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Letter to Editor:

August 28, 2015

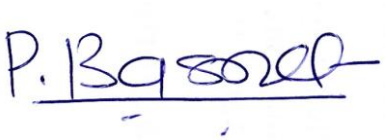
Ref.: Ms. No. **EJPS-D-15-00611** (Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections)

Prof. Jelena Filipovic-Grcic, Ph. D.
Section Editor
European Journal of Pharmaceutical Sciences

Dear Editor,
Please find enclosed the revised version of our manuscript "*Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections*". We highly appreciate valuable comments provided by the Reviewers which helped us to improve our manuscript. We corresponded to all comments and revised the manuscript accordingly. The changes in the revised version of the manuscript are highlighted. In addition, a subheading, "Chemical compounds studied in this article" was added according to your suggestion (page 2, Lines 42-46).

We hope that our response will fulfill the Reviwers' concern (*see the separate page*) and our manuscript will be publishable in the European Journal of Pharmaceutical Sciences,

With our best regards



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Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections

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Abstract

Resveratrol (RES), chemically known as 3,5,4'-trihydroxy-*trans*-stilbene, is a promising multi-targeted anti-oxidative and anti-inflammatory natural polyphenol. Preclinical studies showed its biological activities against the pathogens of sexually transmitted diseases causing vaginal inflammation and infections. Due to its low solubility and poor bioavailability, the optimal therapeutic uses are limited. Therefore, a clinically acceptable topical vaginal formulation of RES exhibiting optimal therapeutic effects is highly desirable. For this purpose, we prepared and optimized chitosan-coated liposomes with RES. The coated vesicles (mean diameter 200 nm) entrapped up to 77% of RES, a sufficient load to assure required therapeutic outcome. *In vitro* drug release study showed the ability of liposomes to provide sustained release of RES. *In vitro* anti-oxidative activities of RES, namely DPPH and $ABTS^{\bullet+}$ radicals scavenging assays, confirmed RES to be as potent as standard anti-oxidants, vitamins C and E. The anti-oxidative activities of RES and its corresponding liposomal formulation were also compared by measuring enhanced superoxide dismutase (SOD) activities in lipopolysaccharide (LPS)-induced J774A.1 cells. *In vitro* anti-inflammatory activities were compared by measuring nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-1β production in LPS-induced J774A.1 cells. Liposomal RES was found to exhibit stronger anti-oxidative and anti-inflammatory activities than RES solution.

Response to the Reviewers:

Reviewer #2:

Please add the conditions at which the zeta potential measurements were performed and check for the proper set up regarding Malvern Zetasizer Nano Z (?Malvern, Oxford, UK).

Corrections were made accordingly as mentioned on page 8, lines 210-217.

Please discuss the possibility for using other polymers instead of chitosan.

Some discussion was added on page 18, lines 551-555

Spelling error - page 7, section 2.7., should be "Characterization…" instead of "characterization…"

It was corrected on page 8, line 201.

Reviewer #3:

I will suggest to include the chemical name of Resveratrol for the pharmaceutical (not phytomedical) audience and to stress the purity of the used compound. This is a typical drawback of natural active compounds. For this reason I found quite odd the choice of Fig.9 as chemical structure of RES and VIT C, E. I would present it as Fig. 1.

The chemical name of resveratrol is added in the manuscript showing on page 2, lines 24, 43 and 48, line 43, page 4, lines 64-65; page 5, lines 115-116.

Figure 9 is changed to Figure 1 (page 12, lines 328-333). Other figure numbers were changed accordingly and changes were highlighted in yellow color in the manuscript.

In my opinion, the subdivision in paragraphs is not respecting Journal format (e.g. 2.7)

Changes were made according to the journal format (lines: 150, 164, 202, 209, 219, 228,)

I would suggest to explain the choice of the 2 marker proinflammatory cytokines, that is not clear in my opinion.

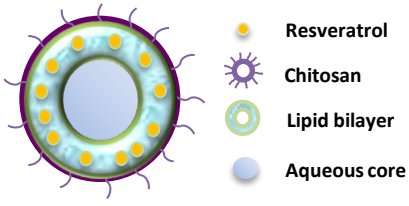
The reason for selecting pro-inflammatory cytokines IL-1β and TNF-α and NO is explained on page 16, lines 487-494.

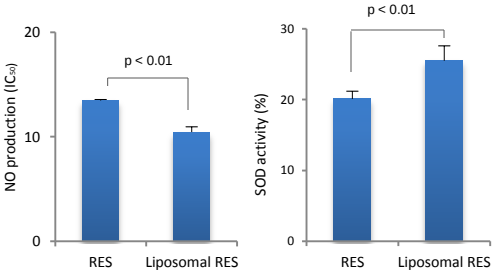
I would suggest to reduce a little bit the discussion session, especially in the first pages, somehow repeating the introduction.

A paragraph in the original version of manuscript was deleted as recommended (pages 17, lines 495-506).

In order to fulfill the Reviewers' comments, we inserted two new references (page 26, line 796 and page 29, lines 891-893) and one original reference was deleted (page 24, lines 743-748).

***Graphical Abstract (for review)**





Decreased NO production and increased SOD activity of Liposomal RES camparing to RES. Results are expressed as mean ± SD (n = 4 or 3). Controls for RES and liposomal RES were 0.2% DMSO and empty liposomes, respectively.

Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections

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Abstract

Resveratrol (RES), chemically known as 3,5,4'-trihydroxy-*trans*-stilbene, is a promising multi-targeted anti-oxidative and anti-inflammatory natural polyphenol. Preclinical studies showed its biological activities against the pathogens of sexually transmitted diseases causing vaginal inflammation and infections. Due to its low solubility and poor bioavailability, the optimal therapeutic uses are limited. Therefore, a clinically acceptable topical vaginal formulation of RES exhibiting optimal therapeutic effects is highly desirable. For this purpose, we prepared and optimized chitosan-coated liposomes with RES. The coated vesicles (mean diameter 200 nm) entrapped up to 77% of RES, a sufficient load to assure required therapeutic outcome. *In vitro* drug release study showed the ability of liposomes to provide sustained release of RES. *In vitro* anti-oxidative activities of RES, namely DPPH and $ABTS^{\bullet+}$ radicals scavenging assays, confirmed RES to be as potent as standard anti-oxidants, vitamins C and E. The anti-oxidative activities of RES and its corresponding liposomal formulation were also compared by measuring enhanced superoxide dismutase (SOD) activities in lipopolysaccharide (LPS)-induced J774A.1 cells. *In vitro* anti-inflammatory activities were compared by measuring nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-1β production in LPS-induced J774A.1 cells. Liposomal RES was found to exhibit stronger anti-oxidative and anti-inflammatory activities than RES solution.

Chemical compound studied in the article:

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), Vitamin C (ascorbic acid), Vitamin E (α-tocopherol), 2,2'-azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Phosphatidylcholine, Chitosan, L-nitro-arginine methyl ester (NAME)

Keywords: Resveratrol; 3,5,4'-trihydroxy-*trans*-stilbene; Liposomes; Anti-inflammatory; Anti-oxidant; Mucoadhesive; Vaginal infections.

Abbreviation:

ABTS, 2,2'-azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified eagle medium; GPX, glutathione peroxidase; HIV-1, human immunodeficiency virus-1; HPV, human papilloma virus; HSV-2, human simplex virus type 2; IL-1β. interleukin-1β; *i*NOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; L-

NAME, L-nitro-arginine methyl ester; MDZ, metronidazole; NO, nitric oxide; PBS, phosphate buffer solution; PM, pig mucin; PC, phosphatidylcholine; PI, polydispersity index; ROS, reactive oxygen species; RES, resveratrol; STD, sexually transmitted diseases; SOD, superoxide dismutase; TH1, T-helper cell type 1; TNF-α, tumor necrosis factor-α;

1. Introduction

Resveratrol (RES), a common natural compound produced by several plants in response to pathogenic infection (Houille et al., 2014), is identified chemically as 3,5,4'-trihydroxy-*trans*-stilbene. RES and its derivatives were reported to exhibit fungicidal and anti-microbial activities (Chan, 2002; Baur and Sinclair, 2006; Adrian and Jeandet, 2012; Houille et al., 2014). It also showed anti-viral effect (Docherty et al., 2005). In addition to this, diverse pharmacological activities such as anti-oxidative, anti-inflammatory, neuro-protective, anti-aging, anti-cancer and cardio-protective effects of RES have been reported which can carry potential therapeutic application to humans (Bhat et al., 2001; Smoliga et al., 2011; Vang et al., 2011; Lu et al., 2013). Due to the multi-targeted microbicide activities and significantly low toxic effects, RES might be the potential candidate in safe topical treatment of vaginal inflammation and infection especially in pregnant women. The vaginal environment and structure are highly vulnerable towards the pathogens such as various bacteria, fungi, viruses or protozoa (*Trichomonas*) which cause vaginal inflammation and infection and are often transmitted easily during sexual intercourse. Contamination and rapid growth of these pathogens lead not only to inflammation and infection but also facilitate human immunodeficiency virus (HIV-1), human simplex virus type 2 (HSV-2) and human papilloma virus (HPV) infections (Nikolic and Piguet, 2010). Therefore the understanding on the cross-links between various diseases and microbicides together with the link between HPV and cervical cancer was recognized by the award of the 2008 Physiology and Medicine Nobel Prize (Abbott and Brumfiel, 2008). Particularly, it is interesting that RES uses different mechanisms to induce cell death in cervical cancer cell lines (Garcia-Zepeda et al., 2013). In addition, pregnant women are vulnerable to vaginal infection because of the reduced T-helper cell type 1 (TH1) activities due to the development of protective mode towards the growing fetus. If they are not treated in time, the pregnancy might result in impaired fetal growth and development or even termination. Although anti-microbial agents are commonly used in the treatment of pathogenic vaginal infection, contemporary normal course of anti-microbial therapy cannot be applied during pregnancy. Moreover, the problem of anti-microbial resistance such as the one linked to metronidazole (MDZ) and other 5-nitroimidazoles (tinidazole, ornidazole, and secnidazole) used against trichomoniasis needs to be taken into consideration (van de Wijgert and Shattock, 2007).

Regarding the pathogen resistance and serious side effects linked to current anti-microbial options in vaginal therapy, especially in pregnant patients, a multi-targeted, less toxic and potential candidate, such as RES could be an ideal molecule. However, due to its low solubility and poor bioavailability, the possible clinical uses against vaginal inflammation and infection remain limited. Clinically applicable and safe formulation of RES assuring its optimal therapeutic value in the treatment of vaginal inflammation and infection in pregnancy is needed. By applying the chitosan-coated liposomal carrier for RES, we aimed to utilize the ability of chitosan not only as microbicide target but also to disrupt bacterial biofilms, which is of great importance in the treatment of vaginal bacterial inflammation and infections (Kandimalla et al., 2013). This paper describes the nanomedicine-based topical formulation of liposomal RES targeted to vaginal inflammation and infection. *In vitro* anti-oxidative and anti-inflammatory effects of free RES were compared with that of the corresponding liposomal formulation.

2. Materials and Methods

2.1. Materials

Lipoid S 100 (PC, >94% phosphatidylcholine) was a gift from Lipoid GmbH, Ludwigshafen, Germany. Vitamin C (ascorbic acid), chitosan [low MW, Brookfield viscosity 20.000 cps, degree of deacetylation (DD) of 92], 1,1-diphenyl-2-picrylhydrazyl (DPPH), mucin from porcine stomach (type III, bound sialic acid 0.5%-1.5%, partially purified), phosphorus standard solution (0.65 mM), sodium chloride, resveratrol (RES: 3,5,4'-trihydroxy-*trans*-stilbene, purity ≥ 99%), vitamin E, 2,2'-azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt (ABTS) and potassium peroxodisulphate were the products of Sigma-Aldrich, Chemie GmbH, Steinheim, Germany. Acetic acid (glacial), anhydrous potassium phosphate, and sodium hydrogen phosphate were purchased from Merck KGaA, Darmstadt, Germany. Ammonium acetate was obtained from BHD Prolab, Leuven, Belgium. Cibacron brilliant red 3B-A was purchased from Santa Cruz Biotech, Dallas, TX, USA. Glycine hydrochloride Plusone® was obtained from Pharmacia Biotec, Uppsala, Sweden. Dulbecco's modified eagle medium (DMEM), trypsin–ethylenediaminetetraacetic acid, lipopolysaccharide (LPS; *Escherichia coli*, 055:B5), L-nitro-arginine methyl ester (L-NAME), sulfanilamide, naphthylethylenediamine dihydrochloride, and phosphoric acid were purchased from Sigma Life Science (Sigma–Aldrich Norway AS, Oslo). Assay kit for SOD activity measurement was from Abnova GmbH EMBLEM, Heidelberg, Germany, and TNF-α, and

IL-1β measured spectrophotometrically with the assay kits were from Cell Biolabs, Inc., San Diego, CA, USA. All chemicals and solvents used were of analytical grade.

2.2. Cell culture

Murine macrophage, J774A.1 (ATCC® TIB67™) cells were purchased from ATCC and used in the *in vitro* anti-oxidative and anti-inflammatory studies. Cells (1×10^5 cells/ml) were cultured in 24-well plates with DMEM medium containing glutamine and 10% calf serum by incubating at 37 °C in 5% CO_2 for 24 h to stabilize and adhere on the plate. After 24 h the cell medium was replaced with the LPS (1 µg/ml) and/or test samples (RES/corresponding liposomal formulations) containing medium. The effects of the test samples on the LPS-induced pro-inflammatory cytokines (TNF-α, IL-1β) and NO expressed in the medium and SOD activity were measured after 24 h according to the instruction provided in the commercial kits.

2.3. Preparation of stock solutions and application to cells

Stock solutions (10 mg/mL) of RES and vitamin E were prepared by dissolving with dimethyl sulfoxide (DMSO). They were diluted with DMEM medium to the desired concentration before the treatment with the cells. In case of DMSO solution (for vitamin E and RES), maximum concentrations of DMSO were not more than 0.2% (v/v). All other samples were prepared and with the medium and applied directly into the cells.

2.4. Anti-oxidative assays

2.4.1. Measurement of DPPH radical scavenging activity

Effect of RES on DPPH free radical was determined by the similar method as reported previously (Basnet et al., 2012). In brief, DPPH solution (60 µM, 0.3 ml) in ethanol was mixed with an equal volume of each sample solution (1, 5, 10 or 20 µg/ml as the final concentrations). The reaction mixture was thoroughly mixed and kept in the dark for 30 min at room temperature. The anti-oxidative activity of RES was expressed by measuring the decreased absorbance intensity at 519 nm with UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) using following formula: Radical scavenging activity (%) = 100 x (A-B)/A, where A is the control (absorbance of DPPH radicals without sample) and B is the absorbance of radicals after reacting with the sample. The anti-oxidative activity of RES was also compared to that of vitamin C and vitamin E under the same experimental conditions. The results are expressed as mean±S.D. of three experiments.

2.4.2. Measurement of $ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ radicals were generated by mixing equal volumes (3 ml) from each of the stock solutions of ABTS (7.4 µM) and potassium peroxodisulphate (2.6 µM) in distilled water. The reaction mixture was allowed to stabilize for 3 h at room temperature and then diluted with ethanol to 100 ml as the $ABTS^{\bullet+}$ radicals working solution. The green colour $ABTS^{\bullet+}$ radicals working solution (0.3 ml) was mixed with an equal volume of sample solutions at the 1, 5, 10 and 20 µg/ml concentration. After mixing, it was kept in the dark at room temperature. After 30 min, optical density was measured with UV spectrophotometer at 731 nm. As the number of $ABTS^{\bullet+}$ radicals decreases, the intensity of green colour reduces. Results were expressed as described for DPPH radical assay. The anti-oxidative activity of RES was compared to that of the vitamin C and vitamin E under the same experimental conditions.

2.5. Preparation of liposomes

Liposomes were prepared by the film hydration method as described earlier (Jøraholmen et al., 2014). Briefly, RES (10 or 20 mg) was dissolved in methanol and mixed with phosphatidylcoline (PC, 200 or 400 mg) in methanol and solvents were evaporated on Büchi rotavapor R-124 (with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for at least 3 h at 50 mm Hg and 50 °C. The remaining film was then re-suspended in distilled water (10 ml). Throughout the preparation RES solution was kept protected from light. Empty liposomes were prepared by similar method. Liposomal suspensions were stored in refrigerator (4-8 °C) for at least 12 h prior to further use.

Liposomal size was reduced through the extrusion through polycarbonate membranes with defined pore sizes (Nuclepore Track-Etch Membran, Whatman House, Maidstone, UK). The extrusion was performed stepwise through the 0.8, 0.4 and 0.2 µm pore size filters and 5 extrusions were executed for each step.

2.6. Coating of liposomes

Coating of liposomes was performed in the presence of unentrapped RES. In brief, chitosan solutions (0.1, 0.3 and 0.6%, w/v) were prepared in 0.1% and 0.5% (v/v) glacial

acetic acid, respectively. The chitosan solution (2 ml) was added drop wise to an equal volume of liposomal dispersion under controlled magnetic stirring at room temperature for 1 h, followed by the incubation in the refrigerator (4-8 °C) overnight. The rate of stirring was kept constant for all preparations (Jøraholmen et al., 2014).

2.7. Characterization of liposomes

2.7.1. Size

The liposomal size distributions were determined by photon correlation spectroscopy (Submicron particle sizer model 370, Nicomp, Santa Barbara, CA, USA) as reported previously (Jøraholmen et al., 2014). The particle intensity was approximately 250-350 kHz the analyses run in vesicle mode and intensity-weight distribution. Three parallels (with a run time of 10 min for each parallel) were determined for each sample measurement.

2.7.2. Zeta potential measurements

Zeta potential measurements were performed on a Malvern Zetasizer Nano ZS (Malvern, Oxford, UK) according to Jøraholmen et al (2014). To assure accuracy, the instrument was calibrated throughout the measurements using the Malvern Zeta Potential Transfer Standard (−50 ± 5 mV). Measurement cell (DTS1060) was cleaned with ethanol and filtrated water (0.2 μm), respectively, prior to loading of sample. The liposomal suspensions were diluted with filtrated water to appropriate concentrations (typically 1:20) before the measurements, to achieve the proper count rate. All measurements were performed at 25 °C and three parallels were measured.

2.7.3. Entrapment efficiency determination

RES liposomes (2 ml) were dialyzed (Mw cutoff: 12–14,000 Da, Medicell International Ltd., London, UK) against distilled water (500 ml) for 4 h at room temperature. The volume of medium was adjusted to assure the solubility of RES. Aliquots of sample and medium were diluted in methanol, and the amount of liposome-associated RES was determined by UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 306 nm. The correlation coefficient for standard curve was 0.9958 and the minimum detectable amount for RES was 1 μg/ml.

2.7.4. Phospholipid assay

The content of PC was measured using the modified Bartlett method as reported previously (Andersen et al., 2015). Phospholipid content was measured by UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 830 nm. The phosphorous standard solution was used to prepare standard curve in concentration range of 1-10 µg/ml.

2.8. Chitosan determination

The surface-available chitosan was determined by a modified colorimetric method (Andersen et al., 2015). In brief, glycine buffer (pH 3.2) was prepared by dissolving glycine (1.87 g) and NaCl (1.46 g) in 250 ml of distilled water; an aliquot of 81 ml was further diluted with 0.1 M HCl to a final volume of 100 ml. Cibacron Brilliant Red 3B-A (150 mg) was dissolved in 100 ml of distilled water. The dye solution (5 ml) was further diluted to 100 ml with the glycine buffer. Vesicle suspensions were diluted with distilled water to desirable concentration (1:6, v/v) before 3 mL of the final dye solution was added. UV-Vis absorbance was measured at 575 nm with a spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The percentage of surface-available chitosan was calculated using the following equation:

$$Percentage\ of\ surface\ available\ chitosan = \frac{Cs}{Cc} \times 100$$

Where, C_s is the concentration of chitosan in the sample and C_c is the concentration of chitosan used to prepare the liposomal formulations.

A chitosan standard solution (0.05 % w/v) was prepared in 0.05 % (v/v) glacial acetic acid. The standard solution was diluted in glycine buffer to desired concentrations. The correlation coefficient for the standard curve was 0.9997 and the minimum detectable amount of chitosan was 2.27 µg/ml.

2.9. In vitro mucoadhesive properties

The mucoadhesive properties were determined by measuring liposomes binding to the pig mucin (PM) as described earlier (Jøraholmen et al., 2014). Briefly, non-coated and chitosan-coated liposomes (1 ml) in original vesicle size were mixed with equal volume of PM suspension (400 µg/ml) in 0.05 M phosphate buffer saline and incubated at room temperature for 2 h, followed by ultracentrifugation (216000 x g) for 1 h at 10 °C (Optima LE-80; Beckman Instruments, Palo Alto, CA, USA). Aliquots of 200 µl (4 from each sample)

of the supernatants were transferred to a microtitre plate (Costar® UV 96-well plate with UV transparent flat bottom, Acrylic, Costar®, Corning, NY, USA) and free PM was measured spectrophotometrically at 251 nm (Microtitre plate reader; Spectra Max 190 Microplate, Spectrophotometer Molecular devices, Sunnyvale, CA, USA). The PM binding efficiency was calculated according to Naderkhani *et al*. (2014).

2.10. In vitro RES release

The *in vitro* RES release experiment was performed by the method described earlier (Jøraholmen et al., 2014). The Franz cell manual diffusion system (Perme Gear Ink, Diffusion Cells and Systems, Hellertown, PA, USA) and the heating circulator (Julabo Laboratechnik, F12-ED, Seelback, Germany) was set to 37 °C. The acceptor chamber (12 ml) was completely filled with acetate buffer (pH 4.6) containing CH_3COONH_4 (77.1 g) and glacial acetic acid (70 ml) and distilled water up to 1000 ml. Cellophane membrane, cut to appropriate size, was pre-soaked in the same buffer prior to fixation. Liposomal samples and controls (RES in propylene glycol) (600 µl) were added to the donor chambers and the system was properly sealed. The RES content in all tested samples was determined spectrophotometrically prior to the experiment to assure the same concentration gradient in the samples and controls, and to assure sink conditions. Samples (500 µl) were withdrawn from acceptor chamber after 1, 2, 3, 4, 6 and 8 h. The collected samples were replaced by an equal volume of buffer in the acceptor chamber. All collected samples, the remaining suspension on and retained in the cellophane membrane were dissolved in methanol and measured spectrophotometrically (Agilent Technologies, Santa Clara, CA, USA) at 306 nm.

2.11. Measurement of SOD activity

The effects of RES and liposomal RES on SOD activity were measured in LPS-induced J774A.1 cells by the similar method as reported previously (Basnet et al., 2012). Cells were cultured by the method as described above. The controls for RES and liposomal formulations were medium containing 0.2% DMSO and empty liposomes, respectively. In both controls, SOD activity induced with LPS (1 µg/ml) after 24 h, were expressed as basal level. The SOD activities (%) exhibited by the LPS-induced macrophages in the presence of test samples (RES, and liposomal RES) at 10 µg/ml were assayed according to the protocol for colorimetric assay kit. The measurements were performed in triplicates.

2.12. Measurement of NO production

The effects of RES and liposomal RES on production of NO in the LPS-induced J774A.1 cells were measured as reported previously (Basnet et al., 2012). The cells were treated with LPS (1 μg/ml) and/or samples at various concentrations. After 24 h, the effects of the samples (RES, and liposomal RES) on the production of NO released in the medium were measured in terms of nitrite formation by Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at 550 nm using $NaNO_2$ as the standard. The measurements were performed in quadruplets. The effects of the RES on the production of NO were also compared to some well-known anti-oxidants (vitamin C and vitamin E) and *i*NOS inhibitor (L-NAME). The controls for RES and liposomal RES were 0.2% DMSO and empty liposomes, respectively. The inhibitory activities of RES and liposomal-RES on NO production were expressed as IC50 (μg/ml).

2.13. Effect on IL-1β and TNF-α production

The effects of RES and liposomal-RES on the production of TNF-α and IL-1β in the LPS-induced J774A.1 cells were measured as reported previously (Basnet et al., 2012). The cell medium were replaced with the medium containing LPS (1 μg/ml) and/or samples (10 μg/mL). After 24 h, the medium (500 μL) was removed and stored at –70 °C until cytokine assays were carried out. Controls for RES and liposomal RES were 0.2% DMSO and empty liposomes, respectively. The production of TNF-α or IL-1β in the medium by the LPS-induced cells were measured and expressed as 100%. Both pro-inflammatory cytokines (TNF-α and IL-1β) were measured in the same set of experiments by the assay kits as described in the manufacturer's protocols. Measurements were performed in triplicates.

2.14. Statistical analyses

Data were analyzed using the SPSS 19.0 software (SPSS Inc. Chicago, IL, USA). Continuous variables are expressed as mean and categorical variables are reported as percentage. Statistical significance of difference between the control and test groups or corresponding groups was determined by one-way ANOVA, and $P < 0.05$ was considered statically significant. Results are expressed as mean ± S.D., where n = 3 or 4.

3. Results

3.1. The effect on DPPH / $ABTS^{+\bullet}$ radicals

Although RES, vitamin C and vitamin E are structurally different (Figure 1) and differ significantly in their physical properties, they show similar chemical reaction towards free radicals and anti-oxidative potentials.

Figure 1

Anti-oxidative activity is expressed as DPPH or $ABTS^{+\bullet}$ radicals scavenging capacity of RES. We examined the radical scavenging activity of RES at 1, 5, 10 and 20 µg/ml concentrations for DPPH or $ABTS^{+\bullet}$ radicals. The anti-oxidative activities of RES were compared to those of vitamin C and vitamin E under the similar conditions. All samples showed concentration-dependent radical scavenging activity and the results are shown in Figure 2 (A and B).

In a separate experiment, the anti-oxidative activities of RES were compared with that of vitamin C or vitamin E by measuring their effective concentrations required for the 50% decrease of radicals (EC50) and the results are shown in Figure 3. EC50 for RES were 17.15 and 3.05 µM against DPPH and $ABTS^{+\bullet}$ radicals, respectively. While vitamin C and vitamin E showed 10.25 and 7.38 µM against DPPH radicals and 7.77 and 6.64 µM against $ABTS^{+\bullet}$ radicals, respectively. Comparing to vitamin C and vitamin E, RES showed stronger reaction towards $ABTS^{+\bullet}$ radicals rather than DPPH (Figure 3).

Figure 2

Figure 3

3.2. Liposomal characteristics

The vesicle sizes, polydispersity index (PI), zeta potential and entrapment efficiency of coated and non-coated liposomes are presented in Table 1. Considering the optimal vesicle size for topical vaginal administration (Vanic and Skalko-Basnet, 2013) and to assure the sufficient entrapment efficiency, the liposomal sizes with the mean diameter of 200 nm were targeted. The coating resulted in an increase in the mean liposomal size; the increase in the vesicle size corresponded to the increasing polymer concentration, as expected. All liposomal dispersions exhibited low PI, indicating a rather monodisperse size distribution. The low PI

confirmed that the extrusion could be a suitable method to obtain liposomes of desired vesicle size with limited polydispersity.

Table 1

The differences in zeta potential on liposomal surface between non-coated and chitosan-coated liposomes also confirmed that liposomes were indeed coated (Table 1). We also observed the differences between liposomes coated with 0.1 and 0.3% (w/v) chitosan, respectively. Moreover, an entrapment efficiency of over 70% of the starting amount of RES was obtained which is sufficient to assure the required RES concentrations for therapeutic effects (Table 1). Although the chitosan-coating was performed in the presence of unentrapped RES, no significant increase in RES incorporation was seen for the chitosan-coated liposomes. A phospholipid assay was applied to assure that the vesicle size reduction via extrusion and the chitosan-coating did not result in a loss of lipids. The loss of lipid was found to be less than 5% and accepted as satisfactory.

3.3. Mucoadhesive properties of liposomal formulation

The increased concentration of polymer is expected to lead to more efficient coating of liposomes (Li et al., 2009). A colorimetric assay with the anionic reactive dye (Cibacron Brilliant Red) was performed in the presence of free chitosan, thus the binding efficiency of chitosan to liposomal surface could not directly be measured. However, for the lower chitosan concentration (0.1%, w/v), 84% of chitosan was found to be surface available, whereas 54% of chitosan was detected on liposomes coated with higher polymer-concentration (0.3%, w/v). The results are in agreement with the findings in the literature (Li et al., 2009, Andersen et al., 2015).

We tested the *in vitro* mucin-binding potential for both coated and non-coated liposomes to confirm the mucoadhesive properties of chitosan-coated liposomal delivery system (Figure 4). Since the vaginal environment varies in pH, the experiments were performed at pH corresponding to healthy vaginal conditions (4.6) and vaginal bacterial infections conditions (7.4). The results indicate that PM-binding properties are significantly ($P < 0.001$) improved for chitosan-coated liposomes compared to non-coated liposomes which are in accordance with the literature (Jøraholmen et al., 2014; Naderkhani et al., 2014). The superior mucin-binding potential of liposomes coated with low chitosan concentration

(0.1%, w/v) was confirmed (Jøraholmen et al., 2014), compared to all other formulations. Importantly, the superiority was confirmed at both pH, suggesting that mucoadhesiveness will be assured regardless of the vaginal pH conditions. Non-coated liposomes are not expected to exhibit mucin-binding activity, however a noticeable binding efficiency was also observed for non-coated liposomes. One possible explanation for observed PM-binding to plain liposomes can be by physical interactions occurring during the ultracentrifugation and not an actual electrostatic interaction between liposomes and mucin. However, the binding was significantly lower than PM-binding of chitosan coated liposomes (0.1 and 0.3%, w/v).

Figure 4

3.4. In vitro release of RES from coated and non-coated liposomes

The Franz diffusion system was employed to assess the RES release from liposomal formulations comparing the release to free RES in propylene glycol solution serving as a control. Liposomes coated with the 0.6% of chitosan showed poor mucoadhesive properties (Figure 4), therefore were not included in the RES release study. An apparent sustained release from all liposomal formulations was determined (Figure 5) and the *in vitro* release studies confirmed prolonged release of RES from both the non-coated and chitosan-coated liposomes as compared to the control. Further, the release of RES was slower from the chitosan-coated liposomes as compared to the non-coated liposomes. This indicates that chitosan-coated liposomes have the ability to prolong the release of RES to a greater extent than non-coated liposomes.

Figure 5

3.5. Effect of RES and liposomal RES on SOD activity

The effects of RES and liposomal RES on SOD activity in LPS-induced J774A.1 cells were evaluated and results are shown in Figure 6. Comparing to the basal SOD activity of the controls, RES increased the SOD activity by 20%. Under a similar condition, liposomal formulation of RES increased the SOD activity by 26%. Liposomal formulations significantly increased (P = 0.009) SOD activity as compared to RES solution.

Figure 6

3.6. Effect of RES and liposomal RES on NO production

LPS treatment induces macrophages to increased amount of NO production by the conversion of L-arginine to L-citrulline. Thus produced unstable NO radical, is rapidly converted to NO_2^- or NO_3^-. Therefore the amount of NO_2^- measured quantitatively by the Griess reagent can be directly correlated to the NO production by the macrophages. We measured the NO_2^- produced by LPS and RES (1, 5 and 10 µg/ml) treated cells and expressed the activity as percentage of NO production and results are shown in figure 7. RES showed a concentration-dependent NO production inhibition. The activities of RES were also compared to that of vitamin C, vitamin E and L-NAME under similar conditions. Vitamin C and vitamin E showed only a weak inhibitory activity at higher concentrations (10 µg/ml).

Figure 7

L-NAME (*i*NOS inhibitor) showed, as expected, concentration-dependent activity at 1, 5, and 10 µg/ml. Under similar conditions, RES was found to be more potent than L-NAME (Figure 7). In another similar experiment, the inhibitory activity of RES and corresponding liposomal formulations against NO production was measured at 1, 2, 5, 10, 15, 20, 25, and 30 µg/ml RES concentrations. The results were expressed in 50% inhibitory concentration (IC50) as µg/ml (Figure 8). RES and corresponding liposomal formulation showed IC50 as 13.5 and 9.6 µg/ml, respectively. Liposomal RES was found to be more potent than the corresponding RES solution ($P = 0.003$), confirming the need for delivery system (Figure 8).

Figure 8

3.7. Effect of RES and liposomal RES on TNF-α and IL-1β production

We measured the TNF-α and IL-1β production by the LPS-induced macrophages in the presence of RES or liposomal RES. As compared to the control group, RES at a concentration of 10 µg/ml inhibited 52% of TNF-α production. Under the similar condition, liposomal RES inhibited 70% of TNF-α production which were significantly different ($P = 0.004$). We also measured the effects of RES and liposomal RES on the production of IL-1β. Compared to the control group, RES and liposomal RES at a concentration of 10 µg/ml inhibited 60% and 62% of IL-1β production, respectively (Figure 9).

Figure 9

4. Discussion

RES acts as a potent defensive anti-oxidant by inhibiting reactive oxygen species (ROS) mainly by activating AMPK (Pangeni et al., 2014). It exhibits stronger anti-radical activity than α –tocopherol, catechin, myricetin and naringenin. RES, vitamin C and vitamin E used in our experiments were supplied commercially (99.0% pure). Their structures are given in Figure 1. Vitamin C is a hydrophilic and vitamin E a lipophilic anti-oxidant with well-defined bioavailability. However, RES remains to be a biopharmaceutical challenge with regard to its solubility limitations (Das et al., 2008). Taken orally RES is relatively well absorbed, rapidly metabolized and generally well tolerated, although limited long-term toxicity studies have also been performed (Cottart et al., 2010). Extensive intestinal and hepatic metabolism is the rate limiting step for the systemic bioavailability resulting in a half-life of only 8-14 min (Das et al., 2008). With logP > 3.1 it is classified as a class-II compound in the Biopharmaceutical Classification System. *Trans*-RES is a pharmacologically active isomer. RES is a photosensitive compound and becomes converted into *cis*-RES after exposure to light for just 1 h in solution (Singh and Pai, 2014). This is an additional reason to incorporate RES in liposomal delivery system enabling the protection against light (Coimbra et al., 2011).

Our interests were in anti-microbial potential of RES, as increasing number of *in vitro* and *in vivo* studies suggest that RES exhibits anti-parasitic (*Trihomonas vaginalis*) (Mallo et al., 2013), anti-fungal (*Candida* species) (Houille et al., 2014), anti-viral (Docherty et al., 2005) and anti-bacterial (Nawrocki et al., 2013) activities. Prior to evaluating the anti-viral potential of liposomal RES, we have focused on the anti-oxidative and anti-inflammatory activities of RES and corresponding formulation. Inflammation is one of the first responses of the immune system to infection. The symptoms of inflammation include redness and swelling, which are caused by increased blood flow into the tissue. Inflammation is caused by eicosanoids and cytokines, which are released by injured or infected cells. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are involved in the process of pathological infection (Dinarello, 2000). Therefore, to express the anti-inflammatory activity, we focused on effect of RES on the production of nitric oxide, IL-1β, and TNF-α.

~~Genital herpes simplex virus (HSV) infection has increasingly burden the sexually active population in spite of the novel synthetic anti-viral agents such as acyclovir. Once infected, the virus remains in the sacral ganglia serving as a source of recurring infections in the afflicted individuals (Docherty et al., 2005). RES was found to inhibit or reduce the HSV replication in the vagina of mice (Docherty et al., 2005), however the dosage regimen used in mice experiments (5 times daily administration) is not applicable in human due to expected low compliance. Viral infection during pregnancy can lead to adverse effects on the developing fetus often resulting in preterm delivery, low birth weight, fetal anomalies or even fetal/infant death. The early treatment with anti-viral agents can reduce the complications and improve outcome, assuming that the anti-viral treatment is safe. Despite the increasing prevalence of use of anti-viral agents, relatively little research was performed focusing on their safety and risk-benefit profiles in pregnant patients (Avalos et al., 2014).~~

4.1. Liposomal RES

In the past ten years, an increasing number of publications dealing with RES in nanosystems have been reported (Amri et al., 2012). Moreover, an extensive list of patents on the use of RES in diverse formulations indicates increasing interest of academia and industry for its commercialization (Singh and Pai, 2014). The limitation of the solubility of RES can be overcome by incorporating RES into delivery system which acts as a solubilizer and improve the delivery of the drug candidate. Several novel delivery systems were proposed in recent years as means to improve its bioavailability, including polyethylene glycol-polylactic acid polymeric nanoparticles (Jung et al., 2014), solid lipid nanoparticles (Teskac and Kristl, 2010; Gokce et al., 2012), polymeric micelles (Lu et al., 2009), RES-loaded poly(*N*-vinylpyrrolidone)-*b*-poly(ε-caprolactone) nanoparticles (Lu et al., 2013). RES self-emulsifying system was shown to increase the uptake by endothelial cells and improve protection against oxidative stress-mediated death (Amri et al., 2014). Phospholipids offer means to improve the poor solubility and consequently low bioavailability of various active compounds (Fricker et al., 2010). In addition, liposomes improved the chemical instability of RES by preventing its inactivation through *cis-trans* isomerization (Coimbra et al., 2011; Scognamiglio et al., 2013). Kristl and co-workers confirmed that liposome-mediated uptake of RES improved the cell-stress response in comparison to free RES (Kristl et al., 2009). The same group (Caddeo et al., 2008) proved the enhanced efficacy of RES incorporated in liposomes on proliferation and UV-B protection of cells. Elastic liposomes incorporating RES and quercetin injected subcutaneously offered a new strategy for reducing the subcutaneous

fat (Cadena et al., 2013). In spite of improving delivery and solubility of RES, its wider therapeutic application is still limited because of the required dose needed to assure therapeutic outcome (Augustin et al., 2013).

Considering localized vaginal therapy, relatively little is known about the effect of vesicle size on the delivery of drugs intended for vaginal mucosal targeting. However, it was suggested that the nanocarriers in the size range of 200-500 nm are superior to both much smaller and also larger nanosystems (das Neves et al., 2011; das Neves et al., 2011). Additionally, it was reported that the number of liposomes penetrating the mucous layer increased when the size of the liposomes was reduced to approximately 100 nm for both non- and chitosan-coated liposomes (Takeuchi et al., 2001). There is usually a correlation between the particle size and drug entrapment efficiency; small vesicles are expected to offer greater surface area to interact with the mucus, but bearing less drug load, whereas larger vesicles enable increased drug loading, nevertheless, reduced mucoadhesion due to less surface contact (Vanic and Skalko-Basnet, 2013).

Liposomes are well-established delivery systems able to incorporate poorly soluble drugs and enable their aqueous medium-based vaginal administration (Pavelic et al., 1999; 2005). Liposomes as carriers for RES were also studied by several groups targeting various routes of administration. For example, transferosomes and ethanol-containing vesicles were used to deliver RES through porcine skin (Scognamiglio et al., 2013).

Chitosan is one of the mucoadhesive polymers recommended for the vaginal delivery (Valenta, 2005). Several other potential mucoadhesive polymers were reported as vaginal delivery systems, such as for example Carbopol (Pavelic et al., 2005), however the known anti-microbial activities were the main reason to focus on chitosan in the present work. Moreover, chitosan is a natural polymer and considered to be biocompatible, biodegradable and bioadhesive. The cationic properties of chitosan contribute to its mucoadhessiveness. When chitosan is used as a coating material for neutral PC liposomes, it is expected that hydrogen bonds be created between the phospholipid head groups and the cationic polysaccharide. In addition to required mucoadhesive properties, chitosan-coating of liposomes may present improved controlled drug delivery and stabilization of liposomes (Joraholmen et al., 2014). Moreover, chitosan also exhibits anti-microbial activity against vaginal pathogens (Kim et al., 2003) and its ability to disrupt vaginal bacterial biofilms makes

chitosan superior polymer in localized vaginal therapy (Kandimalla et al., 2013). Interactions between the cationic chitosan and the negatively charged mucosal surface are shown to render excellent mucoadhesive properties, making this polymer a well suited coating material for drug delivery systems intended for mucosal tissues (Meng et al., 2011). Our results are in agreement with the findings of Meng *et al*. who reported that chitosan-coated nanoparticles in sizes of about 200 and 300 nm exhibited significantly enhanced mucoadhesive properties on porcine tissue (Meng et al., 2011).

The only other report, to the best of our knowledge, on chitosan-coated liposomes containing RES is by Park *et al* who tested potential of this system to provide transdermal delivery of RES as delaying skin-aging substance (Park et al., 2014). They also observed that liposomes coated with lower concentration of polymer maintained their size integrity better than those coated with higher concentrations of polymer (Park et al., 2014).

During the process of chitosan-coating of liposomes, the amount of polymer interacting with the liposomes is expected to increase with an increase in the starting concentration of chitosan (Li et al., 2009). Our findings that lower chitosan concentrations result in more surface-available chitosan are in agreement with Guo *et al*. who reported that chitosan-coating of liposomes reached a saturation state when exceeded 0.1% concentration (Guo et al., 2003).

The effectiveness of drug delivery at the mucosal site is dependent on a series of interconnected actions including i) distribution and retention of nanosystems on the mucosal surface, ii) their penetration into/through the mucus mesh, and iii) release profile of the drug (Vanic and Skalko-Basnet, 2013). The RES release (Figure 5) indicates that chitosan-coated liposomes enabled sustained release. Chitosan-coated liposomes were superior to non-coated liposomes regarding the ability to sustained RES release, confirming our aim that chitosan-coating assures not only the mucoadhesivness but also the prolonged RES release. This is of great importance considering the vaginal administration, as reduced frequency in need for repeated administration correlates with better patient compliance.

4.2. Anti-oxidative activity

Invading pathogens create oxidative stress because of over production of ROS during increased metabolic activities and immune cell reaction. A number of cellular processes

including aerobic metabolism can easily supply one electron to cytoplasmic oxygen to generate $O_2^{\bullet-}$ which is also an essential step for the cellular communication and signalling (Afanas'ev, 2007). Normally when the concentration of $O_2^{\bullet-}$ increases the activity of superoxide dismutase (SOD) and catalase (CAT) increase to dismutate the toxic amount of $O_2^{\bullet-}$ to non-toxic molecules such as water and oxygen. However, as the production of $O_2^{\bullet-}$ and dismutation process are not balanced, there will be the over production of $O_2^{\bullet-}$ or H_2O_2 which can either easily be transformed into the $ONOO^-$ by reacting with $NO^{\bullet}$, the $OH^{\bullet}$ by Fenton reaction (catalysed by Fe^{+++}/Fe^{++}) or the $R^{\bullet}$ by increased peroxidase activity. The free radicals-ROS, especially $ONOO^-$, $OH^{\bullet}$, and $R^{\bullet}$ are very powerful oxidants as compared to $O_2^{\bullet-}$ and H_2O_2. The pathogen-induced ROS and/or free radicals generated *in vivo* together with the pro-inflammatory cytokines can cause significant damage to cells hasten the inflammation and infection. The anti-oxidants can attenuate the direct effect of radicals by deleting or scavenging which may inhibit the inflammatory processes.

RES was exhibited strong reaction to free radicals comparable with those of vitamin C and vitamin E. In addition, its effects on enhancing the SOD activity would make it a stronger candidate as an anti-oxidant. RES showed stronger radical scavenging activity for $ABTS^{+\bullet}$ radicals, comparing to DPPH free radicals (Figures 2 and 3). The superior activity of RES (EC50 of 3.05 µM) compared to the other anti-oxidants in case of $ABTS^{+\bullet}$ radicals, is in agreement with literature (Stojanovic et al., 2001). The reaction with $ABTS^{+\bullet}$ radicals involves an electron transfer process while H-atom transfer mechanism involves in DPPH radical reaction therefore, $ABTS^{+\bullet}$ radicals are more reactive with the RES type polyphenols (Gülçin, 2010). Vanaja and colleagues reported on the enhanced anti-oxidative activities of liposomal RES as compared to free RES based on the inhibition of 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH)-induced luminol enhanced chemiluminescence assay (Vanaja et al., 2013). Only one report found so far describes DPPH radical assay directly used for liposomal suspensions measuring with the UV-Vis spectrophotometer (Caddeo et al., 2013). It might be possible that the alcoholic solution of DPPH radical solubilizes the liposomal suspension by the destructing of liposomal vesicles to release free RES. We do not see any advantage of measuring direct radical scavenging activity for anti-oxidants-associated liposomal suspension unless used lipid is itself anti-oxidants. RES also showed pro-oxidant properties at higher concentration like other common anti-oxidants since it is a redox-chemistry and clinical application depends on the drug concentration (de la Lastra and Villegas, 2007).

RES is reported to exhibit anti-oxidative effect directly either by donating an electron to free radicals and/or providing hydrogen or indirectly by enhancing the SOD activity (Gülçin, 2010; Zheng et al., 2010). We also compared the *in vitro* SOD activities of RES and the corresponding liposomal formulation. RES and corresponding liposomal delivery system significantly enhanced the *in vitro* SOD activities comparing to controls. Moreover, liposomal RES, under similar conditions, was found superior to RES (Figure 6).

4.3. Anti-inflammatory activity

Vaginal inflammation and infection are very common and every woman experiences them at least once in her life. They can be caused by various pathogens such as virus, bacteria, fungi, or parasite (*Trichimonas*). Contamination of such pathogens initiates inflammation and their rapid growth lead to infection. Inflammation is the root cause of severe metabolic dysfunction including loss of cell integrity, enzyme function, genomic stability etc. (Hanahan and Weinberg, 2000). Nitric oxide is known not only as a free radical and vasodilator; it also plays a very important role in the pathways of inflammation and as an immunomodulator (Coleman, 2001). Some free radicals mainly such as $O_2^{\bullet -}$, $OH^{\bullet}$, and $NO^{\bullet}$ radicals along with non-free radical species such as H_2O_2 and HNO_2 are responsible for mediating the inflammation (Khan et al., 2008). In addition, cytokines such as TNF-α and IL-1β are playing important roles in chronic inflammation processes and persistent inflammatory tissue damage leading to each stage of infection. Moreover, pro-inflammatory molecules such as NF-κB and non-steroidal anti-inflammatory gene-1 dominating over the effect of SOD, CAT and glutathione peroxidase (GPX), together with a non-enzymatic system such as glutathione and vitamins (A, C, and E) constitute the defense to overreaction of free radicals (Finkel and Holbrook, 2000). RES showed anti-inflammatory activities by the inhibition of NF-κB activity via multiple mechanisms (Surh and Na, 2008). RES inhibited HSV replication by suppressing NF-κB activity (Faith et al., 2006). NF-κB is a host nuclear transcription factor, activated by multiple stimuli, including inflammatory cytokines, growth factors and bacterial or viral infections (Santoro et al., 2003). RES also showed anti-inflammatory activity by interfering both transcription (Subbaramaiah et al., 1998) and catalytic (Jang et al., 1997) activities of the COX2 enzyme. Therefore, the anti-inflammatory activities of RES can be suggested through ostensibly independent effects on NF-κB, cyclooxygenase and IL-1β (Baur and Sinclair, 2006).

In our experiments, RES was found to be more potent than vitamin C or vitamin E in scavenging $ABTS^{\bullet+}$ radicals (Figure 3). RES was also found to be a stronger inhibitor of NO production as compared to *i*NOS inhibitor L-NAME, which strengthens its potential as an anti-inflammatory agent (Figure 7). In addition, liposomal RES showed superior inhibitory properties on NO production (Figure 8). RES significantly inhibited the production of cytokines TNF-α and IL-1β in the LPS-activated macrophages. In all those set of experiments, liposomal RES was found to be more potent as compared with the corresponding RES especially for TNF-α (Figure 9). The increased inhibitory activity of liposomal RES might be the consequence of enhanced cellular uptake and/or maximized effect of RES. It requires further investigation at cellular and sub-cellular levels. If the finding reported here can be directly correlated to the normal epithelial cells in human, liposomal formulation of RES for topical anti-inflammatory treatment would be an optimal formulation.

Moreover, trichomoniasis is the most common non-viral STD in the worlds. Currently, metronidazole (MDZ) and other 5-nitroimidazoles (tinidazole, ornidazole, and secnidazole), the potent drugs against infections caused by anaerobic or microaerophilic microorganisms, are the only recommended drugs for standard treatment of *T. vaginalis* infection. However, resistance of *T. vaginalis* to MDZ, allergic reactions, and failure to remedy the infection with two consecutive courses of treatment have been reported (Cudmore et al., 2004; Das et al. 2005; Harp and Chowdhury, 2011; Muzny et al., 2012). Studies have shown that at least 5% of clinical cases of trichomoniasis are caused by parasites that are resistant to the above-mentioned drugs. Because of the lack of approved alternative treatments, the only option for patients with resistant infections is to use higher and sometimes toxic doses of MDZ, which leads to an increase in the occurrence of side effects (Cudmore et al., 2004). On the other hand, RES showed *in vitro* anti-parasitic effect against *T. vaginalis* by altering hydrogenosomal dysfunction (Mallo et al., 2013). Therefore, regarding to the potent anti-oxidative and anti-inflammatory activities and multi-targeted mechanisms, RES could be a potential therapeutic candidate especially for pregnant women against common inflammations and infections caused by diverse type pathogens. Although we have not tested the newly developed systems against *T. vaginalis* or particular pathogens, it is to be expected that the developed system can assure the necessary concentration of RES at vaginal site enabling sufficient interaction time and consequent anti-microbial action.

5. Conclusions

Our findings further confirmed RES as a strong anti-oxidant as well as anti-inflammatory compound. The liposomal formulation solubilized RES and enhanced its anti-oxidative and anti-inflammatory properties. With a combination of liposomal carrier as a solubilizer for RES and the mucoadhesive properties of chitosan in chitosan-coated liposomes, development of an optimal vaginal drug delivery system with specific, prolonged and controlled drug release properties might be possible to enable a controlled delivery as well as provide chemical stability for RES. Moreover, anti-microbial properties of chitosan will provide additional advantage against vaginal pathogens. However, further *in vivo* and clinical studies are needed to obtain the direct evidences.

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Conflict of Interest

Authors do not have any conflict of interests.

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Figure(s)

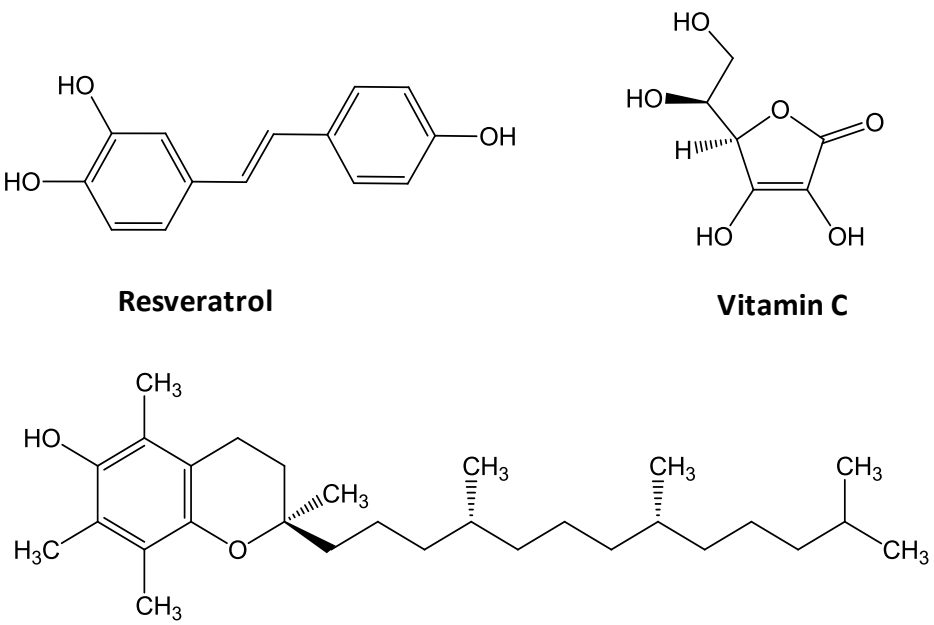

Fig. 1. Chemical structures of resveratrol (*trans*-3,5,4'-trihydroxystilbene), vitamin C (ascorbic acid) and vitamin E (α-tocopherol).

Figure(s)

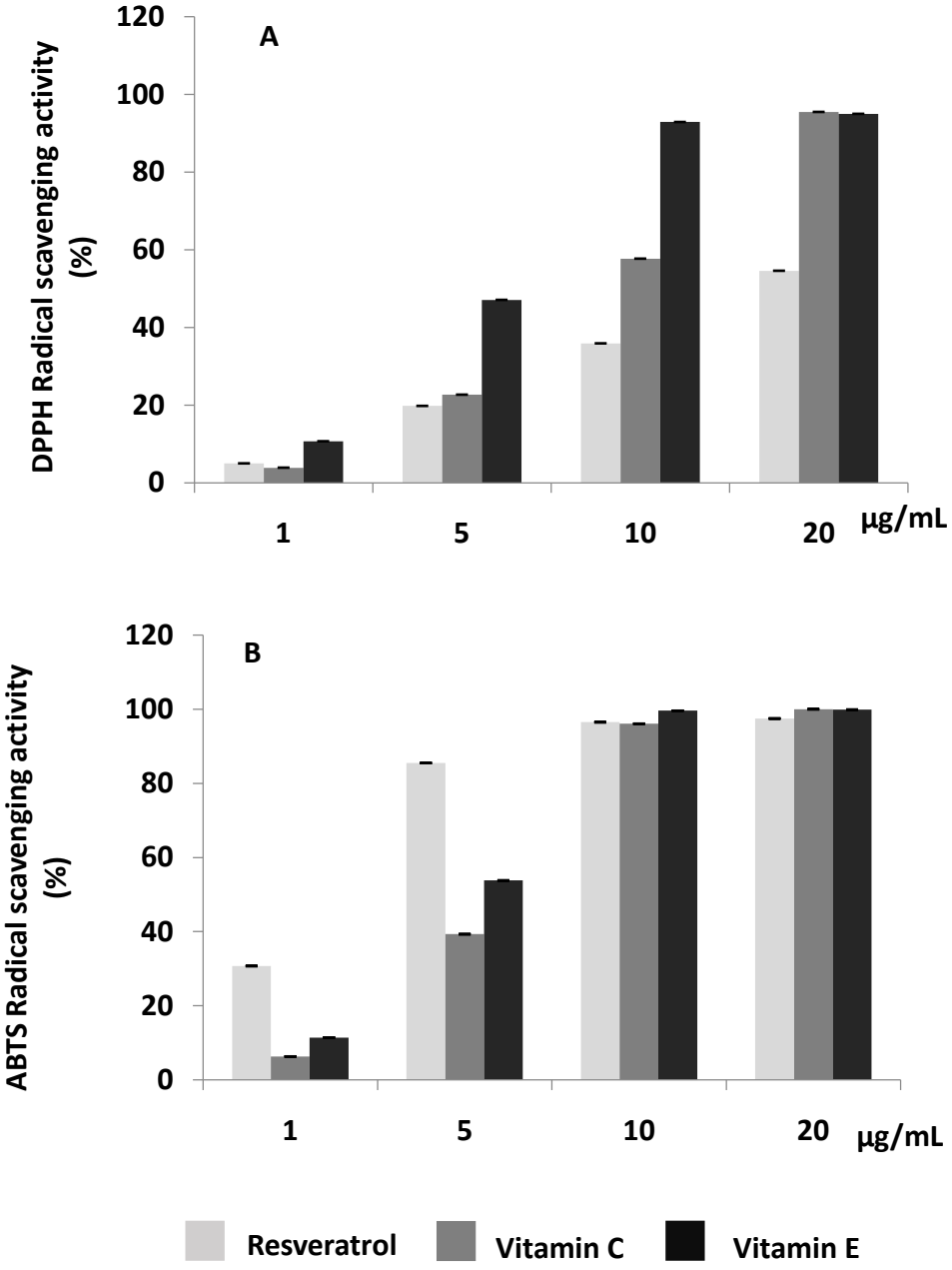

Fig. 2. Anti-oxidative activities of resveratrol, vitamin C and vitamin E.
A) DPPH radical scavenging activity B) $ABTS^{+\bullet}$ radical scavenging activity
Results are expressed as percentage mean ± SD (n = 3)

Figure(s)

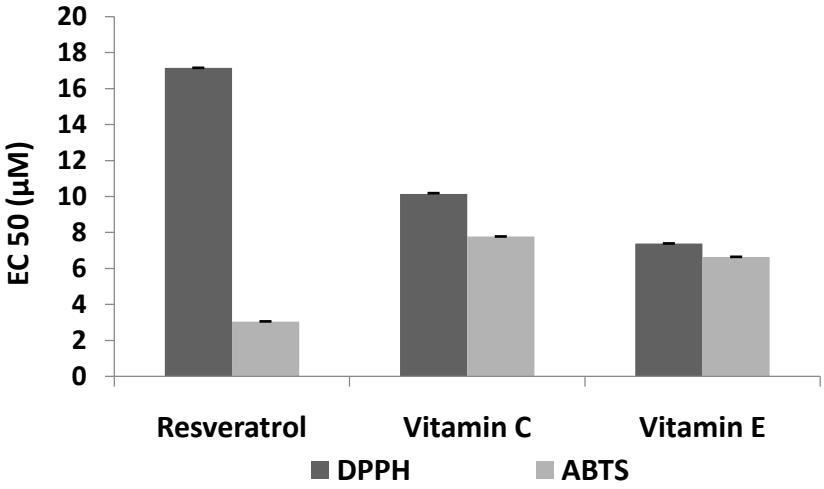

Fig. 3. Anti-oxidative activity of resveratrol, vitamin C and vitamin E expressed as EC_{50} in µM.
Results are expressed as mean ± SD (n = 3).

Figure(s)

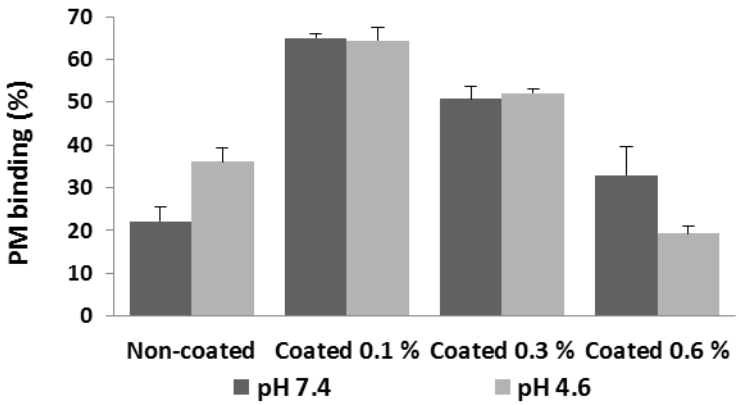

Fig. 4. Pig mucin (PM) binding (%) of non-coated and chitosan-coated liposomes.
The values represents the percentage mean ± SD (n = 3).

Figure(s)

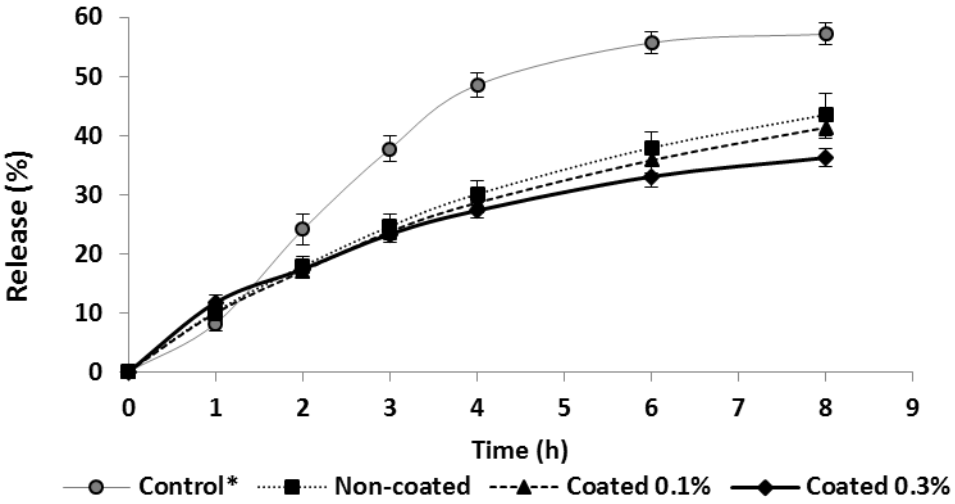

Fig. 5. *In vitro* resveratrol release from non-coated and chitosan-coated liposomes.
*As a control, resveratrol in the same concentration as in liposomes was dissolved in propylene glycol.
Results are expressed as percentage mean ± SD (n = 3).

Figure(s)

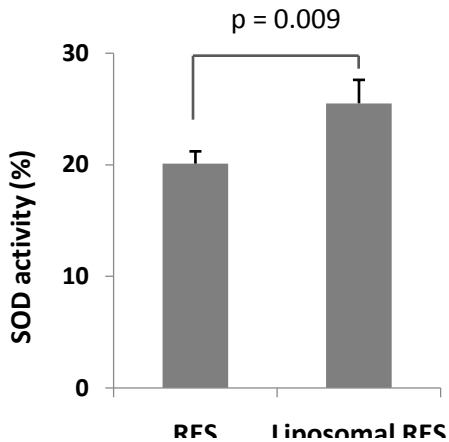

Fig. 6. The effects of resveratrol (RES) and liposomal RES on SOD activity in LPS-induced macrophages.

Stock solution of RES in DMSO was diluted with the cell culture medium euaivalent of RES present in liposomal RES and applied same volume containing 10 µg/mL as the final concentration to cells. Results are expressed as mean ± SD (n=3). Control for RES and liposomal RES were 0.2% DMSO and empty liposomes, respectively.

Figure(s)

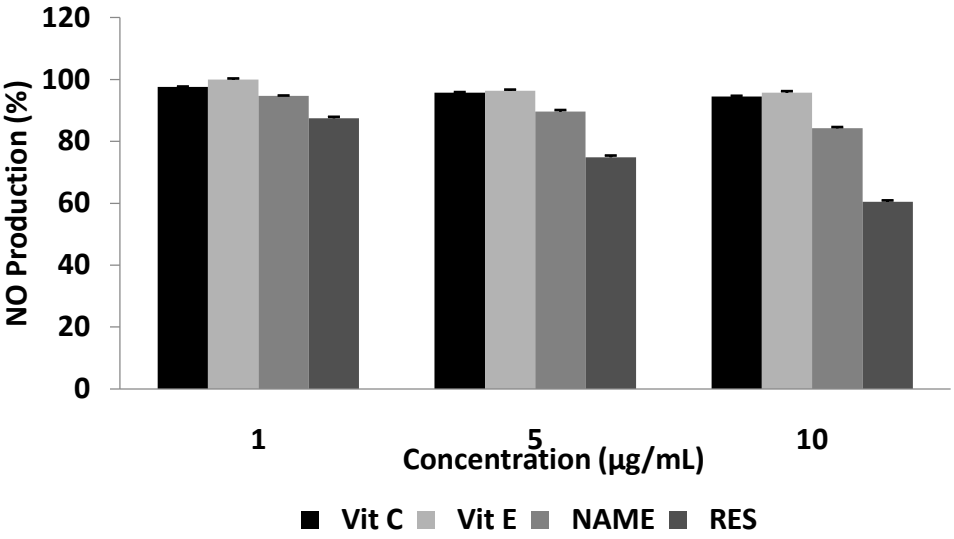

Fig. 7. The effects of resveratrol, vitamin C, vitamin E and NAME on NO production in LPS-induced macrophages.

RES: resveratrol; NAME: L-nitro-arginine methyl ester

Results are expressed as mean ± SD (n = 4).

Figure(s)

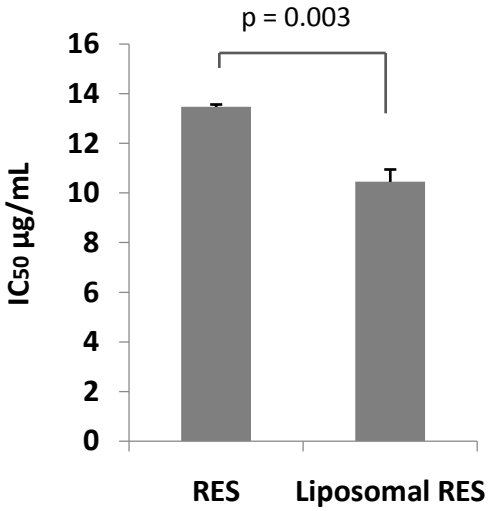

Fig. 8. The effect of resveratrol (RES) and liposomal RES on NO production in LPS-induced macrophages.

Results are mean ± SD (n = 4) showing the amount RES required to show 50% inhibition of NO production (IC50 µg/mL) in J774A.1 cells induced by LPS (1 µg/mL)

Figure(s)

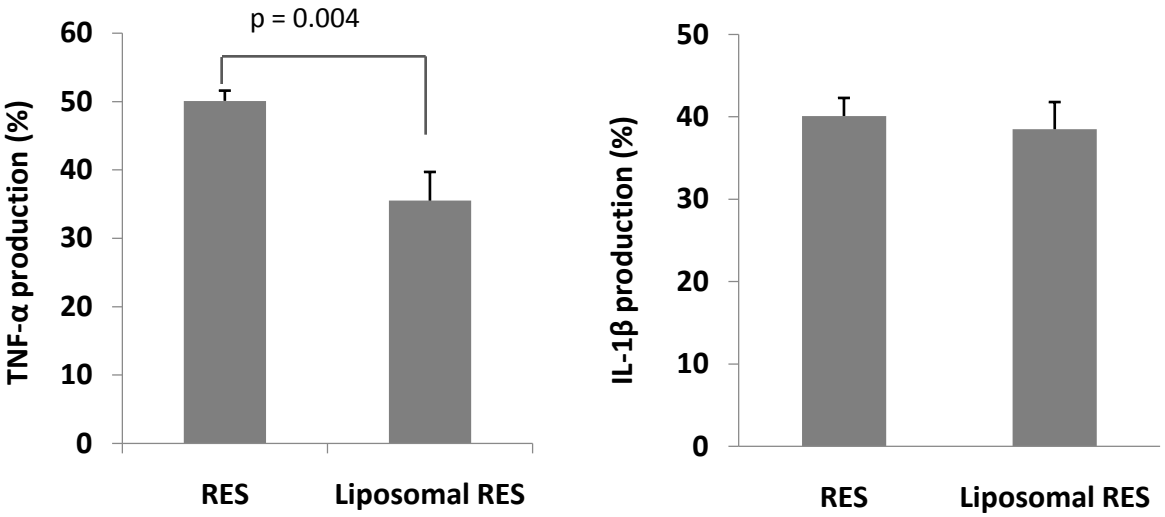

Fig. 9. The effects of resveratrol (RES) and liposomal RES on IL-1β and TNF-α production in LPS-induced macrophages.

Results are expressed as percentage mean ±SD (n = 3)

Table 1
Liposomal size, zeta potential and entrapment efficiency of non-coated and chitosan-coated liposomal resveratrol

	Resveratrol-loaded liposomes			
Chitosan (%)	**Vesicle size (nm)**	**PI***	**Zeta potential (mv)**	**Entrapment (%)**
-	206 ± 10	0.142	-3.17 ± 2.57	80 ± 4
0.1	212 ± 11	0.172	4.15 ± 0.59	77 ± 4
0.3	225 ± 10	0.122	14.77 ± 1.85	74 ± 6

Results are expressed as mean±S.D. (n = 3). Data for chitosan solution (0.1 and 0.3 %, w/v) are presented.
*PI: polydispersity index.