Anti-inflammatory effects of free and liposome-encapsulated natural Algerian thermal water in RAW 264.7 macrophages

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Abstract

The main objectives of this work were to formulate liposomes encapsulating highly mineralized thermal waters (TWs) and to study the potential anti-inflammatory effect of free and encapsulated thermal waters on RAW 264.7 macrophage cells stimulated with lipopolysaccharide (LPS). TWs-loaded conventional and deformable liposomes (TWs-Lip and TWs-DLip) were prepared by sonication and extrusion, respectively. They were characterized for their vesicle size, zeta potential, entrapment efficiency, physical stability and *in vitro* anti-inflammatory effect. Formulated suspensions have a low polydispersity and nanometric size range with zeta potential values closed to zero. The vesicle size was stable for 30 days. Entrapment efficiency of TWs in conventional and deformable liposomes was found to be higher than 90% and 70%, respectively. Pretreatment of LPS-stimulated murine macrophages with TWs resulted in a significant reduction in NO production and modulated TNF- α production suggesting an anti-inflammatory effect which were even more striking with TWs-Lip and TWs-DLip. Liposome formulations may offer a suitable approach for transdermal delivery of TWs indicated in inflammatory skin diseases.

Keywords: Algerian thermal waters, Liposomes, Anti-inflammatory effect, Nitric oxide, Cytokine.

1. Introduction

Skin forms the first barrier and line of immunological defense against pathogens and noxious substances. Extensive crosstalk between epithelial, stromal and immune cells regulates immune responses in the skin to ensure effective host defense and to maintain or restore tissue homeostasis. However, dysregulation of these equilibrium can cause chronic inflammatory skin diseases such as psoriasis, atopic dermatitis, and hidradenitis suppurativa (Pasparakis, Haase et al. 2014). These disorders result in significant morbidity and adversely affect patients quality of life (Merial-Kieny, Mengual et al. 2011). Immune cell populations in the skin are predominantly composed of dendritic cells (DCs) and macrophages (Malissen, Tamoutounour et al. 2014).

Resident skin macrophages are thought to survey the tissue and carry out the early detection of, and response to, antigen entering the body through the skin (Pasparakis, Haase et al. 2014).

The inflammatory response leads to the production of inflammatory mediators including nitric oxide (NO) and inflammatory cytokines (such as tumor necrosis factor- α (TNF- α)). NO is a reactive radical molecule synthesized by a family of enzymes called nitric oxide synthases (NOS) from L-arginine, O₂ and NADPH (Nathan and Xie 1994). In macrophages, the inducible NOS isoform (iNOS or NOS-2) produces a huge amount of NO as a host defense response to pathogens (Xie, Cho et al. 1992, MacMicking, Xie et al. 1997). TNF- α is an important mediator of inflammation, produced and secreted by a variety of cell types, principally by monocytes and macrophages (Liu, Sidiropoulos et al. 2000). But, overproduced, it contributes to the pathogenesis of a variety of skin conditions such as in psoriatic lesions (Dal Pra, Chiarini et al. 2007). Thus, equilibrium between a necessary defense response and too much response is not that easy to find.

Thermal waters (TWs) are natural mineral waters formed under specific geological conditions, and characterized by a rich composition with chemical and physical elements (Ghersetich and Lotti 1996). Over the past decades, the use of thermal waters is gaining popularity in dermatologic practice. They have been extensively used in several skin diseases, such as atopic dermatitis and skin dryness (Nasermoaddeli and Kagamimori 2005). The use of these waters for therapeutic purposes is based on their composition. To date, the mechanisms involved are only partially understood (Nasermoaddeli and Kagamimori 2005). *In vitro* experiments have demonstrated the effect of different thermal waters, in regard to their composition, on membrane fluidity, their antiradical and anti-inflammatory properties, their effects on many mediators involved in the immune response and their stimulating effect on keratinocyte differentiation (Merial-Kieny, Castex-Rizzi et al. 2011, Seite 2013, Tacheau, Weisgerber et al. 2018). Numerous studies have also used cell culture to determine the molecular mechanisms of TWs action at cutaneous level (Dal Pra, Chiarini et al. 2007, Joly, Branka et al. 2014, Karagülle, Karagülle et al. 2018). Furthermore, thermal waters were successfully included as raw materials for dermocosmetic formulations to achieve the cutaneous welfare (Almeida, Madeira et al. 2019, Nunes, Rodrigues et al. 2019, Tan, Spada et al. 2020).

Colloidal lipid-based vesicles, such as liposomes, have shown promising results as drug delivery systems through transdermal route. Liposomes are spherical vesicles, composed of lipid bilayer that encapsulates aqueous phase. This amphiphilic structure allows the incorporation of water-soluble materials into the aqueous core (Wang, Swing et al. 2020). Recent approaches have resulted in the design of deformable liposomes and ethosomes able to improve skin delivery of various drugs, consisted of phospholipids and an edge activator and ethanol, respectively (Elsayed, Abdallah et al. 2006). Further, liposomes were found to deliver efficiently the hydrophilic substances to the deeper skin tissues in higher concentrations than conventional dosage forms, while providing prolonged release (Verma, Verma et al. 2003, Betz, Aeppli et al. 2005). The encapsulation of TWs in liposomes seems to be an interesting alternative to improve

their skin permeation (Araujo, Paiva et al. 2015), as long as the percutaneous penetration of water increased when the stratum corneum is damaged and decreased when it is healthy (Blattner, Coman et al. 2014, Carbajo and Maraver 2018). To date, little scientific works have been published on this subject. Liposomes based on phospholipids and cholesterol, encapsulating an oligomineral TW and stabilized in a DNA gel were proposed, in a published patent in 1992, for a cosmetic and dermatologic applications (Fabre, Cousse et al. 1992). An *in-vitro* study has recorded that TW-encapsulating liposomes can penetrate through the skin barrier until they reached the spinous layer; these formulations have proved an effective moisturizing and anti-inflammatory effects on keratinocyte cells (Nunes and Tamura 2012). Moreover, stable deformable niosomes formulated with surfactants and cholesterol and charged with a TW have increased its cutaneous penetration and consequently increased the skin hydration and mineralization (Manosroi, Witkittilak et al. 2010).

In this work, we were led to encapsulate Algerian highly mineralized thermal waters in conventional and deformable liposomes, investigate their physicochemical characteristics and compare their anti-inflammatory effect to that of non-encapsulated TWs. The anti-inflammatory potential of TWs and liposomal formulations loaded with TWs was carried out in an *in vitro* model, the RAW 264.7 murine macrophage cell line activated by LPS, a molecule that mimics activation by bacteria.

Abbreviations:

AD, atopic dermatitis;
BTW, Baraka thermal water;
CH, cholesterol;
DLip, deformable liposomes;
DLS, dynamic light scattering;
DMSO, dimethyl sulfoxide;
Lip, liposomes;
LPS, lipopolysaccharide;
MLV, multilamellar vesicles;
PBS, phosphate buffer saline;
PC, phosphatidylcholine;
PG, phosphatidyl glycerol;
PdI, polydispersity index;
RAW 264.7, Ralph And William's cell line 264.7;
ROS, Reactive oxygen species;
STW, Salhine thermal water;
SDC, sodium deoxycholate;
T_m, transition temperature;
TNF, tumor necrosis factor;
TWs, thermal waters

2. Materials and methods

2.1. Reagents

2.1.1. Chemical reagents

Soybean L- α -phosphatidylcholine (PC, 99% purity) was purchased from Sigma GmbH Chemical Company (USA). Cholesterol (CH) was provided from Sigma-Aldrich (St. Quentin Fallavier, France) and recrystallized in methanol in the laboratory. Egg yolk L- α -phosphatidyl-DL-glycerol (PG, 99% purity) was purchased from Avanti Polar Lipids, Inc. (Alabama, USA). Stock solutions of PC (25 mM), PG (25 mM) and CH (25mM) were prepared in chloroform/methanol (9/1 (v/v)) and conserved under argon atmosphere at -20°C . Sodium deoxycholate (SDC, 97 % purity) was purchased from Sigma-Aldrich. Spectra/Pore® dialysis membrane (12,000–14,000 molecular weight cut off) was purchased from Spectrum Laboratories, Inc. (USA). Griess reagents were purchased from Sigma (St. Louis, MO, USA). All solvents used were of analytical or HPLC grade or equivalent. All other reagents were commercially available and used without further purifications. Purified 18 M Ω ultrapure-water (Elga, Veolia, UK) was used to prepare reagent solutions.

2.1.2. Biological reagents

Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin, phosphate buffered saline (PBS, pH 7.4), and other supplements for cell culture were purchased from Sigma Aldrich (Saint Louis, MO). Lipopolysaccharide (LPS) extracted from *Escherichia coli* (K12) was purchased from Invivogen (San Diego, CA). Purified anti-mouse TNF- α antibody clone 1F3F3D4 and biotinylated anti-mouse TNF- α antibody clone XT3/XT22 for ELISA testing were purchased from eBioscience/ThermoFischer Scientific (Waltham, MA). Horseradish Peroxidase Avidin (Avidin HRP) was from Jackson (West Grove, PA).

2.2. Thermal waters

TWs were taken from two thermal springs located in the region of Biskra, southeast of Algeria: Baraka (BTW) and Salhine (STW). BTW and STW samples were collected directly from the springs, transported refrigerated to the laboratory and then filtered with a Millipore 0.22 μm vacuum-driven disposable filtration Stericup (Millipore, Billerica, MA) to remove any microorganisms and sand. Later, they were stocked in glass bottles at 4°C until uses. Prior to the analysis of minor elements (Fe, Zn, Mn, Cu, Se), water samples were acidified with 0.5% (v/v) concentrated HNO_3 and measured by inductively coupled plasma – atomic emission spectrometry (ICP-AES). The other physico-chemical parameters were completed by detailed information published in previous works published by Chenaker et al. (Chenaker, Houha et al. 2018). Thermal water osmolality was measured in triplicate using a freezing point osmometer (Advanced Instruments Model 3D3, Norwood, MA). Physiochemical composition and osmolality of BTW and STW are summarized in Table 1.

Table 1

Chemical analysis of the used thermal waters

		STW	BTW
Temperature (°C)		45	42
pH		7.2	6.9
Conductivity (µS/cm) at 25 °C		14780	4410
Calcium	Ca²⁺	339.46	434.23
Magnesium	Mg²⁺	45.56	79.54
Sodium	Na⁺	2936.65	461.76
Potassium	K⁺	79.58	43.75
Chloride	Cl⁻	4512.43	538.67
Sulfate	SO₄²⁻	1485.09	1742.92
Bicarbonates	HCO₃⁻	386.74	134.20
Fluoride	F⁻	1.75	0.66
Bromide	Br⁻	5.69	1.78
Nitrate	NO₃⁻	ND	0.55
Silicon dioxide	SiO₂	34.50	31.90
Iron	Fe²⁺	3.36 ± 0.06	4.56 ± 0.06
Zinc	Zn²⁺	0.007 ± 0.001	0.077 ± 0.001
Manganese	Mn²⁺	0.047 ± 0.7	0.016 ± 0.08
Copper	Cu²⁺	<1	<1
Selenium	Se²⁻	0.05 ± 0.01	0.03 ± 0.01
Osmolality [mOsm/kg]		258.66 ± 6.33	57.33 ± 0.66

Values are expressed in mg/L except SiO₂ in mg/kg. ND: not detected.

2.3. Preparation of TW liposomal formulations

2.3.1. Conventional liposomes preparation

Conventional liposomes loaded with thermal waters (TW-Lip) were prepared by the combined method of thin lipid film hydration with sonication. Liposomes were formulated with PC:PG:CH in 80:20:50 molar ratio, respectively (Ciobanu, Heurtault et al. 2007). Briefly, the desired amounts from each lipid stock solution were introduced into a Pyrex tube and organic solvents were removed on rotary evaporator (Büchi, Switzerland) under reduced pressure, room temperature and moderate speed for 1h. The obtained dried thin lipid film was suspended in thermal water, BTW and STW separately, at a final lipid concentration of 15 mM and agitated on a Vortex mixer for 10 min. The obtained suspension of multilamellar liposomes (MLV) was further sonicated to obtain small unilamellar vesicles (SUV). A Vibra Cell 75041 ultrasonicator (750 W, 20 kHz, Fisher Bioblock Scientific, Illkirch, France) equipped with a 3 mm-diameter tip probe set at 40 % amplitude (sonication strength) was used with a sequence of 1 s of sonication and 2 s rest for 50 min under a continuous flow of argon. The suspension was kept cooled in a water bath to avoid heat generated during sonication. The SUV liposomes were then centrifuged at 13000g to remove titane residues. The resulting samples (BW-Lip and SW-Lip) were stored at 4 °C until use. Control liposomes were formulated with the same method and the same lipid

composition hydrated in 10 mM Hepes buffer (pH 7.4) containing 5 % (w/v) sorbitol. The composition and molar ratio of conventional liposome vesicles are shown in Table 2.

2.3.2. Deformable liposomes preparation

TW loaded deformable liposomes (TW-DLip) were prepared by extrusion through polycarbonate filters as described before by Kakhi and al (Kakhi, Frisch et al. 2016) with slight modifications. Briefly, 15 mM of PC stock solution was evaporated under vacuum in a Pyrex tube for 1 h. The dry lipid film was then dispersed in ethanol (8.5 % ethanol volume/final volume) by mechanical shaking for 2 min. Thermal water containing 5.4 mM SDC was then added to the suspension in order to obtain a final concentration of 2.7 mM of the surfactant. The suspension was agitated for 10 min to hydrate the lipid film, which resulted in the formation of MLVs. These MLVs were manually extruded through a 100 nm pore size polycarbonate membrane, at room temperature (above 25 °C, T_m of PC), for 20 cycles to produce SUVs using an extrusion device (LiposoFast-Basic, Avestin, Canada). The resulting suspensions were then stored at 4 °C until use.

Deformable liposome controls were formulated with the same method and the same membrane composition hydrated in 10mM Hepes buffer (pH 7.4) containing 5 % (w/v) sorbitol. The composition and molar ratio of deformable liposomes is shown in Table 2.

Table 2

Denomination and composition of liposome formulations.

Denomination	Molar ratio				Hydration vehicle
	PC	Lipids PG	CH	Edge activator SDC	
SW-Lip	80	20	50	–	STW
SW-DLip	83	–	–	27	STW; 7 % v/v ethanol
BW-Lip	80	20	50	–	BTW
BW-DLip	83	–	–	27	BTW; 7 % v/v ethanol
Hepes-Lip	80	20	50	–	Hepes
Hepes-DLip	83	–	–	27	Hepes; 7 % v/v ethanol

PC: phosphatidylcholine, PG: phosphatidylglycerol, CH: cholesterol, SDC: sodium deoxycholate, , STW: Salhine thermal water, BTW: Baraka thermal water, Lip: liposomes, DLip: deformable liposomes.

2.4. Physicochemical characterization of TWs loaded liposome formulations

2.4.1. Particle size

The hydrodynamic diameter and the polydispersity index (PdI) of the different liposome formulations were measured in triplicate at 25 °C by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern instruments, Orsay, France) with a scattering angle of 173°. Samples were diluted in the same encapsulated TW at a 1/100 volume ratio and were placed in

disposable plastic micro-cuvettes (Brand GmbH, Germany). The represented data are the average of at least 3 measurements done $\pm$ standard deviation (standard error) (SD).

2.4.2. Zeta Potential

The zeta potential of TWs-loaded liposome formulations was measured at 25°C using Zetasizer Nano-ZS (Malvern instruments, Orsay, France). Samples were diluted at 1/20 volume ratio in purified 18 M Ω ultrapure-water. Measurements were performed in triplicate to determine the zeta potential average values $\pm$ SD.

2.4.3. Physical stability

Formulated suspensions of BTW- and STW-loaded conventional and deformable liposomes were stored at 4 °C during 30 days. Their macroscopic behavior was visually inspected. The particle size and PDI values were checked at 10th, 20th and 30th day of storage. Mean values were used for the analysis of the data.

2.5. Encapsulation efficiency

The efficiency of encapsulation of TWs into liposome formulations (*EE%*) was assessed by determining the concentration of free minerals contained in the non-encapsulated external TWs by dialysis. 1 mL of each liposome formulation (15 mM) was dialyzed using a cellulose membrane with an Mw cutoff of 12–14 000 kD (Spectra/Pore®, Spectrum Laboratories Inc. USA) against 250 mL deionized water for 12 h, under stirring at 4 °C. Chloride was chosen as a trace element to determine the amount of non-encapsulated minerals (Ribeiro, Araújo et al. 2015). Its concentration was determined by Mohr method (Shukla and Arya 2018). Particles size compared before and after dialysis proved their stability. The following equation was used to calculate the Cl⁻ encapsulation efficiency that represents also the efficiency encapsulation of TWs into formulated liposome suspensions:

$$EE\% = \frac{\text{Concentration of entrapped Cl}^{-}}{\text{Concentration of Cl}^{-} \text{ in TW}} \times 100 \quad (1)$$

2.6. Cell culture

RAW 264.7 mouse macrophage cells were obtained from ATCC® TIB-71TM. Cells were cultured in DMEM supplemented with stabilized glutamine, 5 % (v/v) FBS containing 1000 U/mL of penicillin and 100 μ g/mL streptomycin at 37 °C under a 5 % CO₂ 95 % humidified air atmosphere. Cells were harvested at around 90 % confluence using trypsin (0.5 g/L)-EDTA (0.2 g/L) for 5 min at 37 °C and re-suspended in culture medium to the desired dilution. For all experiments, cells were seeded in a 96-well plate at 5×10^4 cells/well.

2.7. In-vitro inflammatory investigation of TWs and TWs-liposome formulations

For the pro-inflammatory experiments, the cells were diluted and dispensed to 96-well plated, 50 μ L/well (5×10^4 cells/well). The cells were allowed to adhere for 24 h at 37 °C with 5 % CO₂ and

the non-adherent cells were washed away. Fresh medium was added to the cells, and the cells were treated with liposome encapsulated (final concentration 3.125 to 100 μ M for conventional and deformable liposomes) or free BTW and STW (final volume 1 to 50 μ L/well). The effects of lipopolysaccharide (LPS, 50 ng/mL) and DMSO (5 and 10 %) were also tested as controls. After an overnight incubation at 37 °C, cell supernatants were used to measure pro-inflammatory compound (nitric oxide (NO)) and inflammation-related cytokine (TNF- α). The cell viability was evaluated with microscopy visualisation.

To evaluate the anti-inflammatory activity after the same first steps until washing the cells, DMEM containing two showed volume of BTW and STW (10 and 50 μ L) and serial concentrations of BTW and STW loaded conventional and deformable liposomes (10, 50 and 100 μ M respectively) was added (final volume 100 μ L/well). After an incubation of 1 , 2 and 4 h at 37 °C, inflammatory response was stimulated by adding 50 ng/mL LPS for 24 h at 37 °C. The negative and positive controls were the cells incubated in culture media non induced and induced with LPS, respectively. The cell supernatants were collected to determine nitrite oxide and TNF- α production.

2.8. Determination of nitrite concentration

The concentration of nitrite (NO_2^-), a stabilized form of nitric oxide (NO), was measured using Griess method (Hardy, Seguin et al. 2018). Briefly, 40 μ L of culture supernatants from each well were mixed with 60 μ L of Griess reagent (equal volumes v/v of 58.1 mM P-aminobenzene-sulfonamide in 30% acetic acid and 3.9 mM N-(1-naphtyl) ethylenediamine dihydrochloride in 60 % acetic acid) in a separate 96-well plate. The absorbance of the colored product was measured at $\lambda=543$ nm, using a microliter plate photometer (Safas SP2000, Xenius 5801, Monaco). The method was standardized with a sodium nitrite curve ranging from 3.125 to 100 μ M.

2.9. Estimation of TNF- α production

TNF- α concentration in culture supernatants was evaluated by ELISA using commercially available reagents and following the manufacturer instructions. Purified anti-mouse TNF α antibody clone 1F3F3D4 and biotinylated anti-mouse TNF α antibody clone XT3/XT22 were purchased from eBiosciences/thermoFischer Scientific (Waltham, MA, USA). Avidin HRP was from Jackson (West Grove, PA, USA). Briefly, capture antibody was diluted to 1 μ g/mL in a 0.05 M pH 9.6 carbonate/bicarbonate buffer and coated 1 night at 4°C before blocking with PBS 0.05% Tween 20 1% BSA (1h,37°C). Samples were then diluted with PBS (1:20 and 1:5 for treated and untreated cells with LPS, respectively) and incubated with capture antibody (2h, 37°C) before addition of the biotinylated detection antibody diluted to 0.5 μ g/mL in PBS 0.05% Tween 20 1% BSA (1 h, 37°C). Avidin HRP was then added (45 min, 37°C) and revelation was conducted by addition of 1.25 mM tetramethylbenzidine and 13.05 mM H_2O_2 in 0.1 M pH 5 citrate buffer. Revelation was finally stopped by addition of 1 M HCl and absorbance was

measured at 450 nm and compared to a calibration curve constructed from standard TNF- α solutions.

3. Results and discussion

3.1.Characterization of thermal waters

Both BTW and STW can be classified into neutral highly mineralized thermal waters, according to their neutral pH, relatively high conductivities and hot temperatures (Table 1) (Ghersetich and Lotti 1996, Nasermoaddeli and Kagamimori 2005). BTW belongs to the sodium-sulfate type ($[Na^+] = 461.79 \text{ mg/L}$, $[SO_4^{2-}] = 1742.92 \text{ mg/L}$) while STW belongs to the sodium-chloride ($[Cl^-] = 4190 \text{ mg/L}$, $[Na^+] = 2943 \text{ mg/L}$) and calcium-sulfate types ($[SO_4^{2-}] = 1220 \text{ mg/L}$, $[Ca^{2+}] = 280 \text{ mg/L}$). Moreover, both BTW and STW are highly enriched in magnesium, potassium and bicarbonate ions. They show an important mineralization in minor trace elements particularly represented by strontium, iron, selenium and manganese. Related to their osmolality values (Table 1), BTW and STW can be classified as hypotonic and isotonic water, respectively. These mineral compositions reflect the possible physiological mechanism of BTW and STW in contact with skin. Several reports have previously demonstrated the relation between rich content of TWs in major and minor minerals and their physiological activities as immune modulatory, antioxidant and anti-inflammatory activities (Celerier, Litoux et al. 1995, Joly, Galoppin et al. 2000, Verdy, Branka et al. 2012). In view of these results, BTW and STW can be considered as potential products for the treatment of skin pathologies, within the framework of dermo-cosmetics.

3.2.Physicochemical characteristics of TW liposome suspensions

Conventional liposomes composed of PC:PG:CH (80:20:50 molar ratio) and encapsulating BTW and STW were produced by lipid film-hydration method, followed by sonication. Both BW-Lip and SW-Lip showed a narrow particle size distribution with a mean diameter around $60 \text{ nm} \pm 12 \text{ nm}$ and a $PdI \leq 0.2$ (Table 3). It was previously reported that the presence of CH in liposomes composition achieve to small molecular geometry and hydrophobicity desirable to form closed small bilayer vesicles (Manosroi, Wongtrakul et al. 2003). In addition, sonication reduced the size of liposomes to the nanorange and allowed to the formation of uniform size (Yamaguchi, Nomura et al. 2009).

Deformable liposomes encapsulating BTW and STW were formulated with PC:SDC (73:27 molar ratio) in presence of ethanol by lipid film-hydration method followed by extrusion. Deformable liposomes (BW-DLip and SW-DLip) showed a mean diameter size of $134 \pm 15 \text{ nm}$ (Table 3), closed to the pore size of the membranes used for the extrusion. The size of deformable liposomes was greater than conventional liposomes. Deformable liposome suspensions were mono dispersed with a $PdI \leq 0.07$. It is well known that extrusion method produces unilamellar liposome suspensions of spectacularly homogeneous size in the nano-range of 30-200 nm (Alves, Cusick et al. 2013).

Zeta potential is an important indicator of liposomes surface charge that allows predicting about physical stability. Conventional liposomes showed anionic charges of -30.23 ± 8.61 mV and -23.00 ± 7.12 mV for SW-Lip and BW-Lip (Table 3), respectively, related to the negative charge of PG present in the formulations. Deformable liposomes exhibited a negative zeta potential ranged between -8.28 ± 8 mV and -11.87 ± 7 mV (Table 2), related to the presence of an anionic surfactants (SDC). Compared to the zeta potential of conventional and deformable liposomes prepared in Hepes medium (around -80 mV and -30 mV, respectively, data not shown) , liposomes formulated in TWs exhibit a less negatively zeta potential, which can be due to the interaction of metal ions present in the TWs with charged phospholipids via Coulombic interactions (Le, Houri et al. 2019). According to literature, the net negative surface charges of membranes of anionic lipids increases cations concentration near the lipid–water interface according to the Gouy–Chapman theory of the electrical double layer (McLAUGHLIN, Mulrine et al. 1981). The counterions do not penetrate the headgroup zone but form a layer over the membrane instead (Le, Houri et al. 2019). As a result, the electric double layer will undergo such a high compression that the zeta potential tends towards zero. The electrostatic shielding of liposome charged surfaces can suppress the electrostatic repulsion and the electrophoretic mobility of particles. In this case, flocculation of formulated liposomes is suspected.

Table 3

Physicochemical characteristics (vesicle diameter, PdI, zeta potential) and encapsulation efficiency of liposome formulations (n = 3).

Formulation	Vesicle diameter (nm) $\pm$ SD	PdI $\pm$ SD	Zeta potential (mV) $\pm$ SD	EE % $\pm$ SD
SW-Lip	66.00 ± 8.00	0.150 ± 0.01	-30.23 ± 8.61	96.51 ± 1.14
SW-DLip	134.10 ± 15.00	0.070 ± 0.04	-11.87 ± 7.03	85.20 ± 1.10
BW-Lip	60.00 ± 9.00	0.181 ± 0.01	-23.00 ± 7.12	95.02 ± 1.89
BW-DLip	134.00 ± 13.00	0.027 ± 0.01	-8.28 ± 8.03	72.52 ± 2.22

3.3. Physical stability

Liposome dispersions are mainly stabilized against sedimentation by electrostatic repulsion (Heurtault, Saulnier et al. 2003). In our case, the strong electrolyte charge in BTW and STW considerably reduces the electrostatic repulsion between the liposomes, which negatively influences their stability by flocculation mechanism (Heurtault, Saulnier et al. 2003). However, the absence of electrostatic repulsion between liposomes will not necessarily result in aggregate formation or fusion of the colloidal particles, regardless the hydration energies (Crommelin 1984). Visual inspection of the prepared liposome dispersions stored at 4° during 30 days did not reveal any change in the appearance of conventional liposome dispersions. However, precipitation appeared in deformable liposome suspensions after 48h of storage. In the case of

conventional liposome suspensions, flocculation probably generated the formation of a three-dimensional network holding the entire volume of the system, which might be responsible of the suspensions stability against sedimentation. In the case of deformable liposomes, the formation of the three-dimensional network is unlikely which may be attributed to the deformability of the liposome membrane.

Table 4 shows that the average vesicles size mainly of SW-Lip slightly increased during the first 10 days of storage and almost stabilized over the subsequent days, which can be induced by the peroxidation of lipids (Heurtault, Saulnier et al. 2003). Other author have observed the same size increase of liposomes prepared with deionized water (Wang, Swing et al. 2020). Stability of liposomes size indicates the stability of encapsulated TWs volume. Manosroi et al. (Manosroi, Wongtrakul et al. 2003) reported that CH is an important constituent of liposomes for the stabilization of the vesicular bilayer membrane and to achieve suitable hydrophobicity. Basically, the stability of ionic strength inside and outside the liposomes maintains the stability of liposomes with high retention rates (Wang, Swing et al. 2020). The PdI values of all formulations were < 0.3 indicating monodispersity with a narrow size distribution and perfect suspension homogeneity.

Table 4

Evolution of the average particle diameter and the PdI of TWs-loaded conventional and deformable liposomes stored at 4 °C as a function of time. Data are mean value $\pm$ standard deviation (n = 3).

Formulation	Particle diameter (nm)				PdI			
	Day 1	Day 10	Day20	Day30	Day1	Day10	Day20	Day30
BW-Lip	59 $\pm$ 8	64 $\pm$ 8	67 $\pm$ 7	65 $\pm$ 7	0.181	0.216	0.182	0.183
SW-Lip	66 $\pm$ 8	83 $\pm$ 9	82 $\pm$ 9	81 $\pm$ 8	0.150	0.158	0.178	0.170
BW-DLip	136 $\pm$ 8	140 $\pm$ 3	140 $\pm$ 2	145 $\pm$ 11	0.074	0.088	0.090	0.104
SW-DLip	136 $\pm$ 3	140 $\pm$ 2	141 $\pm$ 1	142 $\pm$ 8	0.044	0.023	0.071	0.084

3.4. Encapsulation efficiency

BTW and STW encapsulation rates exceeded 90% in conventional liposomes while ranged between 73 and 85 % in the case of deformable liposomes (Table 3). The observed results presented comparatively higher EE% in conventional than deformable liposomes. It may be justified on the basis of the presence of CH in the composition of conventional liposomes, which yielded higher EE% of hydrophilic substances by decreasing membrane permeability (Kulkarni, Betageri et al. 1995). Moreover, the lower EE% of deformable liposomes might be related to membrane fluidity and permeability induced by the intercalation of ethanol into the PC polar head group environment (Fan, Li et al. 2013). The formulation method and the vesicles size might also have influenced the encapsulated volume of TWs.

For further *in-vitro* studies, the encapsulated volume of BTW and STW in liposome dispersions diluted at 10, 50 and 100 μ M was calculated using the EE % values (Table 5).

Table 5

BTW and STW volumes encapsulated in 1.5 mL of 10, 50, 100 and 15 000 μ M of liposomes (concentrations expressed in phospholipids)

Liposomes	EE %	Estimated TWs volume encapsulated in different concentrations of liposomes (μ L)			
		15 mM	100 μ M	50 μ M	10 μ M
SW-Lip	95.90	1438.50	9.59	4.80	0.96
SW-DLip	85.20	1169.37	7.80	3.90	0.78
BW-Lip	93.66	1404.90	9.37	4.68	0.94
BW-DLip	73.90	1014.28	6.76	3.38	0.68

3.5.Effect of TWs and TWs-loaded liposome formulations on NO and TNF- α production by RAW 264.7 macrophages

To evaluate the pro-inflammatory effect of BTW and STW, free and loaded liposome formulations, a model analyzing the activation of RAW 264.7 cells was used. Figure 1 (A, B and C) shows the NO production in RAW 264.7 cells incubated with increased volumes of free BTW and STW, and increased concentrations of BTW and STW loaded conventional and deformable liposomes. We can observe that whatever the added volume of BTW and STW and concentration of liposomes charged with BTW and STW, the cells produced the same level of NO as the untreated cells ($1.03 \pm 0.89 \mu$ M). In contrast, the cells treated with LPS, used as reference of inflammation, produced a high concentration of NO ($17.04 \pm 2.66 \mu$ M). These results indicated that both free and encapsulated BTW and STW did not induced the NO production.

The effect of free and encapsulated BTW and STW on the TNF- α secretion at the used volumes and concentrations is shown in Figure 1 (D, E and F). Low concentrations of TNF- α were produced by cells treated with the used volumes and concentration of free and encapsulated BTW and STW, respectively. These concentrations were approximately the same as the TNF- α concentration produced by untreated cells (84.74 ± 23.69 pg/mL). Whereas in a case of cell inflammation by LPS, the concentration of TNF- α produced is 86 times greater (7242.63 ± 641.00 pg/mL). These results clearly demonstrate that the used volumes and concentrations of free and encapsulated BTW and STW did not induced the TNF- α secretion in RAW 264.7 cells.

The viability of the macrophages after treatment with free and liposomal BTW and STW was visualized under microscopy and compared with the viability of untreated cells and cells treated with DMSO (5 % and 10 %). The microscopic visualization showed no difference in morphology between untreated and RAW cells cultured with different concentrations of TWs and TW-Liposome formulations (data not shown).

These results suggest that free and encapsulated BTW and STW have no pro-inflammatory effect and volumes of 10 and 50 μL (BTW and STW) and concentrations of 10, 50 and 100 μM of conventional and deformable liposomes charged with BTW and STW were chosen for subsequent experiments.

3.6. Effects of TWs, TWs-Lip and TWs-DLip on LPS-activated RAW 264.7 macrophage cell production of NO and $\text{TNF}\alpha$

As thermal waters have shown their positive effect on skin diseases caused by inflammation (Largeau 1881, Boughlali 2003, Ali 2008), the potential effect of TWs, free and encapsulated in various formulations on macrophages activation was evaluated. For this, macrophages were treated for 1 h, 2 h and 4 h with 10 % and 50 % of Baraka and Salhine thermal waters (BTW and STW) compared to Hepes medium, and then activated by LPS (50 ng/mL) for 24 h. Then conventional and deformable liposomes charged with both TWs were prepared (BW-Lip, SW-Lip, BW-DLip and SW-DLip). The potential of encapsulated TWs in various formulations was also tested on macrophages activation with the same protocole. LPS-induced untreated cells were taken as negative control and reference sample in all the experiments.

3.6.1. Effect of BTW and STW on NO production and $\text{TNF}\alpha$ expression in LPS-induced macrophages

As shown in Figure 2A, LPS clearly induced considerable NO production in untreated RAW 264.7 cells (15.34 $\pm$ 2.08 μM). As expected, the pretreatment of cells with BTW and STW shows a clear reduction in NO production in a dose-dependent manner. Interestingly BTW showed a more pronounced inhibitory effect than STW, which might be justified by the difference in the mineral content. Taking as an example, the pretreatment of cells with 10 μL and 50 μL of BTW for 4 h before LPS addition, reduced the NO production by 37 % and 75 %, respectively, when LPS was added after 4 h of TWs addition. On the other hand, NO levels decreased but remained unchanged during incubations with Hepes medium and do not exceeded a reduction by 24 %. In addition, the pre-incubation durations had practically no impact on the inhibitory effect of BTW and STW. Thus, a pretreatment duration of 1h was sufficient for BTW and STW to attenuate the inflammatory effect of LPS. It has been previously revealed that a deep-sea water containing enhanced Mg^{2+} and Ca^{2+} concentrations strengthened the inhibitory effect of chitosan oligosaccharides on iNOS expression in LPS-induced macrophages (Maxwell, Lee et al. 2017). Moreover, an *in vitro* study using human dermal fibroblasts and human skin explants showed that a rich sulphate carbonate-calcium magnesium thermal water prevented the ROS induced damage (Joly, Branka et al. 2014). Numerous studies have also evidenced the effect of TWs on the activities of enzymes involved in the skin's defense system against free radicals inflammatory response (Richard, Guiraud et al. 1990, Cadi, Beani et al. 1991, Bruneau, Bernard et al. 1996). In accordance to these literatures, it can be speculated that the rich composition of BTW and STW with previous mentioned chemical elements might modulate the NO production in LPS induced macrophages.

We next investigated the effects of BTW and STW on the expression of TNF- α in LPS-induced RAW 264.7 cells. The expression of TNF- α cytokine was easily detected in RAW cell supernatants by ELISA. Treatment with both BTW and STW attenuated the LPS-induced increase in TNF- α secretion in a concentration-dependent manner (Fig. 2B). The inhibitory effect of TWs was the same independently of pre-incubation times. It can be also noticed that BTW at 50 μ L seems to prevent more effectively the release of TNF- α than STW at least until 4 h. This observed difference might be attributed to the rich composition of BTW with zinc (0.077 ± 0.001 mg/L) (Table 2). The anti-inflammatory effect of zinc was previously described (Prasad 2014). In addition, BTW and STW have a common composition with trace elements, sodium bicarbonate, and boron ingredients which might be responsible for the suppression of pro-inflammatory cytokines. Selenium and strontium, which are highly present in STW, proved an inhibitory effect on IL-1, IL-6 and TNF- α inflammatory cytokine production by keratinocytes in atopic dermatitis lesions (Celerier, Litoux et al. 1995). Moreover, La Roche-Posay, selenium-rich water, has significantly inhibited TNF- α and IL-1 production in Langerhans cells (LCs) (Wollenberg, Richard et al. 1992), related to the action of containing minerals on the functional mutation of LCs. Other common chemical components in BTW and STW were sodium, chloride and sulphate. Application and balneotherapy with sodium-chloride sulphate mineral waters were found to have potential anti-inflammatory effects on human skin during induced inflammatory reactions and psoriatic plaques (Hercogova, Stanghellini et al. 2002, Tsourelis-Nikita, Menchini et al. 2002). Furthermore, researchers have also demonstrated the effectiveness of an hypotonic thermal water to significantly down regulated the intracellular levels of TNF- α in cultured human psoriatic keratinocytes (Dal Pra, Chiarini et al. 2007). Moreover, a study conducted on human endothelial cells had noted a significant inhibition of the TNF- α -induced E-selectin and ICAM-1 expression in the presence of thermal water (Castex-Rizzi, Charveron et al. 2011). As well, treatment with thermal water from Korea spring decreased the expression of pro-inflammatory cytokines in HaCaT cells under Toll-like receptor (TLR) (Lee, Choi et al. 2012). Overall, data obtained in the current study are in perfect agreement with previous findings which suggest that BTW and STW have anti-inflammatory effects.

3.6.2. Effect of BTW and STW-loaded conventional liposomes on NO production and TNF- α expression in LPS-induced macrophages

First, we investigated the effects of conventional liposome formulations on LPS-induced NO production. BW-Lip and SW-Lip inhibited considerably the NO release in a dose-dependent manner (Fig. 3A). The previous pro-inflammatory study ensures that the reduction in NO production was not due to cells damage. A high inhibitory effect of both BW-Lip and SW-Lip was most noticeable after 4 h of pre-incubation. The data showed also that BW-Lip and SW-Lip at 10 and 50 μ M have practically the same inhibitory effect, while at 100 μ M, BW-Lip showed most noticeable the more efficient effect. The difference in the efficiency of BW-Lip and SW-Lip at 100 μ M might be explained by the more effective inhibitory effect of BTW, previously noted.

We next tested the effect of BW-Lip and SW-Lip on the TNF- α production in LPS-induced RAW 264.7 cells compared to untreated cells (Fig. 3B). Firstly, the results showed that liposome formulations did not affect the expression of TNF- α by RAW 264.7 cells non-stimulated with LPS. Furthermore, BW-Lip and SW-Lip reduced effectively the level of TNF- α expressed in LPS-stimulated RAW 264.7 cells in concentration- and time-dependent manner. As a general trend, the expression of TNF- α was effectively inhibited as the TW-Lip concentration increased and the preincubation time decreased. Importantly, these experiments demonstrate that BW-Lip had a greater inhibitory effect than SW-Lip.

The most remarkable result was observed when comparing the effects of BW-Lip and SW-Lip at 100 μ M (Fig. 3A & B), that encapsulate around 10 μ L of BTW and STW, respectively (Table 5), with the effects of 10 μ L non-encapsulated BTW and STW (10 %, v/v) (Fig. 2A & B). Interestingly, TWs encapsulated in liposomes inhibited more efficiently the NO and TNF- α release than free TWs, which revealed that liposomes strength the anti-inflammatory effect of encapsulated TWs. One of the possible explanations for these results can be the mechanism of encapsulated minerals uptake. The cellular uptake of liposomes was found to be mediated by adsorption of liposomes onto the cytoplasmic membrane and subsequent endocytosis (Allen, Austin et al. 1991). This allows to introduce to macrophage cells, a large quantity of encapsulated drugs, that increases with increasing liposome concentration and incubation time at 37°C (Allen, Austin et al. 1991). In addition to the effect of the TWs compositions with minerals, the lipid composition of vesicles might also participate in the inhibition of TNF- α expression. An earlier study from Treede et al. showed that PC had a broad ability to block many processes linked to the pro-inflammatory response in human Caco-2 cells treated with TNF- α (Treede, Braun et al. 2007). Further, the research group of Klein et al. showed that PG-loaded liposomes significantly decreased the TNF- α production in LPS-induced macrophages (Klein, Mauch et al. 2020). Moreover, the resulting catabolites of liposome membranes are exported from the lysosome and reused as raw materials in cellular metabolism (Kolter and Sandhoff 2010).

Our data show that the encapsulation of TWs in phospholipid vesicles strengthens their anti-inflammatory effect, which could be attributed on the one hand by increasing their bioavailability and on the other hand by adding the anti-inflammatory effect of used phospholipids.

Our findings are in good agreement with previous results reported the antioxidant power of free and encapsulated TWs in a vesicular composition on human fibroblast cell cultures (Laugier, Ribier et al. 1993). It was found that glutathione peroxidase (GPX) and superoxide dismutase (SOD) enzymes, which are two links in the radical detoxification chain, have their activities potentiated in the presence of encapsulated TWs. As for us, authors have concluded that the encapsulation of TWs in lipid vesicles potentiated its effects, particularly its antiradical and antioxidant activities. Further tests carried out human keratinocytes had confirmed the moisturizing and the anti-inflammatory effects of SUV liposomes charged with a strontium rich thermal water (Nunes and Tamura 2012, Tamura, Pedroso et al. 2012).

3.6.3. Effect of BTW and STW-loaded deformable liposomes on NO production and TNF- α expression in LPS-induced macrophages

In this part of experiments, we studied the effects of BTW- and STW-loaded deformable liposomes (BW-DLip and SW-DLip, respectively) on the NO and TNF- α cytokine production in LPS-activated RAW 264.7 cells with the same method previously described. Obtained results showed a reduced nitrite concentration in the presence of BW-DLip and SW-DLip in a concentration-dependent manner (Fig. 4A). However, a low decrease in NO production was observed in cells treated with 10 μ M of deformable liposomes, while the levels of NO produced by cells treated with 100 μ M of deformable liposomes are reduced to nearly the half of NO level produced by control cells. Besides, the NO production was strongly attenuated when the cells are incubated time with deformable liposomes for a short before the LPS-induction. The analysis of TNF- α production (Fig. 4B) has indicated that BW-DLip and SW-DLip inhibited dose-dependently the release of TNF- α . As already observed, deformable liposomes showed a stronger inhibitory effect on the TNF- α production at a short incubation time. In addition, no significant difference related to the encapsulated TWs was observed.

Compared to conventional liposomes, and taking in consideration the standard deviation values, deformable liposomes charged with TWs have practically the same inhibitory effect of NO and TNF- α production on LPS-induced RAW 264.7 cells as conventional liposomes charged with the same TWs. It must be also noted that even in absence of PG and CH from the lipid composition, deformable liposomes exert a marked inhibitory effect. This can confirm that registered anti-inflammatory effects are dominated by the anti-inflammatory effects of minerals containing in encapsulated TWs.

As confirmed in previous *in vitro* studies, deformable liposomes could play significant roles in improving the effects of encapsulated drugs, such as anti-inflammatory (Barone, Cristiano et al. 2020) and antioxidant effects (Liu, Hu et al. 2013). Previous study revealed the efficiency of niosomes, composed of surfactants and CH, entrapped with mineral water to enhance the skin absorption of minerals and the moisturizing effect of the encapsulated mineral water (Manosroi, Witkittilak et al. 2010).

4. Conclusion

In summary, we have successfully incorporated highly mineralized thermal waters into conventional and deformable liposomes using respectively sonication and extrusion techniques. The obtained results confirmed that both TWs-conventional and deformable liposomes have high encapsulation efficiency, nano-size and negative charge. The present study suggests that the high ionic strength of external environment mainly neutralized the zeta potential of the vesicles and affect the suspensions stability. During 30 days of storage, the conventional liposomes showed excellent stability, but sedimentation formation was observed in deformable liposomes.

Moreover, all liposomal formulations were biocompatible and non-toxic for the RAW 264.7 cells. Additionally, the anti-inflammatory potential of the TWs-conventional and deformable liposomes in LPS-induced RAW 264.7 macrophages was studied and compared to those of free TWs. The current study provides clear evidence that TWs-entrapped conventional and deformable liposomes exert anti-inflammatory effects by preventing LPS-induced NO and TNF- α release from RAW 264.7 macrophages. All liposomal formulations exerted concentration-dependent anti-inflammatory activity, superior to activities of free TWs. We confirmed that incorporation of TWs in conventional and deformable liposomes enhances their biological potentials. Our results suggest that the TWs-entrapped phospholipid vesicles can be useful in the treatment of various inflammatory skin diseases.

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