Chemosphere 177 (2017) 93–101

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Evaluation of toxic, cytotoxic and genotoxic effects of phytol and its nanoemulsion

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HIGHLIGHTS

- Preparation of phytol-loaded nanoemulsion.
- High concentration-mediated toxic, cytotoxic and genotoxic effects of phytol and nanoemulsion.
- Adaptive response at low concentration of phytol and its nanoemulsion.
- More toxicogenetic effects of phytol nanoemulsion.

ARTICLE INFO

Article history:
Received 29 August 2016
Received in revised form
24 February 2017
Accepted 27 February 2017
Available online 3 March 2017

Handling Editor: David Volz

Keywords:
Phytol nanoemulsion
Toxicogenetic effect
Adaptive response

ABSTRACT

Phytol (PYT) is a diterpenoid having important biological activity. However, it is a water non-soluble compound. This study aims to prepare PYT nanoemulsion (PNE) and evaluation of toxic, cytotoxic and genotoxic activities of PYT and PNE. For this, the PNE was prepared by the phase inversion method. The cytotoxicity test was performed in *Artemia salina*, while toxicity, cytotoxicity and genotoxicity in *Allium cepa* at concentrations of 2, 4, 8 and 16 mM. Potassium dichromate and copper sulfate were used as positive controls for the tests of *A. salina* and *A. cepa*, respectively. In addition, an adaptation response was detected in *A. cepa* by using the comet assay. The results suggest that both PYT and PNE exhibited toxic and cytotoxic effects at 4–16 mM in either test system, while genotoxicity at 2–16 mM in *A. cepa*. PNE exhibited more toxic, cytotoxic and genotoxic effects at 8 and 16 mM than the PYT. However, both PYT and PNE at 2 and 4 mM decreased the index and frequency of damage in *A. cepa* after 48 and 72 h, suggesting a possible adaptation response or DNA damage preventing capacity. Nanoemulsified PYT

Abbreviations: ATM, ataxia telangiectasia-mutated; ATR, ataxia telangiectasia-mutated-Rad3-related kinase; Casp, caspase; CYP-*c*, cytochrome-*c*; DSBs, double strand breaks; EOs, essential oils; ERK, extracellular kinase; FD, frequency of damage; HRR, homologous recombination repair; ID, index of damage; LDH, lactate dehydrogenase; LMPA, low melting point agarose; MAPKs, mitogen-activatied protein kinases; MDA, malondialdehyde; MI, mitotic index; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDCs, number of dividing cells; NF-κB, nuclear factor kappa B; NHEJ, non-homologous end joining; NMP, N-methyl pyrrolidone; NMPA, normal melting point agarose; O/W, oil-in-water; PBS, phosphate buffered saline; PdI, polydispersity index; PKC, protein kinase C; PNE, phytol nanoemulsion; PPARs, peroxisome proliferator-activated receptors; PYT, phytol; RG, root growth; RL, root length; ROS, reactive oxygen species; RTK, receptor tyrosine kinases; SSBs, single strand breaks; VE, nanovehicle; ZP, zeta potential.

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http://dx.doi.org/10.1016/j.chemosphere.2017.02.145
0045-6535/

(PNE) may readily cross the biological membranes with an increase in bioavailability and produce more toxic, cytotoxic and genotoxic effects in the used test systems.

1. Introduction

The diterpenoid phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol), referred to herein as PYT, has been reported for antioxidant, anti-inflammatory, antimicrobial, cytotoxic, neuroprotective, anti-diabetic and many other biological activities (Islam et al., 2015). It is an essential oil (OE), and can act as a multiple-edged like a sword, since at high concentrations it acts as a pro-oxidant, while anti-oxidant and cytoprotective at low concentrations in several test systems (Islam et al., 2016a). PYT is an agent with well tolerated in rodents (Islam et al., 2015). However, the scientific evidence on its genotoxic potential is very limited.

Drugs may cause genotoxic damage and/or carcinogenic effects and should therefore be considered in the assessment of risk against the benefit (Guzman et al., 2008; Downes and Foster, 2015). Therefore, any product that is the candidate for pharmaceutical consumption should be evaluated for the risks associated with toxicogenotoxic potential (Waters et al., 2010) for the measurement of genetic instability, mutations in genes, chromosomal damage as well as for carcinogenic effects (Zeiger et al., 2015).

It is important to highlight that PYT is a water non-soluble molecule, which limits its application and formulation for human consumption. The nanoencapsulation, liposomes and micelles of EOs can favor their physicochemical, pharmacokinetic and pharmacodynamic properties with oral administration and various biotechnological benefits, emphasizing on the minimizing of toxic, cytotoxic and genotoxic potentials (Onoue et al., 2014; Farcal et al., 2015).

Artemia salina is a widely used test system for the assessment of toxicity of varieties of substances (Baravalia et al., 2012). On the other hand, *Allium cepa* is an important test system for the evaluation of cytogenotoxic effects of pharmaceutical, environmental and biochemical agents, that may affect the genetic materials (Özkara et al., 2015). This study aims to prepare PYT nanoemulsion (PNE) and subsequent evaluate of toxic, cytotoxic and genotoxic potentials of PYT and PNE by using *A. salina* and *A. cepa* along with an adaptation response or damage preventing capacity in *A. cepa* test system.

2. Materials and methods

2.1. Reagents and chemicals

Potassium dichromate ($K_2Cr_2O_7$) and copper sulphate ($CuSO_4 \cdot 5H_2O$) (CS) were used as standards for the *A. salina* and *A. cepa* tests, respectively. The PYT, standards, and all the other necessary reagents and chemicals were purchased commercially from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Source of A. salina cysts and onions (A. cepa)

The *A. salina* cysts were obtained from the Laboratory of Experimental Cancerology of the Postgraduate Program in Pharmaceutical Sciences (UFPI) and the medium-sized onions were obtained from the local market of Teresina, Brazil, in 2016.

2.3. Preparation of the test sample

PYT and PNE were emulsified with 0.05% tween-80 dissolved in saline (0.9% NaCl) to attain final concentrations of 2, 4, 8 and 16 mM. Distilled water was used to dissolve the copper sulphate (2.40×10^{-3} mM) and potassium dichromate (2, 4, 8 and 16 mM). The concentrations tested were chosen according to Islam et al., 2015; 2016a,b.

2.4. Preparation of phytol nanoemulsion (PNE)

PNE was prepared by the emulsion phase inversion method. Briefly, an oil phase composed of medium chain triglycerides (1% w/w) and a surfactant (5% w/w) composed of soy phosphatidylcholine and sodium oleate (1:7 w/w) was slowly titrated with an aqueous solution of N-methylpyrrolidone (2% w/v) at 70 °C under constant magnetic stirring (1500 rpm). This was emulsified using a basic Ultra-Turrax T18 equipped with the S 18 N-19 G dispersing tool at 11,000 rpm for one minute followed by four minutes at 7000 rpm. The surfactant mixture that produced transparent colloidal dispersions was selected in a previous study using a pseudo-ternary phase diagram (Ostertag et al., 2012).

2.4.1. Analysis of size distribution of nano-droplets and determination of zeta potential

The mean droplet diameter of the FNE was calculated using the cumulative method of analysis based on the intensity of the light scattered (DLS) in a particle size analyzer (Brookhaven Instruments, USA) at 25 °C using a wavelength of 659 nm and 90° detection angle. The correlation was performed in parallel mode and the data were analyzed using Zeta Plus® Particle Sizing version software. Zeta potential (ZP) measurements were performed using the same equipment, applying the same field strength (approximately 5.9 V cm^{-1}). Five runs for each sample were used to determine the ZP value using the PALS ZP Analyzer software and the electrophoretic mobility according the Helmholtz-Smoluchowski equation. The samples were diluted 1:100 (v/v) with purified water.

2.4.2. Drug loading

A total 1.55% (v/v) of PYT was loaded into the oil phase. The samples were stored in a thermostatic bath at 25 °C and vortexed for one minute followed by 15 min in an ultrasonic bath every 12 h for 72 h. After centrifugation (1000×*g* for 15 min), the samples were filtered through an acetate membrane (0.45 μm) and loaded PYT was analytically determined by UV–vis spectrophotometry at 239 nm using the equation from the fitted standard curve plot constructed previously.

2.5. Cytotoxicity test in A. salina

The shrimp eggs were incubated in artificial salt water (39.35 mM NaCl, 18.66 mM $MgCl_2 \cdot 6H_2O$, 28.16 mM Na_2SO_4, 8.84 mM $CaCl_2 \cdot 2H_2O$, or 5.93 mM of $CaCl_2 \cdot 6H_2O$ and 9.38 mM of KCl in 1000 mL of water) at 25–30 °C. Sodium bicarbonate (Na_2CO_3) was used as a buffer to attain the pH 9.0 of the brine solution. After 48 h of hatching, 10 live nauplii (*A. salina*) were transferred to the each test tube (Meyer et al., 1982). The final volume of each sample