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Original article

Enhanced anticancer effect of quercetin microparticles formulation obtained by spray drying

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Abstract This study unravels a formulation made of food-based microparticles (MPs) able to control the release of quercetin, a natural anticancer compound, which activity is only limited by its poor aqueous solubility and consequent low bioavailability. To solve this issue, a spray-dried micro delivery system was developed using a bench mini spray dryer B290 Buchi. The resulting MPs were only manufactured with food-derived ingredients such as whey proteins and milk, avoiding the use of any other synthetic material. These microparticles were characterised with a testing campaign encompassing either the physical–chemical characterisation with SEM, DSC and DLS, or the technological and biological features with in vitro, ex vivo and in vivo studies, the latter being characterised by a human colon cancer xenograft model. These studies showed as the quercetin solubility and release rate improved when tested in condition mimicking oral administration, resulting in a general improvement of its bioavailability and the consequent anticancer activity. This study shows as the whey proteins may serves as natural adjuvant able to provide valuable technological features when used to manufactures micro carriers by spray drying.

Keywords Anticancer effect, bioavailability, microparticles, oral administration, quercetin, spray drying, whey proteins.

Introduction

Recently, the administration of quercetin-based supplements has been growing worldwide, particularly in Europe as evidenced by the European Health Product Manufacturers & Distributors (EHPM), the leading European trade association representing the food supplements. Supplements, defined as food products that help maintain a healthy homeostasis, are perceived as a more natural choice to treat pathological conditions linked to deficient levels of vitamins, minerals, fatty acids and other nutrients; they restore the homeostasis and a healthy condition. Among all the natural BACs (BioActive Compound) employed by the supplement industry, quercetin is one of the more clinically studied (Katske *et al*., 2001; National Institute of Diabetes and Digestive and Kidney Diseases,; Vicente-Vicente *et al*., 2019;). Quercetin is a natural flavonoid known for its multiple pharmacological effect, such as nephron-protector (Aldemir *et al*., 2014; Vicente-Vicente *et al*., 2019), anticancer (Vafadar *et al*., 2020; Zang *et al*., 2021), anti-inflammation element (Askari *et al*., 2012) and antibacterial (Hirai *et al*., 2010). Despite the quercetin therapeutic potential, its actual efficacy is limited by its poor water solubility and consequent low bioavailability. To date, quercetin clinical data regarding the bioavailability following oral administration report very low values (1%) (Cai *et al*., 2013; Moon *et al*., 2008). It is also noteworthy as the quercetin elimination of half-life is reported as low as about 0.7 h and absorption following oral administration ranges in the low values in between 0 and ≈ 50% of the dose (Graefe *et al*., 1999). Part of these low technological performances are the consequence of the chemical instability of the glycosides when exposed at the oral physiological condition. These quercetin glycosides, when in the gastrointestinal tract, are indeed degraded by the harsh pH and enzymes operating a partial hydrolysis. In addition, a first-pass effect also affects the quercetin low bioavailability. Quercetin can be absorbed through the small intestine mainly in the form of glucuronides of the parent aglycons or the hydrolysed glycoside. Various

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techniques have been developed to increase the quercetin solubility and absorption, including particle size reduction (Nam *et al*., 2016) as for instance spray-drying techniques (Indra *et al*., 2020; Sansone *et al*., 2011), binary mixtures (Zaini *et al*., 2016) and solid dispersions. All the aforementioned studies employ synthetic adjuvants. The formulation herein described combines the quercetin preservation from gastric degradation, the control over the release rate when in the target tissue and the use of only food ingredients yielding a clean and green controlled delivery system. These features altogether provide a micro delivery system able to sharply increase the percentage of administered dose reaching the absorption site, maximise the bloodstream concentrations and better suit the 'spirit' of supplements with an all-natural formulation, which uses whey proteins as surrogate of synthetic adjuvant as the eudragit. Whey proteins are a natural source of energetic and nutritious foods and people commonly use them as supplements, alongside resistance training, for improving muscle growth. Moreover, there are many benefits associated with the consumption of whey protein, and researchers are constantly finding new possible therapeutic properties, including aiding weight loss (Frestedt *et al*., 2008), anti-cancer properties (Bounous, 2000) and lowering cholesterol (Pal *et al*., 2010). Rarely whey proteins have been used as natural functional adjuvant in pharmaceutical or supplements formulation. In our case, the advantageous physical–chemical properties of whey protein, such as pH depending solubility, site-selective digestibility, drying characteristics, stability and biocompatibility, were used to generate a controlled release microparticles (MPs) formulation without using synthetic materials. Actually, the aim of this study was to develop and characterise a formulation of quercetin-loaded MPs produced by spray drying with only food-derived molecules, and to evaluate its ability to increase the potential anticancer effect of quercetin by oral administration (Davoodvandi *et al*., 2020; Rauf *et al*., 2018; Vafadar *et al*., 2020).

Materials and methods

Materials

Quercetin was purchased by Sigma-Aldrich, Italy. Whey proteins (WPs) were purchased by Bulk Powders, England; all other reagents were of analytical grade.

Human colon cancer cell HCT116 was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v of fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 $mgml^{-1}$), penicillin G (100 units ml^{-1}), amphotericin B (2.5 $\mu g\ ml^{-1}$), all purchased from Euroclone, and incubated al 37 °C in humidified environment with 5% of CO_2.

Animals

Wistar female rats (weighing 250–350 g) and male athymic nude mice (Fox1nu nu^{-1}) (weighing 18–20 g) were purchased from ENVIGO Srl (San Pietro al Natisone UD, Italy).

Preparation of the MPs formulation

The MPs were prepared with a mini spray dryer Buchi B290 (Buchi, Germany). The spray drying processes were operated following parameters: Inlet T: 130 °C; Outlet T: 70 °C; aspiration: 100%; Feed pump: 15%; Atomizer nozzle: 0.7 mm; Used gas: Compressed Air. To prepare the feed, 5.5 g of whey proteins were dispersed for 30 min by continuous stirring at 25 °C in 100 ml of whole milk and then 1 g of quercetin was added and the dispersion was stirred for other 30 min. The feed was then nebulised and dried by the spray dryer following the conditions reported above.

BAC loading

The drug loading (DL%) was calculated according to the below formula, and the amount of quercetin actually embodied in each micro delivery system was assessed by spectrophotometric analysis (Jasco V-760 spectrophotometer) reading at the maximum wavelength (λ max) of the quercetin in ethanol of 373 nm.

$$\%DL = \frac{Mass\ of\ active\ in\ microparticles}{mass\ of\ microparticles} \times 100$$

The incorporation efficiency (%IE) was determined accounting for the embodied quercetin within their respective micro delivery systems and the amount introduced in the feed of the spray dryer.

$$\%IE = \frac{Mass\ of\ active\ in\ microparticles}{mass\ of\ active\ in\ the\ feed\ (excluding\ water)} \times 100$$

All the experiments were carried out in triplicate.

Microscopy

Particle size and morphology were measured by SEM imaging technique, using Phenom ProXSEM (Alfatest, Italy). The SEM analysis was performed at 25.0 °C ± 0.1 °C, accelerating voltage of 10kV and operational distance of 2 mm.

Differential scanning calorimetry (DSC)

Differential Scanning Calorimetry (DSC) analysis was performed with Setaram DSC131 EVO (Setaram, Switzerland), a heating rate of 20 K min^{-1} and 120 µl aluminium crucibles. Pure actives, pure excipients,

physical mixtures of them and final micro delivery systems were analysed.

In vitro drug release

In vitro release of the quercetin embodied in the MPs formulation (MPs) was assessed using a dialysis bag method. MPs (100 mg) were dispersed in 5 ml of 0.1 M HCl, poured into a cellulose ester dialysis membrane, cut-off 12–14 kDa and sealed with appropriate universal closures. The membrane constitutes the donor compartment, which is further immersed in 95 ml of 0.1 M HCl (receiving compartment). The system (receiving + donating compartments) is thus maintained at pH = 1, under stirring and constant temperature (100 rpm, 37 °C) for 2 h. After 2 h, the pH of the receiving compartment is increased up to 6.8, to mimic the first tract of the intestine, and this pH is maintained for the following 4 h. It follows a further pH increase at 7.2, to mime the colon until the last sampling made after 24 h from the beginning of the experiment. Each hour, 1 ml samples were taken, freeze-dried and resolubilised in 1 ml of ethanol in order to extract quercetin, and then the absorbance was monitored using a spectrophotometer Jasco V-760 (cuvette optical path 10 mm, λ max = 373 nm). A calibration curve of pure quercetin was used for quantization.

Cytotoxicity assay

The cytotoxicity studies on human colon cancer (HCT116) cell line were carried out by the tetrazolium salt (MTS) assay, using a commercially available kit (Cell Titer 96 Aqueous One Solution Cell Proliferation assay, Promega). Cells were seeded at a density of 2.5•104 cells/well in 96-well plates with supplemented medium (DMEM) and allowed to adhere for 24 h. Therefore, cells were incubated for 4, 24 and 48 h with quercetin-loaded MPs and free quercetin (dispersed in saline solution), at a drug concentration per well equal to 100, 200 and 300 μM. Untreated cells were used as negative control. After 4, 24 and 48 h of incubation, DMEM was replaced with 100 μL of fresh medium, and 20 μL of MTS solution was added to each well. Plates were incubated for an additional 2 h at 37 °C. Then the absorbance at 492 nm was measured using a microplate reader (PlateReader AF2200, Eppendorf). The cell vitality was expressed as percentage obtained from the ratio between each sample with respect to their negative control (100% of cell vitality).

In vivo bioavailability

Female Wistar rats were randomly divided into 3 groups, 2 treated and one control (n = 5 per group): saline solution (sham group); group (2) (5 mg Kg^{-1} of quercetin); group (3) MPs (25 mg MPs, equivalent to 5 mg Kg^{-1} of quercetin). Aliquots of saline solution or compounds (0.5 ml) were administered by oral gavage and blood samples were collected via the tail vein under isoflurane anaesthesia before (time 0) and at six time points after administration (0.5, 1, 2, 6, 8 and 24 h). Quercetin and MPs were dispersed in saline solution.

Tumour xenograft experiments

To generate a tumour xenograft, $5•10^6$ human colon cancer (HCT116) cells in 200 μL of sterile PBS were subcutaneously injected to the lower back of 4-week-old nude male mice. Mice were randomly divided into treatment or control groups in each experiment after that the tumour masses were appeared and palpable. When tumours became palpable (around 3x3 mm), mice were randomly divided into 3 groups, each group containing five mice. More specifically, animals in group 1 received saline solution (0.05 ml, 0.9% NaCl) by oral gavage. Animals in group 2 received pure quercetin (0.05 ml, 5mg ml^{-1} in saline solution, corresponding to 1 mg Kg^{-1} dye). Animals in group 3 received MPs (0.05 ml, 100 mg ml^{-1} in saline solution, corresponding to 1 mg Kg^{-1} dye of quercetin). Every 3 days, mice received the quercetin and MPs; they were then weighted and the tumours were measured by external calliper. Tumour volume was calculated by the formula $V = L•W^2\ 2^{-1}$, where L is the longest diameter (mm) of the tumour and W (mm) is the longest perpendicular diameter with respect to L. After 10 days, mice were sacrificed and each tumour was excised for final weighting.

Statistical analysis

Results obtained from multiple samples were expressed as mean ± standard deviation (SD). Statistical differences were analysed by student's T-Test. *P-value* < 0.05 (*) was defined as the level of statistical significance.

Results and discussion

Preparation and characterisation of the micro delivery systems

The MPs formulation was developed using spray-drying micronization processes (Modica De Mohac *et al.*, 2019, 2020a, 2020b; Robert *et al.*, 2010) and optimised to improve the quercetin release profile. To achieve this result, several MPs batches were produced to optimise the yields and DL of the manufacturing process. The best yield achieved by testing several process parameters was 55%. This would be a low yield for a pilot-scale processes but, it is known as at lab-scale, such low yields are expected, as widely reported in literature (Prinn *et al.*, 2002). The drug loading of

quercetin was influenced by the BAC/excipients ratio. The last was adjusted in order to obtain, in the optimised formulation, about 100% of IE. For all the studies, the batch with the characteristics reported in Table 1 was chosen. Actually, the preliminary manufacturing studies revealed the relationship between composition of feed dispersion and DL. Differently, MPs morphology was not influenced by the used manufacturing conditions. SEM analysis showed shrinked MPs, due to the quick drying process of the droplets, with a size range between 14 and 22 μm (Fig. 1) and an average diameter of about 18 μm.

The micro delivery system conveying quercetin as active ingredient was analysed by DSC (Figure S1). Quercetin's thermogram exhibits two characteristic peaks: a broad decomposition peak at 141.69 °C and the fusion peak at 320 °C. Da Costa and co-workers reported how the water loss of quercetin starts at 116 °C, confirming that the broad peak at 141 °C in the thermogram of quercetin in Fig. 2 corresponds to a decomposition process of quercetin associated to the water loss (da Costa *et al*., 2002). The DSC showed as the micro delivery system calorimetric profile does not feature the characteristic decomposition peaks of the pure quercetin (141.69 °C) and milk proteins (116 °C). Instead, the complete disappearance of the peak at 141.69 and the shift of the peak at 116 °C of milk proteins, to about 108 °C, show as the quercetin and milk proteins form a specific supramolecular arrangement providing the peculiar release characteristics to the system. Indeed, the DSC of a simple physical mixture of quercetin and milk proteins, not micronized by spray drying, does not interact and rearrange at supramolecular level keeping the characteristic peaks of pure quercetin and milk proteins. Reasonably, the physical interaction of quercetin–proteins generated in aqueous solution remains after dehydration in the solid state, as confirmed by previously shown DSC graph (Mohammadian *et al*., 2020; Wijaya *et al*., 2019). IR analysis does not show any evidences about a chemical (covalent) interaction (Figure S2).

In vitro drug release and cytotoxicity studies

An in vitro drug release study was carried out to highlight the difference of the release profiles between of the quercetin when embodied in the MPs and in the pure form. Furthermore we focused our attention on the pH-sensitive release, miming the gastric environment (HCl 0.1 N) for 2 h and the intestine environment (phosphate buffer pH 6.8 and 7.2) for 22 h. As shown in Fig. 2 at gastric pH (until 2 h of experiment), only 3.5% of the quercetin embodied in MPs is released against the 6.5% of free quercetin. After the change of pH to 6.8, a clear 'burst' in the quercetin released by the MP formulation followed by a steady release until the 24 h, achieving a final released amount equal to ~ 25% of the starting dosage, is

Table 1 Characteristics of micro delivery system batch loaded with quercetin

Batch composition W%	WP/quercetin ratio ± SD	Yield % ± SD	Quercetin DL % ± SD	IE% ± SD	Size μm ± SD
Quercetin 0.94 Whey proteins 5.16 Whole milk 93.9	5.5 ± 0.3	50 ± 3	5.1 ± 0.3	98 ± 5	18 ± 4

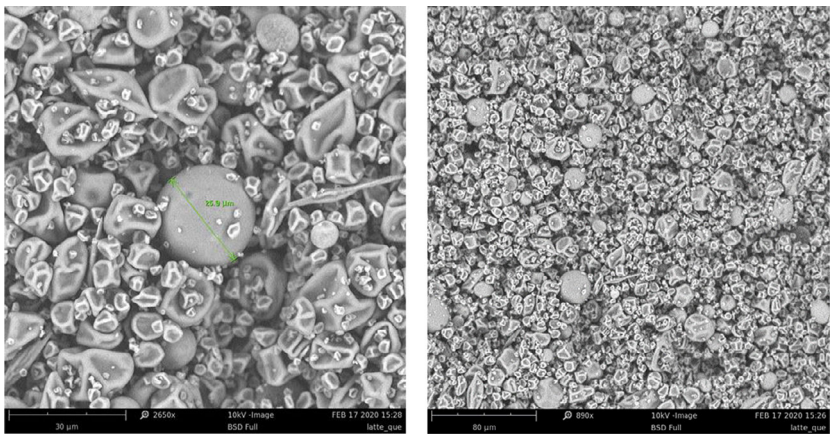

Figure 1 SEM image of the Q-loaded microparticles. Magnifications: 2650x and 890x. Size bars 30 and 80 μm, respectively.

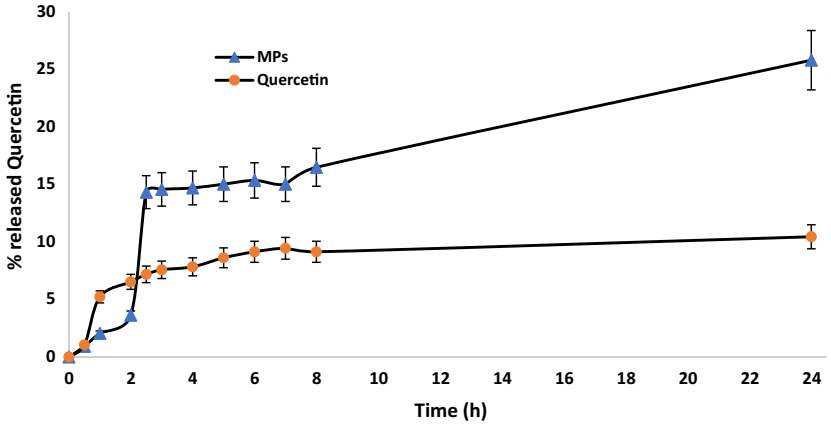

Figure 2 Schematic representation of the pH-sensitive release profile of quercetin embodied in microparticles (MPs) (triangles) and pure Quercetin (circles).

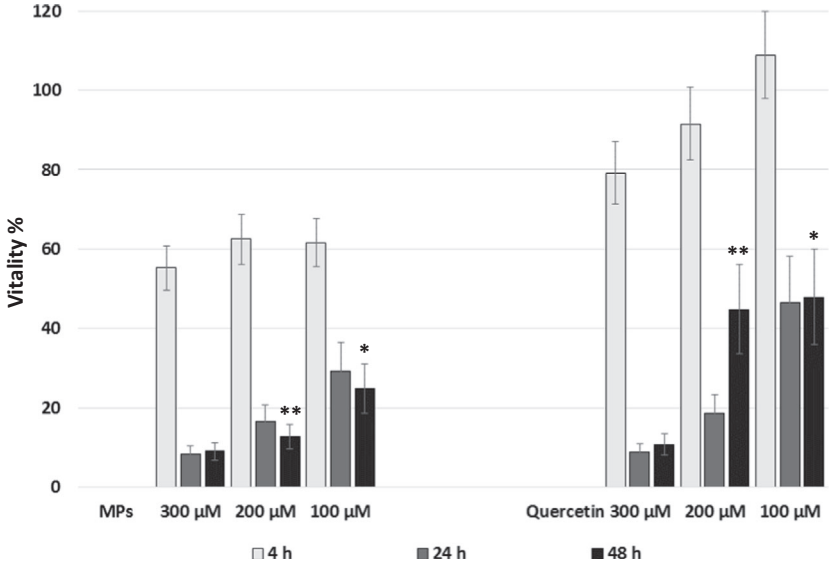

Figure 3 Cell viability of HCT116 cell line after 4-, 24- and 24-h incubation with Q and MPs at concentrations 100, 200 and 300 µM; $p<0.05$ for MPs versus Quercetin group at 200 (**) and 100 (*)µM after 48 h.

visible. It is interesting to underline how the release profile of the MPs owns the typical features of a controlled gastro-resistant release system, although no gastro-resistant excipients or coatings have been used. Differently, the release curve of free quercetin did not show any peculiar improvement of the release operated by the pH change, with a final released amount equal to the ~ 10% of the starting dosage after 24 h.

The cytotoxicity was investigated by an in vitro test with a human colon cancer (HCT116) cell line, a model cell line widely used to evaluate the anticancer activity of pharmaceuticals and drug carriers (Puleio *et al*., 2020; Licciardi *et al*., 2019). As shown in Fig. 3, quercetin appears to be ineffective on cancer cell after 4 h of incubation when tested at concentrations ranging from 100 to 300 µM (Rauf *et al*., 2018). Differently, after longer incubation times (24 and 48 hs), quercetin shows a concentration-dependent cytotoxic effect. The MPs herein unravelled instead resulted more cytotoxic than free quercetin in all tested concentrations and incubation times. These results suggest how these MPs improve the quercetin activity against human colon cancer cells. We further tested the activity of the physical mixture of protein/quercetin as control experiments to better understand the effect of MPs' effect on the quercetin activity, but the physical mixture generates an extremely unstable suspension to be tested. On the other hand, we cannot exclude a synergistic anticancer effect of whey proteins in vitro due to the hypothesis that the whole whey protein system apparently protects against colon and mammary tumours (Madureira *et al*., 2007).

In vivo bioavailability and anticancer efficacy

As expected, also the in vivo bioavailability of resultant quercetin increased when orally administered in the MPs delivery system. Fig. 4 shows the values of

Table 2 AUC relating to the administration of pure Quercetin and Quercetin-loaded microparticles (MPs) and the bioavailability ratio given by the formula = AUC MPs/AUC Q. All values are expressed in mg/ml*h. Maximum observed plasma concentration (Cmax; mg/ml) during the 0–24-hour dosing interval; Time to peak concentration (Tmax)

	Quercetin	MPs	Ratios
AUC ± SD	5.02 ± 0.01 mg ml^{-1}	134.96 ± 0.05 mg ml^{-1}•h	26.88
Cmax ± SD	4.2 ± 0.2 μg ml^{-1}	10.6 ± 0.6 μg ml^{-1}	2.5
Tmax	0.5 h	5 h	10.0

the plasma concentration of quercetin either as pure compound or delivered by the MPs delivery system. Each point of the curves (quercetin plasma concentration vs. time) in Fig. 4 represents the average of the values of the plasma concentrations of quercetin; the respective AUC (area under the curve), Cmax and Tmax values and the relative ratio are shown in Table 2.

When tested *in vivo,* the quercetin-loaded MPs exhibited a higher bioavailability compared to the same active ingredient in its pure form. Actually, the AUC of the loaded active was improved of over 26 times when compared with the pure substance administrated at the same conditions. These results suggest how quercetin, when conveyed by the MPs delivery system, is absorbed to a greater extent than pure quercetin, and mainly in the intestine, as partially evidenced by the visible concentration jump in the bioavailability curve (Fig. 4) starting from about 2 h. Pure quercetin, on the other hand, is immediately absorbed in the stomach and to a lesser extent than the MPs, and consequently it is eliminated from the plasma fluid faster. The above AUC confirms what was predicted by the in vitro release studies, namely that the new release system may increase both the overall bioavailability and the half-life of quercetin. Actually, MPs system constitutes a clear technological progress in the oral dosage forms of quercetin, capable of increasing the bioavailability and let to predict the possibility of reducing the dose or prolong dose intervals in chronic therapy with quercetin.

Finally, the MPs delivery system also provided a notable quercetin efficacy increase against the colon cancer xenograft, proved by a sharp volumetric reduction of the tumour mass when compared to the same model treated with pure quercetin (Fig. 5).

The graph shows that the cancer proliferation was only partially slowed down after quercetin administration

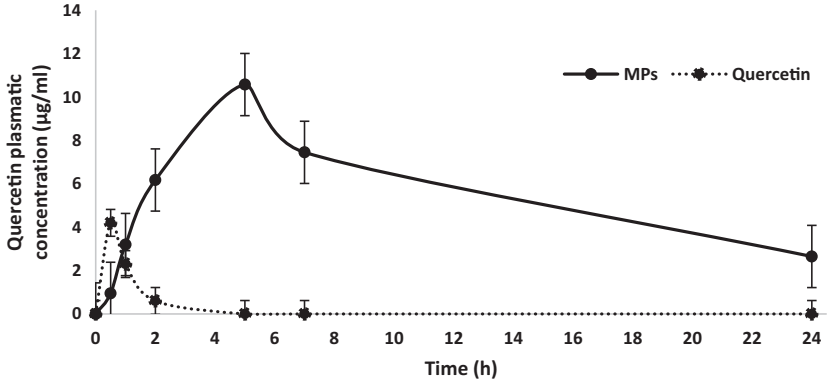

Figure 4 Graphical representation of bioavailability (plasmatic concentration vs. time) of Quercetin in the different groups (2 and 3) after oral administration. Data are expressed as means ± SD of n = 5 for each group; $p<0.05$ versus Quercetin group.

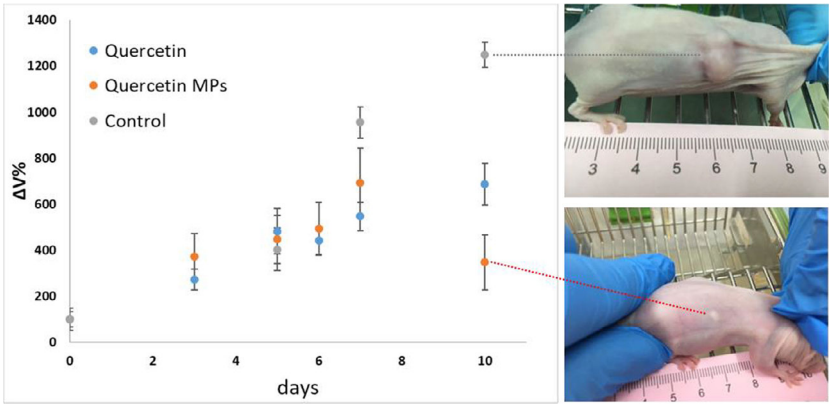

Figure 5 Percentage change in tumour volume after oral treatment with quercetin-containing microparticles (Quercetin MPs), quercetin suspension (Quercetin), compared to untreated group (Control), dosage 1 mg kg^{-1} dye; $p<0.05$ of Quercetin and MPs versus control group, respectively. The pictures were taken ten days after starting administration.

respect the control group, reaching in each case a tumour volume increasing of about 600% on the 10th day of the trial (Figure S3). In contrast, tumour masses treated with MPs showed a growth arrest of the tumour mass, with a trend of volume decrease after the 7th day of evaluation. In fact, although at the end of the experimentation the average tumour volume did not decrease to 0, the values recorded on day 10 indicated that the MPs are able to reverse the tumour growth and induce a significant tumour volume reduction.

Conclusions

In this work, we investigated a novel all-natural green and sustainable MPs formulation. Our results demonstrated how this formulation can be easily prepared by spray drying under mild conditions, featuring a controlled particle size below 30 μm and an optimal dosage form for oral administration of quercetin. This MPs system showed a typical gastro-resistant behaviour and a prolonged release at intestinal pH, higher than three times that of pure quercetin, thus increasing the absorption of the active and improving the expected efficacy, also confirmed by the improvement of anticancer activity in vitro. As a consequence of the improved controlled release profile, the *in vivo* tests showed a dramatic increase of the bioavailability of over 26 times, when compared to the administration of the same dosage of pure quercetin. These features are extremely useful for the reduction of the therapeutic dosage of quercetin supplements. The results of anticancer activity on colon cancer xenograft model show as quercetin embodied in the MPs prevented the tumour mass from increasing in volume compared to the control, highlighting a significant reduction in the tumour after day 7, compared to pure quercetin. The obtained tumour volume growth reduction upon multiple oral administration, let us to predict a potential use of this MPs formulation as supplement to support the common anticancer therapies already in use providing a synergistic effect for the cancer treatment.

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Author Contribution

Roberto Caruana: Formal analysis (equal); Investigation (equal); Methodology (equal); Writing – original draft (equal). **Francesco Montalbano:** Data curation (equal); Validation (equal); Writing – original draft (equal). **Maria Grazia Zizzo:** Data curation (equal); Formal analysis (equal); Investigation (equal). **Roberto Puleio:** Data curation (equal); Formal analysis (equal); Investigation (equal). **Gaetano Caldara:** Data curation (equal); Investigation (equal); Project administration (equal). **Luca Cicero:** Investigation (equal); Methodology (equal). **Giovanni Cassata:** Project administration (equal); Supervision (equal); Validation (equal). **Mariano Licciardi:** Conceptualization (lead); Data curation (equal); Supervision (lead); Writing – review & editing (lead).

Ethical statement

Procedures involving the animals and their care were conducted in the conformity of the Italian D. Lgs 26/2014 and the European directives (2010/63/EU). The animal care and handling were conducted in accordance to the provisions of the European Community Council Directive 210/63/UE, recognised and adopted by the Italian Government. The experiments were approved by the Animal Welfare Committee of the University of Palermo and Istituto Zooprofilattico Sperimentale della Sicilia.

Conflict of interest

Technology Scientific S.r.l. is proprietary of the technology herein disclosed. All other authors declare no conflict of interest.

Peer review

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.15539.

Data availability statement

Data are available in the repository of University of Palermo on request from the authors.

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Note: The above reference highlight the potential positive therapeutic effects of quercetin against a specific cancer cell, such as ovarian, justifying the choice of this natural active substance as the appropriate model molecule for our study.

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Note: The above reference highlight the use of quercetin nanoformulations designed as effective means for the treatment of various types of tumours, validating the strategy approached in our study.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary Material