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To cite this article: Mohamed Sayed Behalo, Ermelinda Bloise, Luigi Carbone, Roberta Del Sole, Diego Lomonaco, Selma Elaine Mazzetto, Giuseppe Mele & Lucia Mergola (2016): Cardanol-based green nanovesicles with antioxidant and cytotoxic activities, Journal of Experimental Nanoscience, DOI: 10.1080/17458080.2016.1212407

To link to this article: http://dx.doi.org/10.1080/17458080.2016.1212407

Published online: 03 Aug 2016.

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Cardanol-based green nanovesicles with antioxidant and cytotoxic activities

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ABSTRACT
This manuscript describes the preparation of green nanovesicles by using cardanol as renewable starting material with embedded minor amounts of phthalazines, a class of heterocyclic bioactive compounds. The nanovesicles were prepared by stirring induced self-assembly in aqueous medium without involvement of any organic solvent. Dynamic light scattering studies and transmission electron microscopy revealed the formation of nanostructure with an average diameter in the range of 227–375 nm and a well defined spherical morphology. Potential antioxidant activity of nanovesicles were evaluated for the first time by 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging assay and bleomycin-dependent DNA damage. Moreover, their cytotoxic effects were also investigated by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay on different tumour cell lines. Unloaded nanovesicles showed moderate antioxidant and antitumoural activity that was further enhanced particularly by embedding the 2-[4-(4-Hydrazinophthalazin-1-yl)-phenyl]-isoindole-1,3-dione compound.

1. Introduction

Fabrication of functional supramolecular structures from renewables is an area of exciting and intensive research. New environmentally friendly materials and processes can be designed combining the green chemistry principles with nanotechnologies for producing biocompatible and bioactive nanotools as new potential drug delivery systems.

There are many examples of drug nanocarriers such as lipid particles and vesicular systems like liposomes, sphingosomes, niosomes etc.[1] Recently, liposomes are used in biomedicine as vehicles to deliver anticancer drugs and genes to targeted cancer cells helping to shield healthy cells from the drug toxicity and preventing concentration in vulnerable tissues.[2, 3]
Although some of these nanosystems are prepared using natural compounds, their preparation is expensive, time consuming and requires the use of organic solvents, incompatible with the physiological environment [4] and resulting in a high environmental impact. To overcome these problems, new types of Cardanol (CA)-based nanosystems were recently prepared from natural bioactive compounds by Bloise and co-workers using an environmental-friendly procedure, without use of any organic solvents.[5,6]

CA is a natural and biobased organic raw material obtained as the major component by vacuum distillation of technical cashew nut shell liquid (CNSL) which is a well-known by-product of the cashew agro-industry.[7,8]

The unique feature of CA is that it contains a hydrophilic reactive phenolic group in the meta position, lipid chains with varying degree of allylic cis double bonds, alkyl chains with odd numbers of carbon atoms, and saturated/unsaturated versions of hydrocarbon chains (C15:n), from which it can be predicted an amphiphilic behaviour in aqueous media. Taking advantage of these features, some researchers reported the development of sulfonic acids starting from CA as amphiphilic dopants for polyaniline nanomaterials.[9,10]

Thanks to these peculiar characteristics, CA and its derivatives can be considered a promising source of green chemicals.[11–14] Apart from current industrial uses, it has been demonstrated that the cashew nut deriving oils may exert antioxidant,[15–17] antifungal,[18] antibacterial [19] and larvicidal [20,21] activities. Moreover, recent studies showed the anti-tumour efficacy of CNSL-nanoemulsions towards breast cancer [22] as well as the biological activities of CA itself, as acetylcholinesterase inhibition against Alzheimer disease [23] and antiproliferative/cytotoxic activity [24] on cancer cell lines, were also studied.

Considering these important properties of CNSL compounds, the choice to use CA as principal component of vesicular nanodispersion obtained by a solvent-free method represents an important innovation in the field of nanotechnology for drug delivery.

Recently, phthalazines, a class of heterocyclic compounds, have drawn a considerable attention in the field of medical research because of their applicability as drugs. Several reports in literature have focused on the pharmacology of phthalazine derivatives thanks to their remarkable biological activity.[25] Their use as efficient antitumoural,[26–29] antimicrobial,[30–32] anticonvulsant [33] and anti-inflammatory agents [34] was reported. Recently, a new series of phthalazines was synthesised and screened for their antitumour activities against human hepatocarcinoma cell lines using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method.[35]

Taking into account both the capability of CA to form stable vesicular nanodispersions and the antitumour properties of variously substituted phthalazines [35] recently demonstrated, we report here the improved environmental-friendly preparation of novel CA-based nanovesicles loaded with four selected phthalazine derivatives (PHTs 1–4, Figure 1) as well as the promising results concerning their antioxidant and cytotoxic activities against different cancer cell lines.

### 2. Materials and methods

#### 2.1. Materials

CA was kindly furnished by Oltremare S.r.l. Cholesterol (CH), KCl, H3BO4, and NaOH were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received. Ultra pure (UP) water delivered by a Zeneer UP 900 Human Corporation system.
Borate buffer solution pH 9.0 is a UP water solution of H$_3$BO$_4$ 30 mM, KCl 70 mM and NaOH 18 mM.

Antioxidant assays were performed using different reagents: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), MnO$_2$, phosphate buffer solution, DNA (Calf Thymus type 1), bleomycin sulphate, penicillin, thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), L-ascorbic acid, FeCl$_3$ and HCl. All reagents and solvents with analytical grade were purchased from Sigma Chemical Company, St. Louis, MO.

For cytotoxicity studies, the cell lines (HepG2, HeP2, PC3, MCF-7) were obtained from American Type Culture Collection (ATCC) via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. RPMI-1640 and fetal bovine serum (FBS) were purchased from GIBCO, Waltham, MA, dimethylsulfoxide (DMSO), MTT and 5-Fluorouracil (5-Fu) were supplied from Sigma Chemical Company, St. Louis, MO.

### 2.2. General procedure of nanovesicles preparation and characterisation

A mixture of CA, CH and PHTs (molar ratio 1:0.7:0.2, respectively) was heated at 90 °C for about 20 h, then mixed by mechanical stirring in the presence of glass beads (10 g) for 1 h to form a lipidic film on the flask’s wall. Hydration of lipidic films was carried out adding 40 mL of borate buffer (pH 9.0, preheated at 50 °C) under mechanical stirring (800 rpm) and heating at 90 °C for 1 h. Then, obtained vesicular nanodispersions were submitted to sonication step for 45 min at 60 °C.

A sample without PHTs has been also prepared with the same procedure as a blank reference (CA-CH).

Characterisation of nanodispersions was carried out by dynamic and electrophoretic light scattering (DLS) measurements on a Malvern Zetasizer Nano ZS90. The hydrodynamic diameter (d) of nanodispersions has been determined at 25 °C measuring the auto-correlation function at a 90° scattering angle. Cells have been filled with 200 μL of sample.
solution and diluted to 2 mL with UP water. Each d value is the average of five separate measurements. The same diluted solutions were used to fill the Zeta meter cells and to determine Z-potential (ZP) values of vesicle dispersions. The voltage ramps were performed according to the indications given by the purveyor.

The morphology of nanovesicles were examined using transmission electron microscopy (TEM). Low-magnification TEM analyses were performed on a Jeol JEM-1011 electron microscope operating at 100 kV, equipped with a CCD camera ORIUS 831 from Gatan, Pleasanton, CA. Samples were prepared by initially staining dilute vesicle dispersions with a few µL of osmium tetroxide aqueous solution (1% w/v) and then drop-casting them onto carbon-coated copper grids. Hence, each grid is twice rinsed in pure water, then the deposited samples are completely dried at 60 °C for one night before examination.

2.3. Antioxidant activity assay by ABTS method

A mixture of ABTS screening antioxidant assay was used to evaluate the antioxidant properties of all the prepared nanovesicles. This technique involves the direct production of the blue/green ABTS radical cation (ABTS$$^{++}$$) chromophore generated by oxidation of ABTS with MnO$_2$ that shows a maximum adsorption capacity at 734 nm. The addition of specific hydrogen-donating antioxidants to the pre-formed radical cation causes a reduction to ABTS decreasing the colour intensity. Thus, the extent of decolourisation as percentage inhibition of the ABTS$$^{++}$$ was determined relative to the reactivity of Ascorbic acid used as standard, under the same conditions. Briefly, the method was performed as follows: for each of the investigated samples, 2 mL of ABTS solution (60 µM) were added to 3 mL of MnO$_2$ solution (25mg mL$^{-1}$), all prepared in 5 mL of aqueous phosphate buffer solution (pH 7, 0.1 M). The resulting mixture was shaken and stabilised in the dark at room temperature for 12–16 h before use. Afterwards, the mixture was centrifuged, filtered and the absorbance ($A_{\text{control}}$) of the resulting green blue ABTS$$^{++}$$ solution ($\lambda$ 734 nm) was adjusted to approximately 0.5. Then, 50 microliter (µL) of nanovesicles or positive control sample (L-ascorbic acid, 2 mM) in spectroscopic grade MeOH/phosphate buffer (1:1) were added. The absorbance ($A_{\text{test}}$) was measured and the reduction in colour intensity was expressed as % inhibition. The % inhibition for each sample was calculated from the following equation:

$$\frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100$$ (1)

Blank sample was run without ABTS and using MeOH/phosphate buffer (1:1) instead of tested compounds. Negative control was run with ABTS and MeOH/phosphate buffer (1:1) only.

2.4. Bleomycin-dependent DNA damage assay

The bleomycin assay has been adopted for assessing the pro-oxidant of food antioxidants. The antitumour antibiotic bleomycin binds iron ions and DNA. The complex obtained degrades DNA that, upon heating with TBA, yields a pink chromogen. Upon the addition of suitable reducing agents, antioxidant competes with DNA and diminishes formation of chromogen.[36,37]
To the reaction mixtures in a final volume of 1.0 mL, the following reagents at the final concentrations stated were added: DNA (0.2 mg mL\(^{-1}\)), bleomycin (0.05 mg mL\(^{-1}\)), FeCl\(_3\) (0.025 mM), MgCl\(_2\) (5 mM), KH\(_2\)PO\(_4\)-KOH buffer pH 7.0 (30 mM), and L-ascorbic acid (0.24 mM) or the tested samples diluted in MeOH to give a concentration of 0.1 mg mL\(^{-1}\). The reaction mixtures were incubated in a water bath at 37 \(^{\circ}\)C for 1 h. Afterwards, 0.1 mL of EDTA (0.1 M) was added to stop the reaction. DNA damage was assessed by adding 1 mL of 1% (w/v) TBA and 1 mL of 25% (v/v) HCl followed by heating in a waterbath maintained at 80 \(^{\circ}\)C for 15 min. The chromogen formed was extracted into 1-butanol, and the absorbance was measured at 532 nm.[36,38]

2.5. Cytotoxic activities assay by MTT method

This colorimetric assay is based on the conversion of the yellow tetrazolium bromide to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. The cells were seeded in a 96-well plates at a density of 1.0 \(\times\) \(10^4\) cells/well at 37 \(^{\circ}\)C for 48 h under 5% CO\(_2\). After incubation, the cells were treated with different amounts of nanovesicles (100 – 5 \(\mu\)g mL\(^{-1}\)) and incubated for 24 h. Then, 20 \(\mu\)L of MTT solution (5 mg mL\(^{-1}\)) was added and the cells were incubated for 4 h. Finally, 100 \(\mu\)L of DMSO were added into each well to dissolve the purple formazan formed and the absorbance at 570 nm (A570) was measured by means of Enzyme-labeling instrument (EX-800).

3. Results and discussion

3.1. Preparation and characterisation of nanovesicles

In this work CA-based vesicular nanodispersions were loaded for the first time with four different PTHs derivatives (PTHs 1–4), attractive heterocyclic compounds with biological activities, in order to produce a new multifunctional tool as potential anticancer system. The innovation of this methodology provides the absence of organic solvents commonly used during the nanodispersion preparation by lipidic film formation. In this case, CA acts as solvent as well as main component of the mixture, which when combined with opportune amounts of PTHs and CH, is able to produce stable nanovesicles (CA-CH-1, CA-CH-2, CA-CH-3 and CA-CH-4). The influence of the embedded PHTs was estimated comparing its performance with the blank sample CA-CH. To evaluate the outcome of preparation procedure, size distribution and stability of nanosystems were determined by DLS measurements of hydrodynamic diameter (\(d\)) and Z-potential (\(ZP\)) and results are reported in Table 1.

As it can be seen, stable nanodispersions were obtained with mean diameters in the range of 227–375 nm and monodisperse size distributions, confirmed by low values of

|          | \(d\) (nm) | PDI | \(Z-P\) (mV) |
|----------|------------|-----|-------------|
| CA-CH    | 237 ± 54   | 0.04| −73.6 ± 7.7 |
| CA-CH-1  | 227 ± 80   | 0.08| −62.8 ± 7.3 |
| CA-CH-2  | 298 ± 89   | 0.04| −67.5 ± 7.4 |
| CA-CH-3  | 375 ± 138  | 0.22| −60.9 ± 7.9 |
| CA-CH-4  | 280 ± 98   | 0.09| −62.4 ± 6.8 |
polydispersity index (PDI). Moreover, ZP analysis described a good stability of all vesicular nanosystems with values higher than $-60$ mV.

The morphology of nanodispersions was also examined using TEM confirming the formation of spherical vesicular structures for all samples. Figure 2 shows the transmission electron micrographs of CA-based nanovesicles embedded with PHTs 1 (a) and 4 (b).

### 3.2. Antioxidant and cytotoxic activities of nanovesicles

Having regard to biological properties of single system components (CA and PHTs), the evaluation of antioxidant activity of loaded and unloaded nanovesicles was assessed by ABTS scavenging assay. The percentage of inhibition for each sample was calculated as reported in previous works.[38–40] As it can be seen in Table 2, unloaded nanovesicles (CA-CH) showed a good antioxidant activity with 65.32% of inhibition, pointing out the important antioxidant action of CA that is the main component of nanocarriers. On the other hand, its antioxidant properties are well known as reported in many works.[41–44] Comparing the antioxidant properties of loaded and unloaded nanovesicles, it has been seen that the embedding of PHTs 1 and 4 enhanced the antioxidant action of the nanosystem.

To confirm these results, all samples were also assessed by their protective effect against bleomycin-induced DNA damage using a well-known method. [36–38,40] Also in this case, CA-CH-1 and CA-CH-4 proved the best protective effect against DNA damage as it can be noted from the low absorbance values obtained (Table 3), confirming the defensive/antioxidant action of the aforementioned nanosystems. In particular, higher

#### Table 2. Antioxidant assays by ABTS method of CA-based nanovesicles embedded with PHTs.

|                  | % Inhibition |
|------------------|--------------|
| Ascorbic acid    | 88.10 ± 0.78 |
| CA-CH            | 65.32 ± 2.18 |
| CA-CH-1          | 73.79 ± 1.34 |
| CA-CH-2          | 48.58 ± 1.15 |
| CA-CH-3          | 53.62 ± 1.40 |
| CA-CH-4          | 78.83 ± 1.18 |

*Figure 2.* Transmission electron micrographs of CA-based nanovesicles embedded with PHTs 1 (a) and 4 (b).
A protection effect was obtained with CA-CH-4 sample with absorbance value very close to L-ascorbic acid used as reference.

Probably, the presence of methoxy group -OCH$_3$ or hydrazine group -NHNH$_2$ in conjugated aromatic heterocycles 1 and 4, may increase the antioxidant activity of these molecules.[39] The improved antioxidant properties of vesicles loaded with the compounds 1 and 4 reflect the combination of resonance and inductive effects of the ring substituents being the +M and -I effects of -OCH$_3$ or -NHNH$_2$ groups considerably influent compared to substituents of compounds 2 and 3. It can be assumed that supramolecular interactions established between CA, PHT1 or PHT4 and CH produce specific chemical conditions strengthening the biological activity of individual compounds, producing specific tools with interesting features in cancer treatment. In this regard, the cytotoxic activity of each CA-based vesicular nanodispersion was evaluated. In particular, the in vitro antitumour activity of loaded and unloaded nanocarriers was determined by MTT assay method [38] using four tumour human cell lines: hepatocellular carcinoma (HepG2), mammary gland (MCF-7), epidermoid laryngeal carcinoma (HeP2) and human prostate cancer (PC3). The 50% antitumour activity dose (IC$_{50}$), defined as the concentration of samples that reduce the absorbance of treated cells by 50%, was evaluated. The results obtained were compared with the antitumour activity of 5-Fu, a typical anticancer drug commonly used as reference.

Figure 3 shows a different behaviour of tumoural cell lines when treated with each vesicular nanodispersion. Cancer cells have different abilities to respond to cancer treatments related with the aggressiveness of the tumour and its ability to resist to a specific therapy, then, its not surprising to observe different behaviours.

A first finding of this study highlights the moderate cytotoxicity of unloaded nanovesicles CA-CH on all cancer cell lines. Moreover, all nanosystems tested on MCF-7 cell line showed no increase in cytotoxicity compared to CA-CH, displaying IC$_{50}$ values between 32.69 and 39.33 $\mu$g mL$^{-1}$, except for CA-CH-2 (87.80 $\mu$g mL$^{-1}$). Indeed, the addition of PHT 2 in vesicular nanosystem caused a drastic reduction of cytotoxicity on all tumoural cell lines tested compared to unloaded vesicles. A similar trend was observed for CA-CH-3. Instead, CA-CH-1 reduced the viability of HepG2 and PC3 cell lines with IC$_{50}$ = 28.39 and 27.37 $\mu$g mL$^{-1}$, respectively. CA-CH-4 nanosystem showed a stronger cytotoxic effect on all cell lines, especially for PC3 cells (IC$_{50}$ = 15.86 $\mu$g mL$^{-1}$), probably due to a synergic action of antioxidant activity previously discussed, an intrinsic cytotoxicity of 'bare' nanosystem and the cytotoxic effect of the embedded PHT 4 recently demonstrated by Wasfy and co-workers.[35]

From these results, it can be deduced that CA-CH-1 and especially CA-CH-4 showed the most cytotoxic effect strengthened by their greatest antioxidant activity, previously proved.
4. Conclusions

New CA-based vesicular nanodispersions loaded with new PHTs derivatives were prepared by a solvent-free method and their antioxidant and cytotoxic activity was evaluated for the first time. The antioxidant action and the moderate cytotoxic effect of unloaded nanovesicles CA-CH conferred by CA itself represent an important novelty of these new nanosystems compared with common nanocarriers.[4,46] The properties showed by blank vesicles, were improved by loading with PHTs 1 and 4. In particular, the embedding of PHT 4 allowed the preparation of a nanosystem (CA-CH-4) that showed a strong cytotoxic effect on all tested tumoural cell lines. The antiproliferative power associated with antioxidant action of these innovative green-multifunctional tools could lead to the development of new potential therapies in cancer treatment, in order to provide a support to medical research.

Acknowledgements

The authors wish to thank Benha University, Ministry of Scientific Research (Egypt), University of Salento, Ministero degli Affari Esteri e della Cooperazione Internazionale (Italy), Brazilian FUNCAP, CAPES and CNPq, Apulia Region Programs Future in Research and Cluster ATS–SISTEMA for the financial support.

Disclosure statement

No potential conflict of interest was reported by the authors.
Funding

Ministero degli Affari Esteri e della Cooperazione Internazionale [grant number EG13MO4]; Apulia Region Programs Future in Research [grant number XYA7HY5]; Cluster ATS — SISTEMA [grant number T7WGSJ3]; Brazilian FUNCAP, CAPES and CNPq [grant number PVE 401359/2014-0 2015-2017].

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