Scientific Research Publishing

Journal of Cancer Therapy, 2026, 17(8), 428-447
https://www.scirp.org/journal/jct
ISSN Online: 2151-1942
ISSN Print: 2151-1934

Synthesis of Encapsulated Oroxylin A Nanoparticles and Their Effect on Cancer Cell Viability and Apoptosis

Michael A. Pitt[1*], Paul Davis[2], Catherine Davis[2], Sean M. Mackay[3,4], Eng Wui Tan[3]

[1]Paraparaumu, New Zealand
[2]Trinity Bioactives Ltd., Wellington, New Zealand
[3]Department of Chemistry, University of Otago, Dunedin, New Zealand
[4]Massey Ventures Limited, Massey University, Tiritea Campus, Palmerston North, New Zealand
Email: *pitt@xtra.co.nz, p.davis@trinityboiactives.com

How to cite this paper: Pitt, M.A., Davis, P., Davis, C., Mackay, S.M. and Tan, E.W. (2026) Synthesis of Encapsulated Oroxylin A Nanoparticles and Their Effect on Cancer Cell Viability and Apoptosis. *Journal of Cancer Therapy*, **17**, 428-447.
https://doi.org/10.4236/jct.2026.178037

Received: May 26, 2026
Accepted: August 25, 2026
Published: August 28, 2026

Abstract

Cancer cells shift their energy metabolism (ATP) production from Mitochondrial oxidative phosphorylation observed in non-tumour cells to cytosolic aerobic glycolysis. This change, known as the Warburg Effect, is mediated by increased expression of uncoupling protein-2 (UCP_2), resulting in the uncoupling of oxidative phosphorylation of ATP in the mitochondria. It has been proposed that mitochondrial recoupling would be a novel therapeutic strategy for cancer. Oroxylin A has been observed to be an inhibitor of the uncoupling protein-2 (UCP_2). This report details the synthesis of nanoparticles containing Oroxylin A encapsulated into PLGA, Solutol or nanofibrils. The effect of Oroxylin A alone and encapsulated Oroxylin A on the cell viability of mouse melanoma cells is studied, as well as the effect of Oroxylin A alone and encapsulated Oroxylin A on apoptosis (determined by caspase-9 activity). It was found that Oroxylin A alone had little effect on melanoma cell viability or apoptosis but that encapsulation of Oroxylin A into PLGA, Solutol or nanofibrils had a marked inhibitory effect on melanoma cell viability. Oroxylin A encapsulated into Solutol markedly stimulated caspase-9 while Oroxylin A encapsulated into PLGA strongly inhibited caspase-9 activity. The effect of PLGA or Solutol alone on melanoma, human colorectal carcinoma cells or human gastric adenocarcinoma cell viability was studied. PLGA showed little inhibition or was even, somewhat, stimulatory. Solutol, particularly at higher concentrations, was inhibitory.

Keywords

Oroxylin A, Nanoparticles, Cancer Cells, Viability, Apoptosis

1. Introduction

In this study, we target a fundamental difference in metabolism between cancer cells and normal cells. Cancer cells shift their energy metabolism (ATP production) from mitochondrial oxidative phosphorylation observed in non-tumour cells to cytosolic aerobic glycolysis. This change is known as the Warburg Effect [1]. The Warburg Effect is mediated by increased expression of uncoupling protein-2 (UCP_2) [2]. UCP_2 uncouples the oxidative phosphorylation from ATP in the mitochondria.

Oroxylin A is an o-methylated flavone obtained from the root-bark of *Oroxylum indicum*, the dried root of *Scutellaria baicalensis* and several other plants.

Oroxylin A has been documented to have preventative and therapeutic effects in cancer [3] [4]. The studies of Lu *et al.* [3], Sajeev *et al.* [4] and Wang *et al.* [5] detail the mechanisms of the anti-cancer activity of Oroxylin A. Also, Tuli *et al.* [6] have reviewed the anti-cancer effects of Oroxylin A in both *in vitro* and *in vivo* studies.

Pertinent to the present study, Oroxylin A has been observed to be an inhibitor of uncoupling protein-2 (UCP_2) [7].

The role of UCP_2 in cells is the uncoupling of mitochondrial oxidative phosphorylation. Baffy *et al.* [8] have suggested that mitochondrial recoupling would be a novel therapeutic strategy for cancer.

This study details the synthesis of nanoparticles containing Oroxylin A encapsulated into PLGA, Solutol or nanofibrils. The primary aim of the present work is to study the effect of these encapsulated Oroxylin A nanoparticles on cancer cell viability and apoptosis. In addition, the effect of PLGA or Solutol alone on cancer cell viability is also studied in this work.

The high molecular weight of these encapsulated Oroxylin A nanoparticles, in PLGA, Solutol or nanofibrils, will enable them to be taken up and retained by the tumour vasculature and also accumulated in tumours to a greater extent than they do in normal tissue. This is known as the Enhanced Permeability and Retention Effect (EPR effect) [9], creating the potential for a novel enhanced and targeted cancer therapy based on mitochondrial recoupling.

2. Materials and Methods

2.1. Materials

The mouse melanoma (B16/F10), human colon carcinoma cells (HCT-116), human gastric adenocarcinoma cells (AGS) and human skin fibroblasts (Detroit 551) were from American Type Cell Culture (ATCC)/(Bethesda, MD, USA). Dulbecco's Modified Eagle's medium (DMEM) was provided by Gibco (Auckland, New Zealand), Eagle's Minimum Essential Medium (EMEM), McCoy's 5A medium and Ham's F-12K medium were from ATCC. Oroxylin A was supplied by MedChem Express (Monmouth Junction, NJ, USA). The caspase-9 activity assay kit was from R&D Systems (Minneapolis, MN, USA). Poly (D,L-Lactide-co-glycolide) (lactide:glycolide) (1:1) ester terminated (MW 24,000 - 38,000) (PLGA) and Solutol®/Kolliphor HS15 (Macrogol) (15)-hydroxystearate, and all other reagents were obtained from

Sigma-Aldrich (St Louis, MO, USA). Information of the properties of (PLGA) may be found in Rezvantalab *et al.* [10]. Information of the properties of Solutol® HS15 is described in a review by Ali *et al.* [11].

The Oroxylin A-PLGA at 4.26 μg of Oroxylin A/mL in 1% PLGA and the Oroxylin A-Solutol at 1.99 μg of Oroxylin A/mL in 0.1% Solutol were prepared by Dr. Sean Mackay, University of Otago, Dunedin, New Zealand. Also, the preparation of Oroxylin A in whey protein-derived nanofibrils was donated by Hi-Aspect Ltd., Wellington, New Zealand.

2.2. Methods

Mouse melanoma cells (B16/F10) were cultured in DMEM, human colon carcinoma cells (HCT-116) in McCoy's 5A medium, human gastric adenocarcinoma cells (AGS) in Hams F-12K medium and human skin fibroblasts in EMEM. All cultures were supplemented with 10% foetal bovine serum and penicillin (100 U/mL)/streptomycin (100 μg/mL). The test samples were added to the cells (5×10^4 cells/mL), which were incubated using 96-well flat-bottomed multi-well plates for 24 hours at 37˚C in a 5% CO_2/95% air atmosphere. Each sample was assayed at least in triplicate. The carrier used was 1.5% dimethyl sulphoxide in Hank's Balanced Salt Solution. Cells were then lysed and the cell concentrations were determined at 570 nm following reaction with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [12]. Caspase-9 activity was measured using an assay kit supplied by R&D Systems. The caspase activity per cell in each culture was also determined by dividing the caspase activity by the absorbance value from the MTT assay.

Oroxylin A-PLGA nanoparticles were prepared using a double emulsion method (**Figure 1**) similar to that described by Ramalho *et al.* [13]. Initially, as proof of concept for the double emulsion method, a hydrophobic organic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarboryamine perchlorate, was used as a substitute to Oroxylin A for PLGA encapsulation. Using this model, it was established that PLGA nanoparticles could be repeatedly formed using the double emulsion method, whereby insoluble 1,1'-dioctadecyl-3,3,3',3'-trimethylindocarboryamine perchlorate could be suspended in phosphate-buffered saline (pH 7.4) to produce a pink suspension.

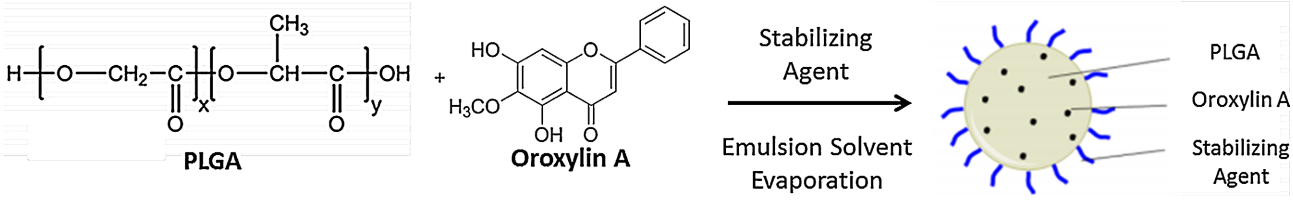


Figure 1. Schematic illustration depicting the preparation of PLGA nanoparticles encapsulating Oroxylin A.

Following this successful demonstration, we undertook the encapsulation of the desired agent, Oroxylin A. PLGA (100 mg) and Oroxylin A (approximately 1 mg)

were dissolved in acetone (10 mL), while a 1% aqueous solution of Pluronic F127® (1%, 200 mg in 20 mL) was prepared. The organic phase was added dropwise to the aqueous phase with continuous stirring (~1000 rpm). The solution was continuously stirred for a further 6 hours under a light air flow to allow the acetone to evaporate. Control PLGA nanoparticles were prepared in an identical manner, but without the inclusion of Oroxylin A. The PLGA suspensions were subsequently purified by centrifugation at 2500 rpm for 5 minutes, followed by 3500 rpm for 5 minutes. The pelleted material (larger micron-sized PLGA/Oroxylin A) was discarded. The remaining suspension was then dialysed against phosphate-buffered saline for 24 hours to remove any excess Pluronic F127®.

A spectrophotometric assay for the sensitive detection and quantification of Oroxylin A was developed based on the strong absorbance of Oroxylin A observed at 374 nm under alkaline conditions, which is due to the deprotonation of Oroxylin A to form the Oroxylin A anion (Figure 2).

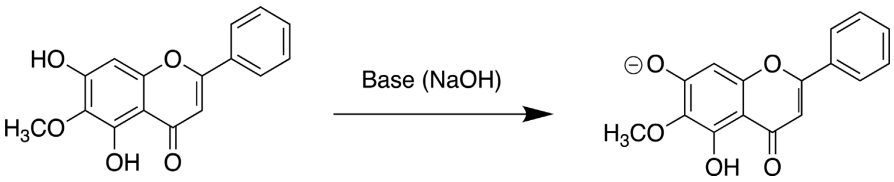


Figure 2. Reaction scheme for the deprotonation of Oroxylin A under alkaline conditions.

To determine the concentration of Oroxylin A in the PLGA nanoparticle suspension, a standard curve of Oroxylin A was prepared by dissolving Oroxylin A in a mixture of acetone and pH 10 sodium borate buffer (1:1 acetone: buffer; 100 mM sodium borate adjusted with NaOH; 1 mM Oroxylin A). The stock solution was serially diluted to produce a concentration range between 0 and 0.33 mM and the absorbance was measured at 374 nm (Table 1, Figure 3).

Characterisation of the PLGA nanoparticle suspensions was performed primarily by Dynamic Light Scattering (DLS) (Figures 4-7). DLS was performed on all suspensions. Control PLGA nanoparticles displayed a hydrodynamic diameter of approximately 150 nm with excellent polydispersity (Figure 4). PLGA nanoparticles encapsulating Oroxylin A tended to have a slightly greater hydrodynamic diameter of approximately 160 nm with good polydispersity (Figure 5). The potential

Table 1. Absorbance of Oroxylin A at 374 nm under alkaline conditions (~pH 9) with increasing Oroxylin A concentration.

Absorbance	Concentration (mM)
0	0
0.062	0.0024
0.215	0.011
1.068	0.061
2.459	0.33

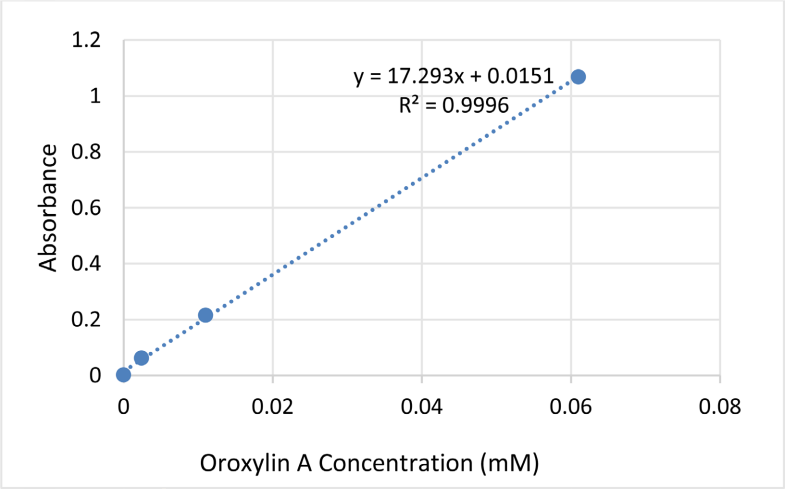


Figure 3. Standard curve of Oroxylin A alkaline conditions (pH ~9) with absorbance measured at 374 nm.

for the PLGA/Oroxylin A nanoparticle suspensions to be lyophilised and stored as a dry powder for re-suspension when required was also investigated. After lyophilisation, PLGA nanoparticles containing Oroxylin A could be easily re-suspended in deionized water or phosphate-buffered saline (pH 7.4) with only a small degree of aggregation observed by DLS (**Figure 6**). It was noted however, that re-suspending the PLGA nanoparticles containing Oroxylin A by ultrasonication (bath ultrasonicator, 40 kHz, 100 W, 60 seconds) caused significant aggregation by DLS (**Figure 7**). Therefore, PLGA nanoparticles containing Oroxylin A can be lyophilised and stored as a dry powder but are not recommended for re-suspension to be facilitated by ultrasonication.

The absorbance of control and Oroxylin A PLGA nanoparticles was measured under identical conditions by diluting (1:10) an aliquot of nanoparticle suspension with acetone:borate buffer (pH 10; 1:1 acetone:buffer; 100 mM sodium borate) and measuring absorbance. Under these conditions, it was observed that the cloudy appearance of the nanoparticle suspension clarified, suggesting either that PLGA is more soluble under these conditions, or that the PLGA is hydrolysed into lactic acid and glycolic acid. Absorbance of control PLGA nanoparticles (no Oroxylin A) had little absorbance at 374 nm (0.07, within error of baseline), while the absorbance of the Oroxylin A PLGA nanoparticle suspension corresponded to an *Oroxylin A concentration of approximately* 60 μM (A = 0.124; c = ~0.063 mM; batch variation ~0.057 - ~0.063 mM). Increasing the concentration of Oroxylin A used during the manufacturing process produced little benefit to the final Oroxylin A concentration in suspension using this method. To maximise the concentration of Oroxylin A, the PLGA/Oroxylin A nanoparticle suspension was concentrated by lyophilisation resulting in an increased *Oroxylin A concentration of approximately* 150 μM.

Attempts to solubilise Oroxylin A as a microemulsion were made using Kolliphor HS15 (Solutol). A solid quantity of Oroxylin A was dispersed in 1% Solutol

via ultrasonication (60 minutes) and stirred continuously for 15 hours at 40˚C. *The maximum concentration of Oroxylin A achieved using a 1% Solutol emulsion was approximately* 70 μM.

Attempts to produce a liposome suspension of Oroxylin A were also made. Oroxylin A and soy phosphatidylcholine were dissolved in chloroform. The chloroform was removed under vacuum and the residual phospholipid/Oroxylin A film was rehydrated with pH 7.4 PBS to produce a final concentration of 1 mM Oroxylin and 10 mM soy phosphatidylcholine in suspension. The lipid suspension was subsequently extruded 15 times through 1000 nm followed by 100 nm polycarbonate membranes at 60˚C. The liposome suspension was then purified via dialysis against pH 7.4 PBS for 12 hours, during which the liposome suspension aggregated from solution. The process was repeated unsuccessfully using 0.5 mM Oroxylin A to 10 mM soy phosphatidylcholine.

Cell viability was measured by the MTT assay. The MTT assay is a colorimetric assay that detects the conversion of the yellow tetrazolium salt, MTT, to a purple formazan product produced by active mitochondria in living cells.

3. Results

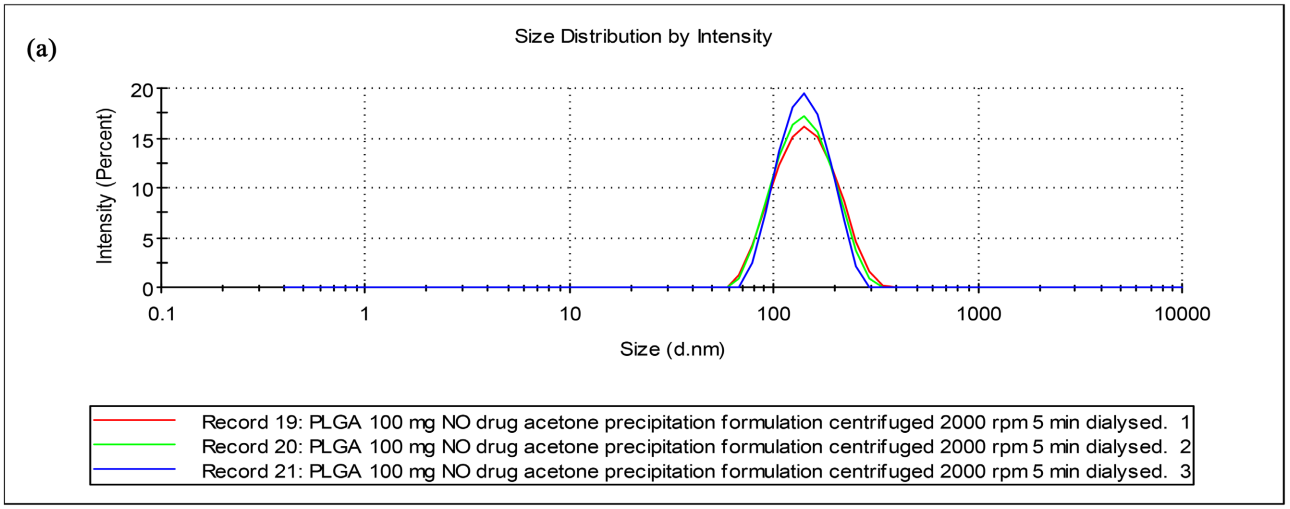
Figures 4-7 show the size distribution of PLGA nanoparticles, PLGA nanoparticles encapsulated with Oroxylin A and the further treatment of PLGA encapsulated with Oroxylin A.

Figure 4 shows the size distribution of nanoparticles containing PLGA alone.

Figure 5 shows the size distribution of PLGA nanoparticles encapsulating Oroxylin A.

Figure 6 shows the size distribution of PLGA nanoparticles encapsulating Oroxylin A after freeze drying and re-suspension in deionized water.

Figure 7 shows the size distribution of PLGA nanoparticles encapsulating Oroxylin A after freeze drying and re-suspension in deionized water and using bath sonication to re-disperse the nanoparticles.



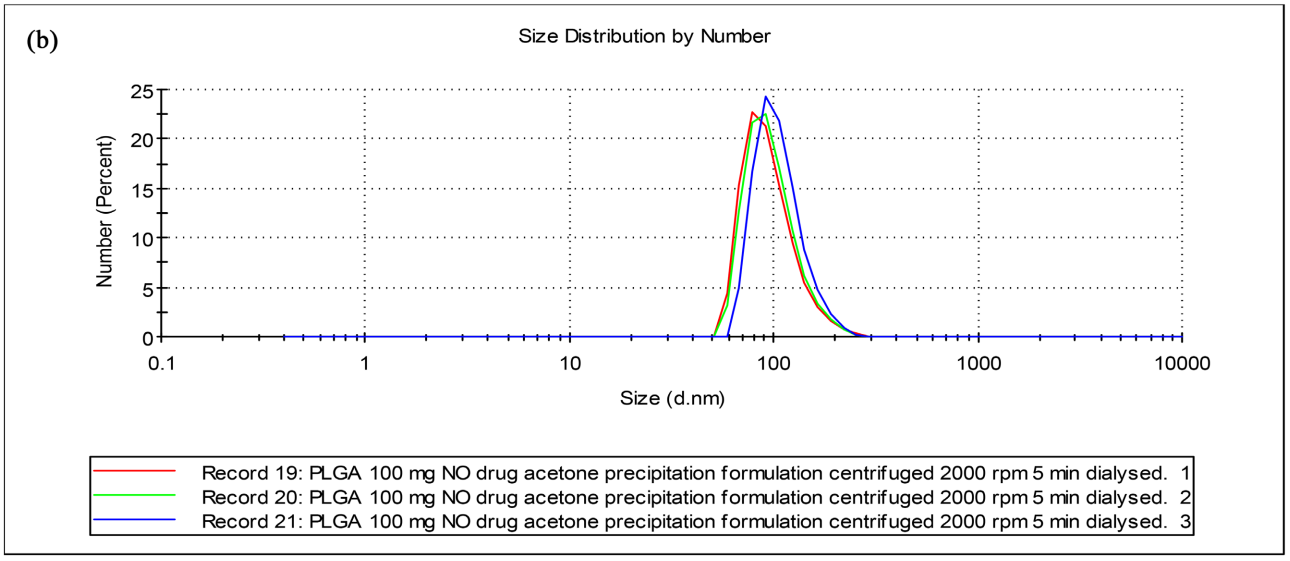


Figure 4. PLGA nanoparticles made by acetone precipitation. Average size = 148 ± 3 nm. Polydispersity index = 0.07 ± 0.03. (a) Size distribution by intensity. (b) Size distribution by number. The results of three preparations (Record 19, Record 20, Record 21) determined by Dynamic Light Scattering (DLS).

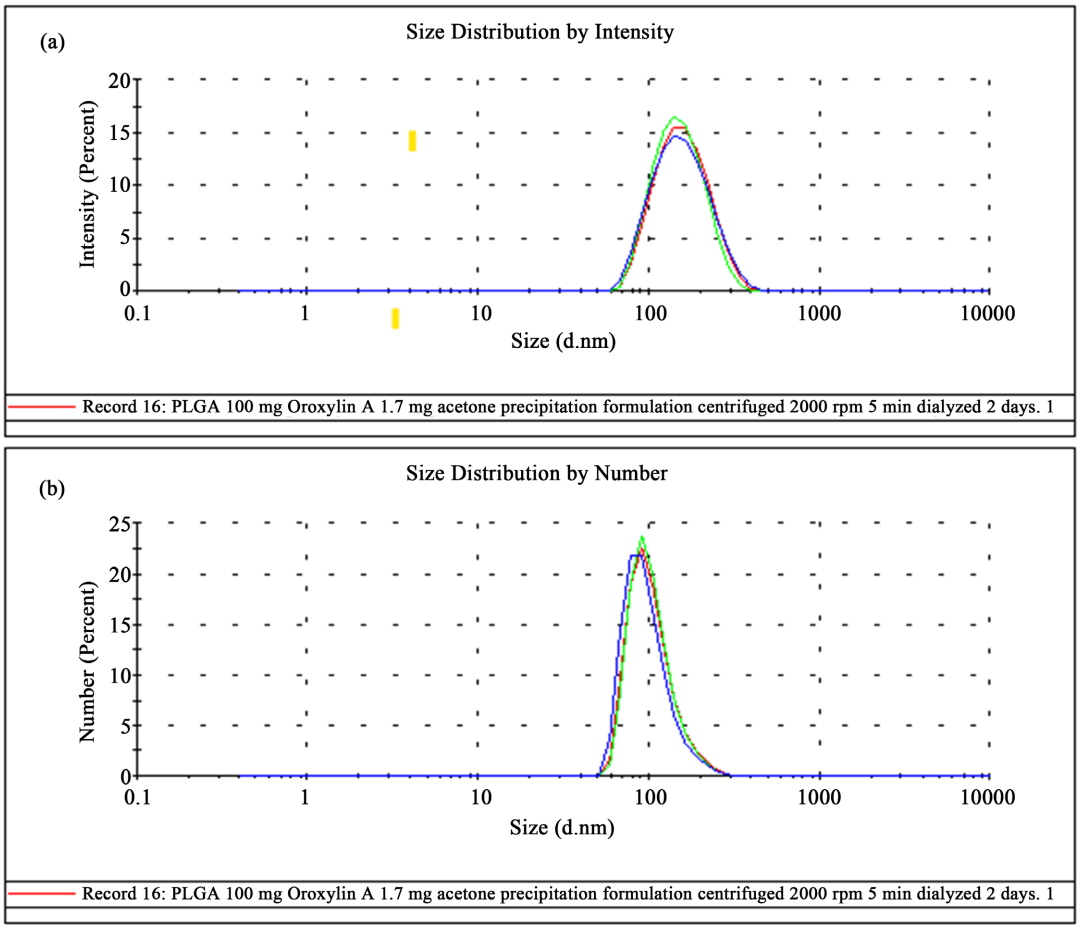


Figure 5. PLGA nanoparticles encapsulating Oroxylin A produced by acetone precipitation. Average size = 162 ± 5 nm. Polydispersity index = 0.12 ± 0.02. (a) Size distribution by intensity. (b) Size distribution by number. The results of three preparations (Record 16, Record 17, Record 18) determined by Dynamic Light Scattering (DLS).

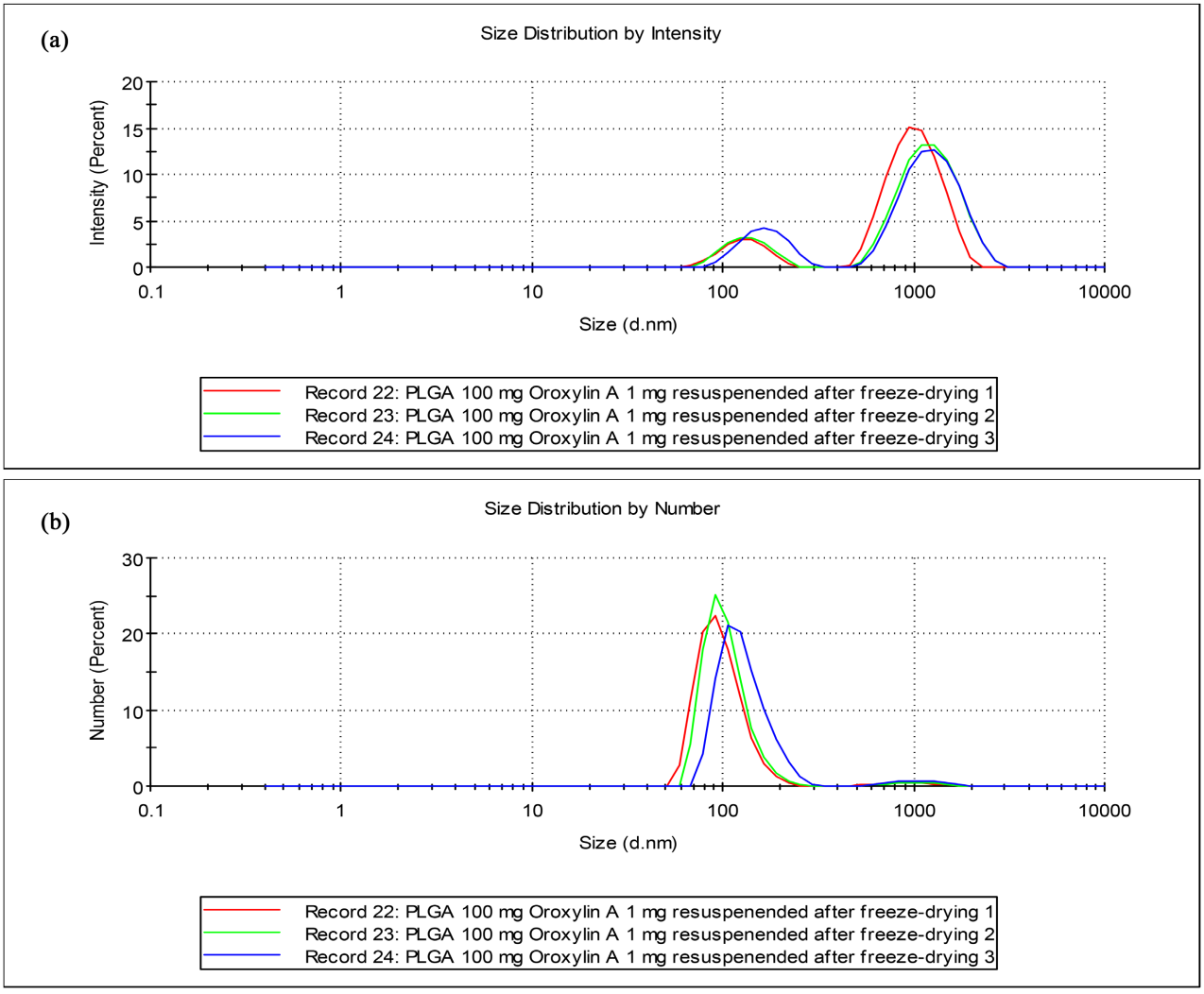
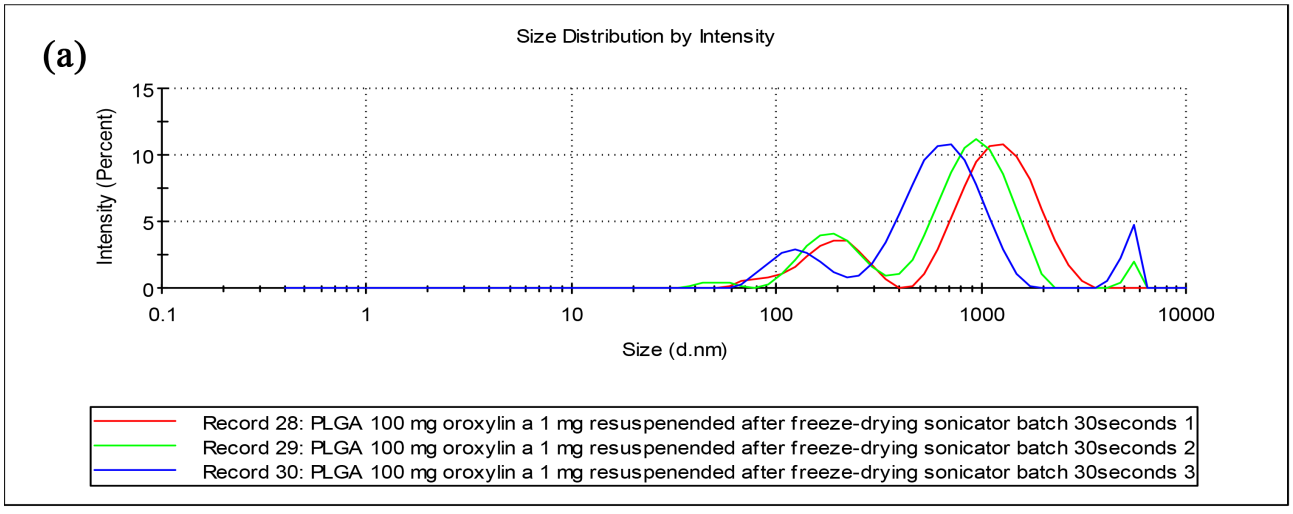


Figure 6. PLGA nanoparticles encapsulating Oroxylin A by acetone precipitation after freeze-drying and re-suspensing in deionised water. Average size = 1202 ± 138 nm (peak 1) and 147 ± 20 nm (peak 2). Polydispersity index = 0.64 ± 0.04. (a) Size distribution by intensity. (b) Size distribution by number. The results of three preparations (Record 22, Record 23, Record 24) determined by Dynamic Light Scattering (DLS).



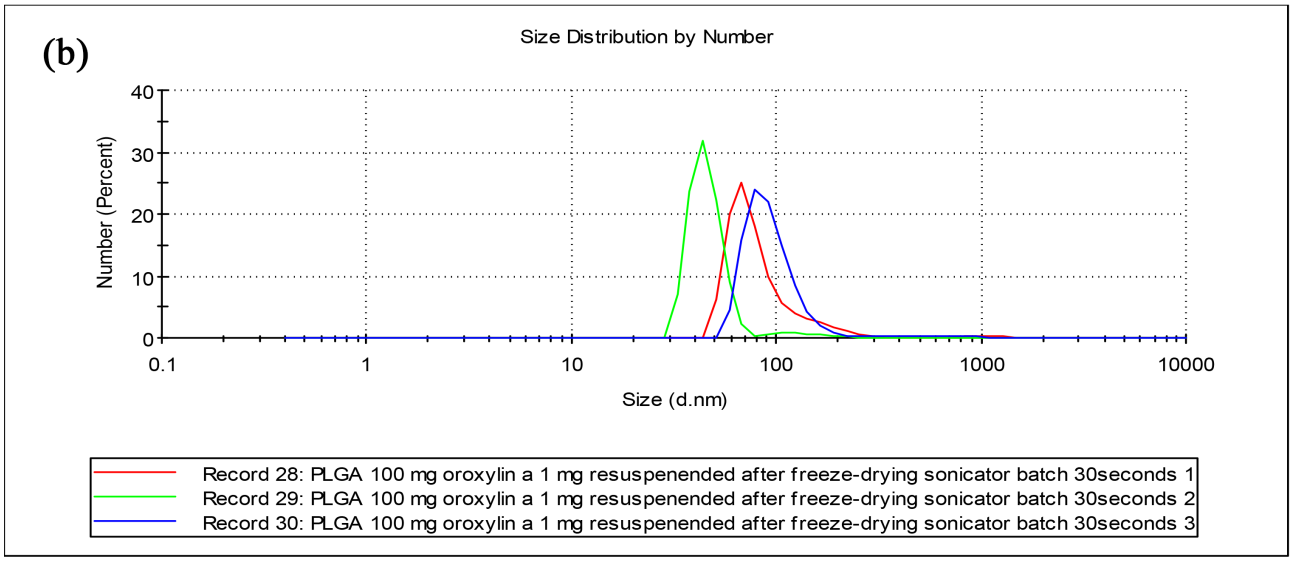


Figure 7. PLGA nanoparticles encapsulating Oroxylin A by acetone precipitation after freeze-drying, re-suspending in deionised water, and using bath sonication (40 kHz) to re-disperse the nanoparticles. Average size = 995 ± 312 nm (peak 1), 171 ± 35 nm (peak 2), 3547 ± 3073 nm (peak 3). Polydispersity index = 0.64 ± 0.13. (a) Size distribution by intensity. (b) Size distribution by number. The results of three preparations (Record 28, Record 29, Record 30) determined by Dynamic Light Scattering (DLS).

In this study, we measured the effect of Oroxylin A and encapsulated Oroxylin A on cell viability in melanoma cells in culture and apoptosis in melanoma cells in culture. The effects of PLGA and Solutol on melanoma cells, colon carcinoma cells and gastric adenocarcinoma cells were also studied. Initially, the effects of different Oroxylin A preparations on the viability of melanoma cells were determined (Table 2).

Table 2. Melanoma cell concentration after 24 hours of incubation in the presence of different Oroxylin A preparations. Viability determined by absorbance at 570 nm (mean ± SEM) following MTT reaction. Statistical significance (T-test) ($p < 0.05$). NS = Not significant. The stimulation or inhibition in the presence of the test samples is presented. The concentration of Oroxylin A in nanofibrils was not determined, but it is estimated that it would be of a similar order to the concentration of Oroxylin A in PLGA or Solutol.

Test Culture	Absorbance (MTT) at 570 nm (mean ± SEM)	Statistical Significance $p < 0.05$	% Inhibition	% Stimulation
Cells in 1.5% DMSO (control)	0.972 ± 0.012	-	-	-
Cells + Oroxylin A (40 μg/mL)	1.055 ± 0.052	NS	-	8.58%
Cells + Oroxylin A (20 μg/mL)	0.981 ± 0.033	NS	-	0.93%
Cells + Oroxylin A in PLGA (4.26 μg/mL in 1% PLGA)	0.362 ± 0.018	<0.0001	62.75%	-
Cells + Oroxylin A in Solutol (1.99 μg/mL in 0.1% Solutol)	0.356 ± 0.018	<0.0001	63.34%	-
Cells + Oroxylin A in Nanofibrils	0.289 ± 0.018	<0.0001	70.27%	-

The effect of Oroxylin A on melanoma cell viability showed that Oroxylin A alone was slightly stimulatory to melanoma cell growth at a higher concentration (40 μg/mL) (Table 2). However, incorporation of Oroxylin A into PLGA, Solutol

or nanofibrils markedly inhibited the growth of the cells (Table 2). This suggests the incorporation of Oroxylin A into the above carriers may be a useful means of inhibiting tumour growth. It is noted that the effective concentration of the Oroxylin A is lower when incorporated into the carriers.

Caspase-9 activity is a marker of apoptosis with increased caspase-9 activity being associated with increased apoptosis. When caspase-9 activity was calculated per viable melanoma cell, there was a decrease in activity using Oroxylin A alone at both 20 μg/mL and 40 μg/mL and also Oroxylin A incorporated into PLGA (Table 3). However, there was increased caspase-9 activity with Oroxylin A incorporated into Solutol and also into the nanofibrils, with the effect in the former being much stronger.

Table 3. Caspase-9 activity per melanoma cell after 24 hours of incubation in the presence of different Oroxylin A preparations. The caspase-9 activity was divided by the cell concentration (MTT absorbance) (mean ± SEM). Statistical significance (T-test) ($p < 0.05$). NS = Not Significant. The stimulation or inhibition in the presence of the test samples is presented.

Test Culture	Caspase Activity per Cell (mean ± SEM)	Statistical Significance $p < 0.05$	% Inhibition	% Stimulation
Cells in 1.5% DMSO (control)	0.019 ± 0.008	-	-	-
Cells + Oroxylin A (40 μg/mL)	0.018 ± 0.040	NS	5.26%	-
Cells + Oroxylin A (20 μg/mL)	0.016 ± 0.026	0.0016	15.83%	-
Cells + Oroxylin A in PLGA (4.26 μg/mL in 1% PLGA)	0	<0.0001	100.00%	-
Cells + Oroxylin A in Solutol (1.99 μg/mL in 0.1% Solutol)	0.030 ± 0.013	0.0001	-	58.00%
Cells + Oroxylin A in Nanofibrils	0.020 ± 0.014	0.0001	-	5.31%

In the course of the studies on the effect of Oroxylin A and of Oroxylin A encapsulated on cell viability, it was possible that PLGA or Solutol alone affected the viability of cancer cells. The results of the effects on cell viability are summarised in Table 4.

Solutol (particularly at the highest concentration used) inhibited cell viability of these cell cultures. Interestingly, PLGA did not have this effect. Therefore, the potential use of Solutol alone may be considered to have activity as a single agent in the treatment of cancer and therefore may provide a potentially additive effect over the encapsulated anti-cancer agent, particularly when the Solutol-drug particle retains its nanoparticulate form and the Enhanced Permeability and Retention (EPR) effect is taken into account [9].

In order to establish whether the results presented for Solutol and PLGA are confined to melanoma, the effects of these two carriers on colorectal carcinoma and gastric adenocarcinoma cells were determined.

Similar to the results with melanoma cells (Table 4), Solutol (particularly at the highest concentration used) inhibited cell viability of colorectal carcinoma cells and gastric adenocarcinoma cells (Table 5).

Table 4. Melanoma cell concentration after 24 hours of incubation in the presence of different concentrations of PLGA and Solutol. Viability determined by absorbance at 570 nm (mean ± SEM) following MTT reaction. Statistical significance (T-test) ($p < 0.05$). NS = Not Significant. The stimulation or inhibition in the presence of the test samples is presented.

Test Culture	Absorbance (MTT) at 570 nm (mean ± SEM)	Statistical Significance $p < 0.05$	% Inhibition	% Stimulation
Cells	0.199 ± 0.001			
Cells in 1.5% DMSO (control)	0.197 ± 0.004	-	-	-
Cells + PLGA (10 mg/mL)	0.250 ± 0.026	NS	-	26.76%
Cells + PLGA (3 mg/mL)	0.214 ± 0.013	NS	-	8.13%
Cells + PLGA (1 mg/mL)	0.179 ± 0.004	0.015	9.14%	-
Cells + PLGA (0.3 mg/mL)	0.220 ± 0.005	0.007	-	11.71%
Cells + PLGA (0.1 mg/mL)	0.191 ± 0.003	NS	3.02%	-
Cells + Solutol (10 mg/mL)	0.015 ± 0.001	<0.0001	91.16%	-
Cells + Solutol (3 mg/mL)	0.155 ± 0.007	<0.0001	21.71%	-
Cells + Solutol (1 mg/mL)	0.160 ± 0.002	<0.0001	19.09%	-
Cells + Solutol (0.3 mg/mL)	0.206 ± 0.005	NS	-	4.31%
Cells +Solutol (0.1 mg/mL)	0.204 ± 0.006	NS	-	3.28%

Table 5. Colorectal carcinoma cells (a) and gastric adenocarcinoma cells (b) concentration after 24 hours of incubation in the presence of different concentrations of PLGA and Solutol. Viability determined by absorbance at 570 nm (mean ± SEM) following MTT reaction. Statistical significance (T-test) ($p < 0.05$). NS = Not Significant. The stimulation or inhibition in the presence of the test samples is presented.

(a) HCT-116—Colorectal Carcinoma Cells				
Test Culture	**Absorbance (MTT) at 570 nm (mean ± SEM)**	**Statistical Significance $p < 0.05$**	**% Inhibition**	**% Stimulation**
Cells	0.707 ± 0.009	-	-	-
Cells in 1.5% DMSO (control)	0.615 ± 0.011	-	-	-
Cells + PLGA (10 mg/mL)	0.776 ± 0.021	<0.0001	-	26.31%
Cells + PLGA (3 mg/mL)	0.646 ± 0.019	NS	-	5.17%
Cells + PLGA (1 mg/mL)	0.623 ± 0.007	NS	-	1.37%
Cells + PLGA (0.3 mg/mL)	0.609 ± 0.010	NS	0.93%	-
Cells + PLGA (0.1 mg/mL)	0.549 ± 0.008	0.002	10.69%	-
Cells + Solutol (10 mg/mL)	0.011 ± 0.002	<0.0001	98.26%	-
Cells + Solutol (3 mg/mL)	0.537 ± 0.009	0.0006	12.55%	-
Cells + Solutol (1 mg/mL)	0.554 ± 0.007	0.002	9.80%	-
Cells + Solutol (0.3 mg/mL)	0.552 ± 0.016	0.015	10.22%	-
Cells +Solutol (0.1 mg/mL)	0.605 ± 0.007	NS	1.62%	-

Continued

(b) AGS—Gastric Adenocarcinoma Cells				
Test Culture	**Absorbance (MTT) at 570 nm (mean ± SEM)**	**Statistical Significance $p < 0.05$**	**% Inhibition**	**% Stimulation**
Cells	0.709 ± 0.023	-	-	-
Cells in 1.5% DMSO (control)	0.682 ± 0.006	-	-	-
Cells + PLGA (10 mg/mL)	0.876 ± 0.044	0.003	-	28.23%
Cells + PLGA (3 mg/mL)	0.783 ± 0.014	0.0002	-	14.70%
Cells + PLGA (1 mg/mL)	0.720 ± 0.008	0.005	-	5.49%
Cells + PLGA (0.3 mg/mL)	0.677 ± 0.026	NS	0.75%	-
Cells + PLGA (0.1 mg/mL)	0.646 ± 0.009	0.012	5.29%	-
Cells + Solutol (10 mg/mL)	0.013 ± 0.002	<0.0001	98.10%	-
Cells + Solutol (3 mg/mL)	0.650 ± 0.014	NS	4.71%	-
Cells + Solutol (1 mg/mL)	0.609 ± 0.008	0.0001	10.75%	-
Cells + Solutol (0.3 mg/mL)	0.651 ± 0.010	0.030	4.61%	-
Cells +Solutol (0.1 mg/mL)	0.724 ± 0.012	0.019	-	5.89%

The preparation of Oroxylin A incorporated into PLGA, Solutol or nanofibrils enables the impact of the EPR effect on tumours to be assessed. While the results of the studies carried out here are concerned with cell culture and not tumours *in situ*, it may be useful to consider the possible EPR effect of nanoparticle encapsulated Oroxylin A, as macromolecular drugs play a key role in tumour selective targeting [9].

In the *in vitro* studies reported here, a range of concentrations of Oroxylin A and incorporated Oroxylin A concentrations have been used, both in cell viability and apoptosis studies. As well, a range of PLGA and Solutol concentrations has been used in the studies on the cell viability of these macromolecular carriers. In a recent review, Graham *et al.* [14] caution the danger of translating the effect of *in vitro* drug concentrations to *in vivo* situations, especially as many drug studies have used supratherapeutic drug concentrations.

An experiment was undertaken to determine the IC_{50} of Solutol on melanoma cells and human skin fibroblasts, aimed to distinguish the action of this carrier on cancerous cells (melanoma) and non-cancerous cells (human skin fibroblasts). The IC_{50} value for the melanoma cells was 5.6 mg/mL and for the skin fibroblasts, it was 6.4 mg/mL. The fact that the IC_{50} for the two cell types was similar indicates that the effect of Solutol is not specific to cancer cells but also applies to normal skin cells. It is considered that the similar effect of Solutol on cancer cells and normal cells may be due to a detergent effect of Solutol on these cells.

These studies show that Oroxylin A enhances melanoma cell viability and inhibits apoptosis. However, cell viability is inhibited by Oroxylin A when integrated into all three carriers evaluated. Apoptosis in the melanoma cells is inhibited by

Oroxylin A and Oroxylin A incorporated into PLGA but is stimulated by Oroxylin A incorporated into Solutol. Thus, nanoparticle-encapsulated Oroxylin A may be useful as an anti-cancer drug. This effect may be both through affecting cell viability and inducing apoptosis. As well, Solutol alone may be a useful anti-cancer drug as it reduces the viability of melanoma cells, colorectal carcinoma cells and gastric adenocarcinoma cells. However, as stated in the above paragraph, the effect of Solutol may be due to its detergent action.

4. Discussion

The results obtained in the present study are clearly distinguished from those reported in the published literature. In all cases, findings by others are clearly identified with the relevant references. The reference [2] is a commentary on the overexpression of uncoupling protein-2 in cancer and contains many references to studies in this area.

4.1. Characteristics of PLGA and SOLUTOL

Poly (lactic-co-glycolic acid) (PLGA) was used as the platform delivery system for the encapsulation of Oroxylin A due to its widespread use in pharmaceutical science for the formulation of poorly water-soluble drugs [15] [16]. The metabolic products of PLGA produce lactic acid and glycolic acid, which are subsequently eliminated as carbon dioxide and water via the Krebs cycle. Therefore, PLGA nanoparticles are biodegradable, biocompatible, and approved by the FDA as a pharmaceutical excipient.

Poly (lactic-co-glycolic acid) (PLGA) is a copolymer of lactic acid and glycolic acid [10]. Solutol HS 15 (Solutol) is a non-ionic surfactant and is composed of polyglycol mono- and di-esters of 12-hydroxystearic acid and free polyethylene glycol [11]. There is little evidence that PLGA or Solutol nanoparticles have intrinsic anti-cancer effect.

PLGA decomposes into H_2O and CO_2 that are eliminated in the body. Its polymeric nanoparticles degrade *in vivo* through hydrolysis of the ester bonds to its monomeric anions (lactate and glycolate). While D-Lactate is not further metabolised before excretion, L-Lactate is converted into CO_2, which is excreted through the lungs and is converted to pyruvate, which then enters the Krebs cycle. Glycolate is either directly excreted through the renal system or it can be oxidised to glyoxylate, which is subsequently converted into glycine, serine and pyruvate. The latter can again enter the Krebs cycle and be metabolised into CO_2 and H_2O [10].

PLGA-based nanoparticles encapsulating anti-cancer drugs have been widely used [10]. Notwithstanding the above, Solutol is known to reverse multi-drug resistance of cancer cells [17]. Also, Solutol-based nanoparticles encapsulating anti-cancer drugs have been used in several studies [18]-[22]. In these studies, Solutol is mixed with other polymers to form mixed micelles. Despite the limited evidence of Solutol alone affecting cancer cells, we found in this study that Solutol alone does decrease the viability of cancer cells (**Table 5**).

4.2. Changes on Cancer Cells and Some Metabolic Effects of Uncoupling Protein-2 (UCP_2) (Table 6 and Figure 8)

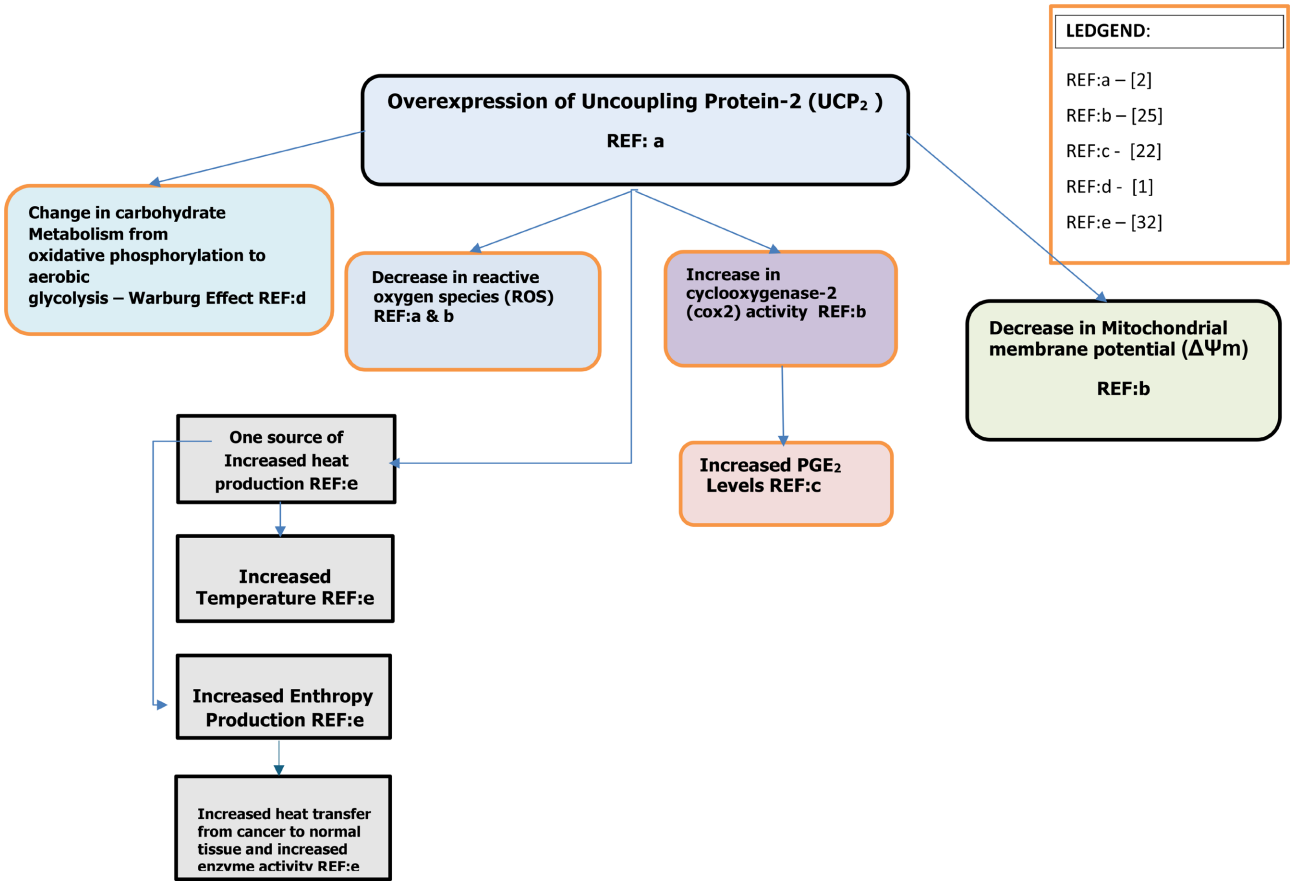


Figure 8. Some metabolic changes due to overexpression of UCP_2 in cancer cells. These changes include decrease in ROS, increase in COX2 activity and increase in PGE_2 levels.

Table 6. Oroxylin A reverses these changes. Hence, Oroxylin A inhibits UCP_2 expression, stimulates ROS, inhibits COX2 expression and lowers PGE_2 levels in cancer cells.

Effect of Oroxylin A on Metabolic Changes in Cancer	
Inhibition of UCP_2 Expression	[7]
Stimulation of ROS	[25]
Inhibition of COX-2 Expression	[25]
Lower PGE_2 Levels	[25]

4.3. Effects of Oroxylin A and Encapsulated Oroxylin A on Cancer Cells

In this study, we observed that melanoma cell viability is not affected following administration of Oroxylin A to cancer cells. However, Zhu *et al.* [23] observed decreased cell viability of squamous cell carcinoma cells following Oroxylin A treatment after a lengthy incubation of 48 hours. Also, Zhao *et al.* [24] and Ha *et al.* [25] demonstrated decreased cell viability of human hepatocellular carcinoma cells

and human colon cancer cells respectively following administration of Oroxylin A. However, our studies showed decreased cell viability following administration of Oroxylin A encapsulated nanoparticles to melanoma cells. We did not observe decreased cell viability following administration of non-encapsulated Oroxylin A (**Table 2**). The difference of our findings for Oroxylin A treatment of cancer calls and those of Zhu *et al.* [23], Zhao *et al.* [24], Ha *et al.* [25] may be due to the fact that different cancer cells have been used in each of these studies. We showed increased apoptosis following treatment of cancer cells with Oroxylin A encapsulated with Solutol but not with PLGA. Interestingly, Zhu *et al.* [23] showed Oroxylin A-loaded PDots caused increased apoptosis in squamous cells carcinoma.

4.4. Effect of Oroxylin A on UCP_2 and Changes in Carbohydrate Metabolism in Cancer Cells

Oroxylin A is an inhibitor of UCP_2 [7]. We consider that the effects outlined above of Oroxylin A encapsulated nanoparticles on cancer cells are due, at least in part, to the inhibition of UCP_2 by Oroxylin A. UCP_2 converts the carbohydrate metabolism of cancer cells from mitochondrial oxidative phosphorylation to aerobic glycolysis. It does this by uncoupling mitochondrial oxidative phosphorylation. Baffy *et al.* [8] have suggested that recoupling mitochondrial oxidative phosphorylation would be a novel way to treat cancer. As Oroxylin A is an inhibitor of UCP_2, this agent would be a suitable candidate to cause the recoupling of mitochondrial oxidative phosphorylation.

4.5. Effect of Oroxylin A and Oroxylin A Encapsulated Nanoparticles on Tumour Size and Weight of Cancer Cells. Synergistic Effect of Oroxylin A and 5-Fluorouracil on Cancer Cells

Recently, Zhu *et al.* [23] investigated the effect of Oroxylin A-Loaded PDots on squamous cell carcinoma cells. Both Oroxylin A alone and Oroxylin A-Loaded PDots inhibited cell growth, however Oroxylin A-Loaded PDots were more potent. Zhu *et al.* [23] also found that Oroxylin A-Loaded PDots increased apoptosis and that Oroxylin A and Oroxylin A-loaded PDots were effective in decreasing size and weight of implanted tumours in mice.

Zhao *et al.* [24] studied synergism of Oroxylin A and fluorouracil on liver cancer viability and observed that Oroxylin A alone inhibited cell growth, increased apoptosis and reduced growth of implanted tumours. Similarly, Ha *et al.* [25] carried out parallel studies on the synergism of Oroxylin A and 5-fluorouracil on colon cancer cells. They found Oroxylin A inhibited cell growth, enhanced apoptosis and reduced growth of implanted tumours. When combined with 5-fluorouracil, Oroxylin A was more effective than Oroxylin A alone in decreasing weight and size of implanted tumours [24] [25]. Other studies have shown that other inhibitors of UCP_2, when combined with anti-cancer agents, are more effective in decreasing cell viability and increasing ROS production and apoptosis than the inhibitors alone [2] (reference therein).

In non-small cell lung cancer, Oroxylin A reverses hypoxia-induced cisplatin resistance [26]. The combination of Oroxylin A and gefitinib in A549 cells instigated more apoptotic cells than other studied combinations and gefitinib alone [27].

4.6. Oroxylin A and Encapsulated Oroxylin A Nanoparticles and the EPR Effect

The effects of encapsulated Oroxylin A nanoparticles and Oroxylin A PDots on cancer cells have been demonstrated in cancer cells in culture. Maeda [9] has shown that certain high molecular weight substances are more effective in the treatment of whole tumours (Enhanced Permeability and Retention Effect—EPR Effect). Although the studies carried out by us have been on cultured cancer cells, Zhu *et al.* [23] have shown that Oroxylin A-Loaded PDots do shrink tumour size and weight. Despite our studies with cultured cancer cells, it is interesting that we only achieved a decrease in cell viability and an increase in apoptosis using Oroxylin A nanoparticles and not using Oroxylin A alone.

4.7. Effect of PLGA or Solutol Alone on Cancer Cell Viability and Apoptosis

In this work, we treated cancer cells with PLGA or Solutol alone. We found that Solutol decreased cancer cell viability, but PLGA did not. It appears that the effect of Solutol alone may be due to its properties as a detergent.

4.8. UCP_2 Releases Heat and the Effect of Increased Temperature, Heat and Entropy Production on Cancer Cells

Lawson *et al.* [28], Lawson *et al.* [29] and Stefanadis *et al.* [30] [31] have shown that there is an increase in the temperature of cancers compared to normal tissue. This temperature increase has been attributed to increased heat production by these tumours [32]. UCP_2 has been shown to release heat [8] and so its overexpression in cancer is at least one source of the extra heat production in cancer [32]. Conversely, inhibition of UCP_2 by Oroxylin A will reduce the heat production in cancers. The heat production in cancer also results in increased entropy production in these tumours. Increased entropy production is a feature of a departure from the steady state, where entropy production is a minimum. Growing tumors are a departure from the steady state of normal tissue [32].

4.9. Apoptosis Studies in Cancer Cells

Tuli *et al.* [6] have reviewed the anti-cancer potential of Oroxylin A and noted that apoptosis is lacking in cancer cells, resulting in immortal malignant cells. They then proceed to cite numerous studies showing that Oroxylin A enhances apoptosis in cancer cells. Consistent with these findings, our studies have shown that Oroxylin A encapsulated in Solutol enhances apoptosis as shown by increased caspase-9 activity.

Consent for Publication

All authors have read and approved this manuscript.

Availability of Data and Material

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Funding

No external financial support or grants were received from any public, commercial, or not-for-profit entities for the research, authorship, or publication of this article.

Author Contributions

The design of the experiments was formulated by Paul Davis, Catherine Davis and Michael A. Pitt. Paul Davis and Catherine Davis carried out the tests on cancer cell viability and apoptosis studies. Sean Mackay and Eng Wui Tan synthesised the Oroxylin A Encapsulated Nanoparticles. The discussion, particularly, is a collaborative effort.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Warburg, O. (1956) On the Origin of Cancer Cells. *Science*, **123**, 309-314. https://doi.org/10.1126/science.123.3191.309

[2] Pitt, M.A. (2015) Overexpression of Uncoupling Protein-2 in Cancer: Metabolic and Heat Changes, Inhibition and Effects on Drug Resistance. *Inflammopharmacology*, **23**, 365-369. https://doi.org/10.1007/s10787-015-0250-3

[3] Lu, L., Guo, Q. and Zhao, L. (2016) Overview of Oroxylin A: A Promising Flavonoid Compound. *Phytotherapy Research*, **30**, 1765-1774. https://doi.org/10.1002/ptr.5694

[4] Sajeev, A., Hegde, M., Daimary, U.D., Kumar, A., Girisa, S., Sethi, G., *et al.* (2022) Modulation of Diverse Oncogenic Signaling Pathways by Oroxylin A: An Important Strategy for Both Cancer Prevention and Treatment. *Phytomedicine*, **105**, Article 154369. https://doi.org/10.1016/j.phymed.2022.154369

[5] Wang, P.X., Mu, X.H., Huang, S.H., *et al.* (2024) Cellular and Molecular Mechanisms of Oroxylin A in Cancer Therapy: Recent Advances. *European Journal of Pharmacology*, **969**, Article 176452. https://doi.org/10.1016/j.ejphar.2024.176452

[6] Tuli, H.S., Garg, V.K., Kumar, A., Aggarwal, D., Anand, U., Parashar, N.C., *et al.* (2023) Anticancer Potential of Oroxylin A: From Mechanistic Insight to Synergistic Perspectives. *Naunyn-Schmiedeberg's Archives of Pharmacology*, **396**, 191-212. https://doi.org/10.1007/s00210-022-02298-0

[7] Qiao, C., Wei, L., Dai, Q., Zhou, Y., Yin, Q., Li, Z., *et al.* (2015) UCP2-Related Mitochondrial Pathway Participates in Oroxylin A-Induced Apoptosis in Human Colon Cancer Cells. *Journal of Cellular Physiology*, **230**, 1054-1063. https://doi.org/10.1002/jcp.24833

[8] Baffy, G., Derdak, Z. and Robson, S.C. (2011) Mitochondrial Recoupling: A Novel Therapeutic Strategy for Cancer? *British Journal of Cancer*, **105**, 469-474. https://doi.org/10.1038/bjc.2011.245

[9] Maeda, H. (2001) The Enhanced Permeability and Retention (EPR) Effect in Tumor Vasculature: The Key Role of Tumor-Selective Macromolecular Drug Targeting. *Advances in Enzyme Regulation*, **41**, 189-207. https://doi.org/10.1016/s0065-2571(00)00013-3

[10] Rezvantalab, S., Drude, N.I., Moraveji, M.K., Güvener, N., Koons, E.K., Shi, Y., *et al.* (2018) PLGA-Based Nanoparticles in Cancer Treatment. *Frontiers in Pharmacology*, **9**, Article 1260. https://doi.org/10.3389/fphar.2018.01260

[11] Ali, S. and Kolter, K. (2019) Kolliphor® HS 15—An Enabler for Parenteral and Oral Formulations. *American Pharmaceutical Review*, **22**. https://www.americanpharmaceuticalreview.com/Featured-Articles/358749-Kolliphor-HS-15-An-Enabler-for-Parenteral-and-Oral-Formulations/

[12] Mosmann, T. (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, **65**, 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

[13] Ramalho, M.J. and Pereira, M.C. (2016) Preparation and Characterization of Polymeric Nanoparticles: An Interdisciplinary Experiment. *Journal of Chemical Education*, **93**, 1446-1451. https://doi.org/10.1021/acs.jchemed.5b00837

[14] Graham, G.G. and Scott, K.F. (2021) Mechanism of Action of Paracetamol. *American Journal of Therapeutics*, **12**, 46-55. https://doi.org/10.1097/00045391-200501000-00008

[15] Makadia, H.K. and Siegel, S.J. (2011) Poly Lactic-Co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polymers*, **3**, 1377-1397. https://doi.org/10.3390/polym3031377

[16] Mirakabad, F.S.T., Nejati-Koshki, K., Akbarzadeh, A., Yamchi, M.R., *et al.* (2014) PLGA-Based Nanoparticles as Cancer Drug Delivery Systems. *Asian Pacific Journal of Cancer Prevention*, **15**, 517-535. https://doi.org/10.7314/apjcp.2014.15.2.517

[17] Coon, J.S., Knudson, W., Clodfelter, A., *et al.* (1991) Solutol HS 15, Nontoxic Polyoxyethylene Esters of 12-Hydroxystearic Acid, Reverses Multidrug Resistance. *Cancer Research*, **51**, 897-902.

[18] Oh, K.S., Kim, K., Yoon, B.D., Lee, H.J., *et al.* (2016) Docetaxel-Loaded Multilayer Nanoparticles with Nanodroplets for Cancer Therapy. *International Journal of Nanomedicine*, **11**, 1077-1087. https://doi.org/10.2147/ijn.s100170

[19] Hou, J., Sun, E., Sun, C., Wang, J., Yang, L., Jia, X., *et al.* (2016) Improved Oral Bioavailability and Anticancer Efficacy on Breast Cancer of Paclitaxel via Novel Soluplus®-Solutol® HS15 Binary Mixed Micelles System. *International Journal of Pharmaceutics*, **512**, 186-193. https://doi.org/10.1016/j.ijpharm.2016.08.045

[20] Bergonzi, M.C., Vasarri, M., Marroncini, G., Barletta, E. and Degl'Innocenti, D. (2020) Thymoquinone-Loaded Soluplus®-Solutol® HS15 Mixed Micelles: Preparation, *in Vitro* Characterization, and Effect on the SH-SY5Y Cell Migration. *Molecules*, **25**, Article 4707. https://doi.org/10.3390/molecules25204707

[21] Yan, H., Zhang, Z., Jia, X. and Song, J. (2016) D-α-Tocopheryl Polyethylene Glycol Succinate/Solutol HS 15 Mixed Micelles for the Delivery of Baohuoside I against Non-Small-Cell Lung Cancer: Optimization and *in Vitro*, *in Vivo* Evaluation. *International Journal of Nanomedicine*, **11**, 4563-4571. https://doi.org/10.2147/ijn.s112204

[22] Surh, Y.J., Chun, K.S., Cha, H.H., Han, S.S., *et al.* (2001) Molecular Mechanisms Underlying Chemopreventive Activities of Anti-Inflammatory Phytochemicals: Down-

Regulation of COX-2 and iNOS through Suppression of NF-κB Activation. *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, **480**, 243-268. https://doi.org/10.1016/s0027-5107(01)00183-x

[23] Zhu, Y., Guo, Y., Liu, M., Wei, L. and Wang, X. (2020) An Oroxylin A-Loaded Aggregation-Induced Emission Active Polymeric System Greatly Increased the Antitumor Efficacy against Squamous Cell Carcinoma. *Journal of Materials Chemistry B*, **8**, 2040-2047. https://doi.org/10.1039/c9tb01818b

[24] Zhao, L., Chen, Z., Wang, J., Yang, L., Zhao, Q., Wang, J., *et al.* (2010) Synergistic Effect of 5-Fluorouracil and the Flavanoid Oroxylin a on HEPG2 Human Hepatocellular Carcinoma and on H22 Transplanted Mice. *Cancer Chemotherapy and Pharmacology*, **65**, 481-489. https://doi.org/10.1007/s00280-009-1053-2

[25] Ha, J., Zhao, L., Zhao, Q., Yao, J., Zhu, B., Lu, N., *et al.* (2012) Oroxylin A Improves the Sensitivity of HT-29 Human Colon Cancer Cells to 5-FU through Modulation of the COX-2 Signaling Pathway. *Biochemistry and Cell Biology*, **90**, 521-531. https://doi.org/10.1139/o2012-005

[26] Liu, Y., Wang, X.P., Li, W., Xu, Y., *et al.* (2020) Oroxylin A Reverses Hypoxia-Induced Cisplatin Resistance through Inhibiting HIF-1*α* Mediated XPC Transcription. *Oncogene*, **39**, 6893-6905. https://doi.org/10.1038/s41388-020-01474-x

[27] Hirata, Y., Tatsu, M., Amano, C., Kawaratani, Y., Hirata, M., Ohmomo, Y., *et al.* (2017) Synergistic Antitumor Effect of Genitinib (Iressa®) with Flavonoids from the *Scutellaria baicalensis* Root on the Non-Small Cell Lung Cells. *The Open Plant Science Journal*, **10**, 62-69. https://doi.org/10.2174/1874294701710010062

[28] Lawson, R.N. and Gaston, J.P. (1964) Temperature Measurements of Localized Pathological Processes. *Annals of the New York Academy of Sciences*, **121**, 90-98. https://doi.org/10.1111/j.1749-6632.1964.tb13688.x

[29] Lawson, R.N. and Chughtai, M.S. (1963) Breast Cancer and Body Temperature. *Canadian Medical Association Journal*, **88**, 68-70.

[30] Stefanadis, C., Chrysochoou, C., Markou, D., Petraki, K., Panagiotakos, D.B., Fasoulakis, C., *et al.* (2001) Increased Temperature of Malignant Urinary Bladder Tumors *in Vivo*: The Application of a New Method Based on a Catheter Technique. *Journal of Clinical Oncology*, **19**, 676-681. https://doi.org/10.1200/jco.2001.19.3.676

[31] Stefanadis, C., Chrysohoou, C., Panagiotakos, D.B., Passalidou, E., Katsi, V., Polychronopoulos, V., *et al.* (2003) Temperature Differences Are Associated with Malignancy on Lung Lesions: A Clinical Study. *BMC Cancer*, **3**, Article No. 1. https://doi.org/10.1186/1471-2407-3-1

[32] Pitt, M.A. (2015) Increased Temperature and Entropy Production in Cancer: The Role of Anti-Inflammatory Drugs. *Inflammopharmacology*, **23**, 17-20. https://doi.org/10.1007/s10787-014-0224-x

Abbreviations

ATP	Adenosine Triphosphate
UCP_2	Uncoupling Protein-2
EPR EFFECT	Enhanced Permeability and Retention Effect
B16/F10	Mouse Melanoma (B16/F10) Cells
HCT-116	Human Carcinoma Cells
AGS	Adenocarcinoma Cells
ATCC	American Type Cell Culture
DMEM	Dulbecco's Modified Eagles Medium
EMEM	Eagles Minimum Essential Medium
PLGA	Poly (D,L-Lactide-co-glycolide) (lactide:glycolide) (1:1) ester terminated (MW 24,000 - 38,000)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DLS	Dynamic Light Scattering
COX2	Cyclooxygenase 2
PGE_2	Prostaglandin E_2
PBS	Phosphate-Buffered Saline
ROS	Reactive Oxygen Species
HCT-116	Human Colon Carcinoma Cells
AGS	Human Gastric Adenocarcinoma Cells