytoprotection in PC12 cells confirms and extends recent studies showing that biochanin A protects PC12 cells from toxicity induced by

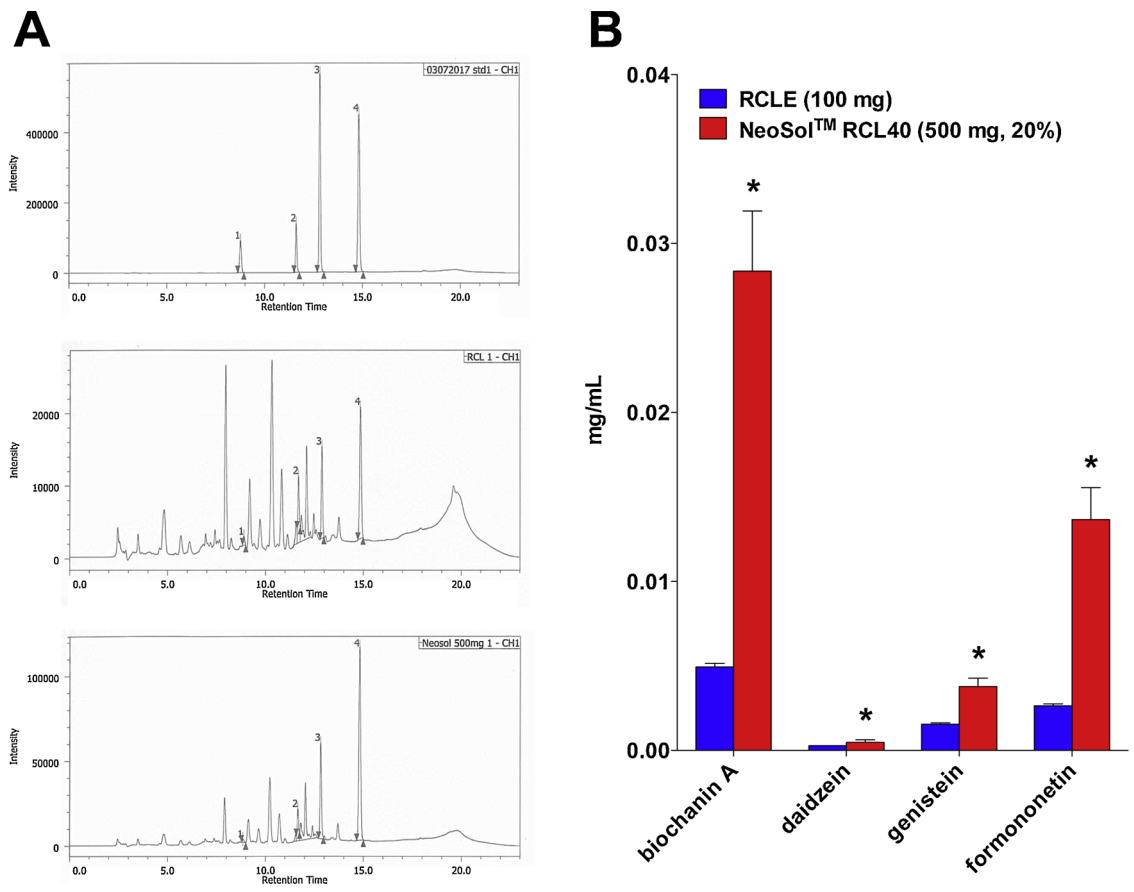

Fig. 3. NeoSol™RCL40 increases the water solubility of RCL isoflavone aglycones. Panel A: typical HPLC assays of daidzein (peak 1), genistein (peak 2), biochanin A (peak 3) and formononetin (peak 4) in a standard solution (upper panel), and in solutions obtained by dissolving in water (100 mg/50 mL, final concentration 2 mg/mL) RCLE (middle panel), or NeoSol™RCL40 (lower panel). Panel B: concentrations of isoflavone aglycones in aequous solutions of RCLE and of NeoSol™RCL40 (both at the concentration of 2 mg/mL. Data are shown as means ± SD of at least 15 separate determinations. * = P < 0.0001 vs RCLE.

glutamate [33] or by β-amyloid [34]. Remarkably, biochanin A has been shown to protect dopaminergic neurons from proinflammatory microglia-induced damage [35], and supplementation with RCL extracts protect nigrostriatal dopaminergic neurons in a rodent model of Parkinson's disease (PD) [36], in line with the traditional use of RCL to treat PD-related symptoms by some traditional communities [37]. Remarkably, in our experiments only NeoSol™RCL40 reduced oxidative damage in PC12 cells, while standard RCLE was ineffective, suggesting that isoflavone aglycone availability is a key issue in RCL-dependent cytoprotection. Increased isoflavone solubility and activity provided by

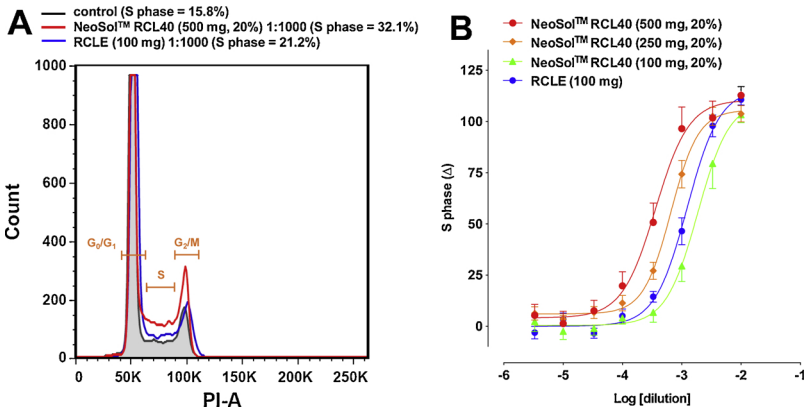

Fig. 4. Estrogenicity comparison of NeoSol™RCL40 and RCLE in MCF-7 cells. Panel A: typical histogram of cell distribution according to DNA content (as assessed by PI staining). Panel B: effect of NeoSol™RCL40 and of RCLE on ΔS phase in MCF-7 cells. Each point is the mean ± SEM of at least 6 experiments. Data expressed as % of the effect of E2 0.1 nM.

Table 1
EC_{50}, E_{max} and Hill coefficient values of the concentration-response curves of Neosol RCL solution in comparison to a standard RCL extract (RCLE) solution on ΔS phase in cultured MCF-7 cells. Data expressed as % of the effect of E2 0.1 nM.

Solution	EC_{50} (dilution factor x 1000)		E_{max} (ΔS phase) *% of E2 0.1 nM*		Hill coefficient	
	mean	95% CI	mean	95% CI	mean	95% C.I.
NeoSol™ RCL 500 (500 mg, 20%)	0.361	0.248-0.527	110.6	98.2-123.0	1.54	0.70-2.38
NeoSol™ RCL 250 (250 mg, 20%)	0.657	0.539-0.801	105.7	98.5-112.8	1.87	1.29-2.46
NeoSol™ RCL 100 (100 mg, 20%)	1.857	1.217-2.835	110.1	88.6-131.7	1.62	0.81-2.44
RCLE (100 mg)	1.237	0.981-1.558	116.0	104.6-127.3	1.59	1.09-2.08

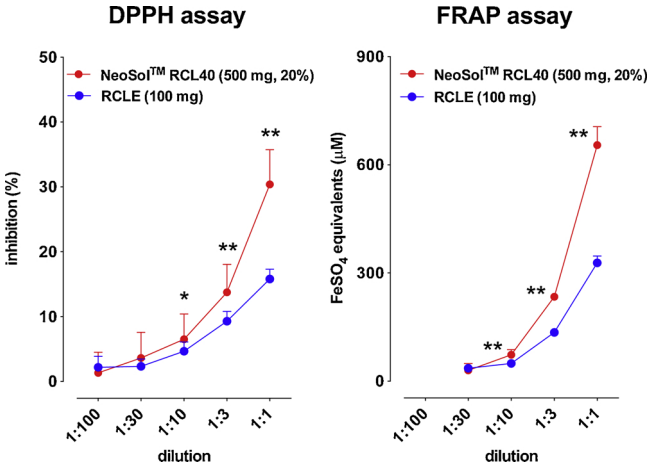

Fig. 5. Concentration-response relationships for NeoSol™RCL40 500 and RCLE antioxidant activity in the DPPH (left) and in the FRAP assay (right). Each point is the mean ± SEM of five to six separate observations. * = P < 0.05 and ** = P < 0.01 vs RCLE.

NeoSol™RCL40 thus opens up novel potential therapeutic applications which might be tested in clinical studies, including neuroprotection in neurodegenerative disease.

In conclusion, this is the first study reporting the ability of the novel NeoSol™ technology to promote solubilization and availability of active compounds from a herbal extract, resulting in enhanced functional activity. In comparison to the standard RCLE, NeoSol™RCL40 was more estrogenic and more antioxidant, and antioxidant activity resulted in cytoprotective effects. NeoSol™RCL40 is therefore an interesting novel preparation providing improved availability of active isoflavones aglycones. Benefits of improved availability could in principle include increased absorption of isoflavones after standard RCLE dose as well as standard absorption after lower dosage administration. Standard RCLE used in the present study contains isoflavones 90–95% in the form of aglycones, thus possibly reducing variable absorption due to biotransformation from β-glycosides in the gut lumen [11]. No information exists so far concerning the molecular structure and the chemical interactions occurring in the host-guest NeoSol™RCL40 complex. Host-guest chemistry is based on non-covalent interactions, including van der Waals forces, ionic bonds, hydrophobic interactions, and hydrogen bonds. Biological effects of isoflavones and in particular their antioxidant properties depend on mechanisms such as hydrogen atom transfer, single-electron transfer, and sequential proton-loss electron-transfer [38]. The bonding pattern of isoflavones in the host-guest complex may therefore significantly affect their biological activity, therefore detailed structural information about the resulting NeoSol™RCL40 multicomposite will likely provide clues for further

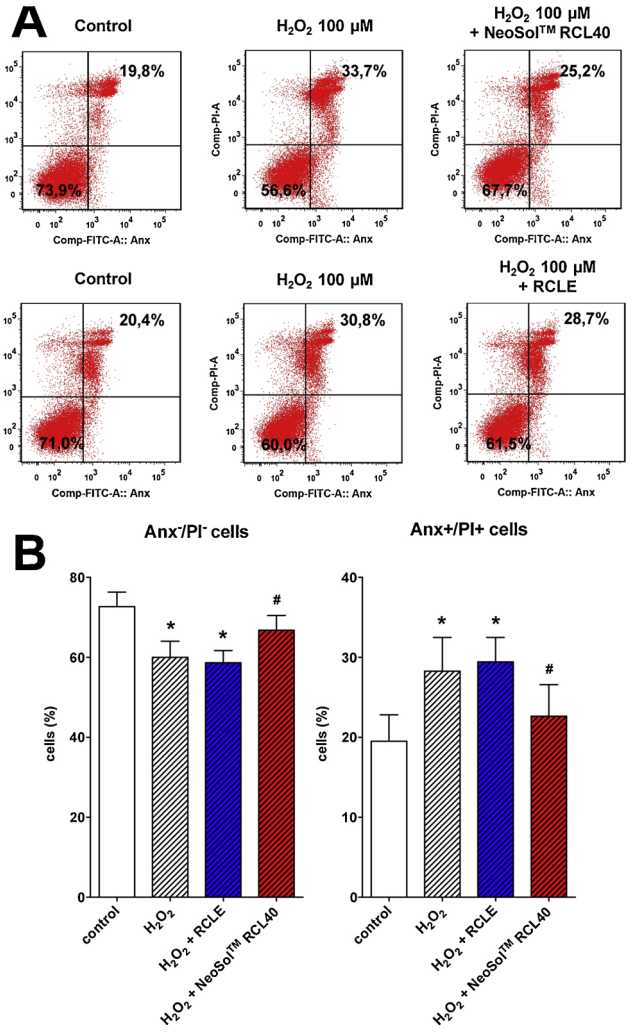

Fig. 6. Cytoprotective assay of NeoSol™RCL40 and RCLE in PC2 cells. Cells were treated with H_2O_2 100 μM and NeoSol™RCL40 500 or RCLE were added at 1:100 final dilution. Panel A: flow cytometric analysis of living cells (Anx^-/PI^-, lower left box) and of late apoptotic/necrotic cells (Anx^+/PI^+, upper right box). Panel B: effect of NeoSol™RCL40 500 or RCLE 1:100 on H_2O_2-induced cytotoxicity. Columns are means ± SEM of four separate experiments. * = P < 0.05 vs control and # = P < 0.05 vs H_2O_2.

optimization of the preparation. Nonetheless, taken as a whole the results of the present study showing increased solubility and availability provided by the NeoSol™RCL40 suggest that this preparation could in principle also reduce variability due to limited water solubility of

isoflavones. Such possibility awaits proper testing in bioavailability studies.

Author contribution and declaration

Study conception and design: MC, FM, BP.
Acquisition of data: ER, ML, RB, AL, BP.
Analysis and interpretation of data: MC, FM, ER, ML, RB, AL, BP.

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, and declare to have confidence in the integrity of the contributions of their co-authors.

Competing financial interests

BP is employee of Linnea SA (CH). All the other Authors declare no conflict of interest.

Availability of data and materials

All the data presented in the manuscript should be available to readers.

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References

[1] S.P. Myers, V. Vigar, Effects of a standardised extract of Trifolium pratense (Promensil) at a dosage of 80mg in the treatment of menopausal hot flushes: a systematic review and meta-analysis, Phytomedicine 24 (2017) 141–147.
[2] M. Ghazanfarpour, R. Sadeghi, R.L. Roudsari, I. Khorsand, T. Khadivzadeh, B. Muoio, Red clover for treatment of hot flashes and menopausal symptoms: a systematic review and meta-analysis, J. Obstet. Gynaecol. 36 (2016) 301–311.
[3] P. Gartoulla, M.M. Han, Red clover extract for alleviating hot flushes in post-menopausal women: a meta-analysis, Maturitas 79 (2014) 58–64.
[4] J. Kolodziejczyk-Czepas, Trifolium species-derived substances and extracts–biological activity and prospects for medicinal applications, J. Ethnopharmacol. 143 (2012) 14–23.
[5] T. Sabudak, N. Guler, Trifolium L.–a review on its phytochemical and pharmacological profile, Phytother. Res. 23 (2009) 439–446.
[6] A. Jungbauer, S. Medjakovic, Phytoestrogens and the metabolic syndrome, J. Steroid Biochem. Mol. Biol. 139 (2014) 277–289.
[7] S.E. Geller, L. Studee, Soy and red clover for mid-life and aging, Climacteric 9 (2006) 245–263.
[8] V.S. Lagari, S. Levis, Phytoestrogens for menopausal bone loss and climacteric symptoms, J. Steroid Biochem. Mol. Biol. 139 (2014) 294–301.
[9] A.E. Katz, Flavonoid and botanical approaches to prostate health, J. Altern. Complement. Med. 8 (2002) 813–821.
[10] P. Spagnuolo, E. Rasini, A. Luini, M. Legnaro, M. Luzzani, E. Casareto, M. Carreri, S. Paracchini, F. Marino, M. Cosentino, Isoflavone content and estrogenic activity of different batches of red clover (Trifolium pratense L.) extracts: an in vitro study in MCF-7 cells, Fitoterapia 94 (2014) 62–69.
[11] D.C. Vitale, C. Piazza, B. Melilli, F. Drago, S. Salomone, Isoflavones: estrogenic activity, biological effect and bioavailability, Eur. J. Drug Metab. Pharmacokinet. 38 (2013) 15–25.
[12] S.A. Linnea, NeoSol™RCL40 - Improved Isoflavones for Pure Efficacy (Product Sheet, 2018) (URL: (2018)) (Last accessed: august 14, 2018) https://www.linnea.ch/media/63869/Neosol_RCL.pdf.
[13] F. Carli, P. Corvi Mora, T. Canal, Co-grinding Process for the Preparation of a Ternary Composition (URL: European patent office, 2003) (Last accessed: august 14, 2018) http://www.wipo.int/patentscope/search/en/WO2003097012.
[14] P. Corvi Mora, T. Canal, F. Ruzzier, Composition Containing Micronutrients With Improved Anti-oxidant Activity and the Use Thereof (URL: European patent office, 2008) (Last accessed: august 14, 2018) http://www.wipo.int/patentscope/search/en/WO2007009997.
[15] USP-NF Dietary Supplement Monographs: Red Clover Aerial Parts Isoflavone Aglycones Dry Extract. U.S. Pharmacopeial Convention, USP Dietary Supplements Compendium, (2018), pp. 4814–4815.
[16] D.J. Cram, J.M. Cram, Host-guest chemistry: complexes between organic compounds simulate the substrate selectivity of enzymes, Science 183 (1974) 803–809.
[17] D.J. Cram, The design of molecular hosts, guests, and their complexes, Science 240 (1988) 760–767.
[18] P.A. Kollman, The nature of molecular recognition: examples from Host/Guest chemistry, Computational Approaches in Supramolecular Chemistry, Kluwer Academic Publishers, 1994, pp. 175–181 ©.
[19] R. Stancanelli, A. Mazzaglia, S. Tommasini, M.L. Calabrò, V. Villari, M. Guardo, P. Ficarra, R. Ficarra, The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: a spectroscopic investigation with implications in the pharmaceutical analysis, J. Pharm. Biomed. Anal. 44 (2007) 980–984.
[20] J. Yuan, M. Antonietti, Poly(ionic liquid)s: Polymers expanding classical property profiles, Polymer 52 (March (7)) (2011) 1469–1482 23.
[21] T. Laurent, I. Kacem, N. Blanchemain, F. Cazaux, C. Neut, H.F. Hildebrand, B. Martel, Cyclodextrin and maltodextrin finishing of a polypropylene abdominal wall implant for the prolonged delivery of ciprofloxacin, Acta Biomater. 7 (2011) 3141–3149.
[22] N. Sharma, A. Baldi, Exploring versatile applications of cyclodextrins: an overview, Drug Deliv. 23 (2016) 739–757.
[23] S. Wang, J.M. Aarts, N.M. Evers, A.A. Peijnenburg, I.M. Rietjens, T.F. Bovee, Proliferation assays for estrogenicity testing with high predictive value for the in vivo uterotrophic effect, J. Steroid Biochem. Mol. Biol. 128 (2012) 98–106.
[24] M. Cosentino, F. Marino, M. Ferrari, E. Rasini, R. Bombelli, A. Luini, M. Legnaro, M.G. Delle Canne, M. Luzzani, F. Crema, S. Paracchini, S. Lecchini, Estrogenic activity of 7-hydroxymatairesinol potassium acetate (HMR/lignan) from Norway spruce (Picea abies) knots and of its active metabolite enterolactone in MCF-7 cells, Pharmacol. Res. 56 (2007) 140–147.
[25] V. Bondet, W. Brand-Williams, C. Berset, Kinetics and mechanisms of antioxidant activity using the DPPH free radical method, Lebensm-Wiss u Technol. 30 (1997) 609–615.
[26] I. Parejo, C. Codina, C. Petrakis, P. Kefalas, Evaluation of scavenging activity assessed by Co(II)/EDTA-induced luminol chemiluminescence and DPPH* (2,2-diphenyl-1-picrylhydrazyl) free radical assay, J. Pharmacol. Toxicol. Meth. 44 (2000) 501–512.
[27] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[28] H. Sekikawa, M. Nakano, T. Arita, Inhibitory effect of polyvinylpyrrolidone on the crystallization of drugs, Chem. Pharm. Bull. 26 (1978) 118–126.
[29] M. Yoshioka, B.C. Hancock, G. Zografi, Inhibition of indomethacin crystallization in poly(vinylpyrrolidone) coprecipitates, J. Pharm. Sci. 84 (8) (1995) 983–986.
[30] S.H. Kim, M.G. Lee, Pharmacokinetics of ipriflavone, an isoflavone derivative, after intravenous and oral administration to rats hepatic and intestinal first-pass effects, Life Sci. 70 (2002) 1299–1315.
[31] A.E. Daruházi, L. Szente, B. Balogh, P. Mátyus, S. Béni, M. Takács, A. Gergely, P. Horváth, E. Szoke, E. Lemberkovics, Utility of cyclodextrins in the formulation of genistein part 1. Preparation and physicochemical properties of genistein complexes with native cyclodextrins, J. Pharm. Biomed. Anal. 48 (2008) 636–640.
[32] A.E. Daruházi, T. Kiss, M. Vecsernyés, L. Szente, E. Szőke, E. Lemberkovics, Investigation of transport of genistein, daidzein and their inclusion complexes prepared with different cyclodextrins on Caco-2 cell line, J. Pharm. Biomed. Anal. 84 (2013) 112–116.
[33] J.W. Tan, C.L. Tham, D.A. Israf, S.H. Lee, M.K. Kim, Neuroprotective effects of biochanin A against glutamate-induced cytotoxicity in PC12 cells via apoptosis inhibition, Neurochem. Res. 38 (2013) 512–518.
[34] J.W. Tan, M.K. Kim, Neuroprotective effects of biochanin A against β-Amyloid-induced neurotoxicity in PC12 Cells via a mitochondrial-dependent apoptosis pathway, Molecules 21 (April (5)) (2016) E548, https://doi.org/10.3390/molecules21050548.
[35] H.Q. Chen, Z.Y. Jin, G.H. Li, Biochanin A protects dopaminergic neurons against lipopolysaccharide-induced damage through inhibition of microglia activation and proinflammatory factors generation, Neurosci. Lett. 417 (2007) 112–117.
[36] K.P. Datla, V. Zbarsky, D. Rai, S. Parkar, N. Osakabe, O.I. Aruoma, D.T. Dexter, Short-term supplementation with plant extracts rich in flavonoids protect nigrostriatal dopaminergic neurons in a rat model of Parkinson's disease, J. Am. Coll. Nutr. 26 (2007) 341–349.
[37] A. de Rus Jacquet, M.A. Tambe, S.Y. Ma, G.P. McCabe, J.H.C. Vest, J.C. Rochet, Pikuni-Blackfeet traditional medicine: neuroprotective activities of medicinal plants used to treat Parkinson's disease-related symptoms, J. Ethnopharmacol. 206 (2017) 393–407.
[38] M.K. Tiwari, P.C. Mishra, Scavenging of hydroxyl, methoxy, and nitrogen dioxide free radicals by some methylated isoflavones, J. Mol. Model. 24 (2018) 287.