



## Pharmaceutical nanotechnology

## Nanoemulsion formulation of fisetin improves bioavailability and antitumour activity in mice

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## ABSTRACT

The natural flavonoid fisetin (3,3',4',7-tetrahydroxyflavone) has shown antitumour activity but its administration is complicated by its low water solubility. Our aim was to incorporate fisetin into a nanoemulsion to improve its pharmacokinetics and therapeutic efficacy. Solubility and emulsification tests allowed to develop an optimal nanoemulsion composed of Miglyol® 812N/Labrasol®/Tween® 80/Lipoid E80®/water (10%/10%/2.5%/1.2%/76.3%). The nanoemulsion had an oil droplet diameter of  $153 \pm 2$  nm, a negative zeta potential ( $-28.4 \pm 0.6$  mV) and a polydispersity index of 0.129. The nanoemulsion was stable at 4 °C for 30 days, but phase separation occurred at 20 °C. Pharmacokinetic studies in mice revealed that the fisetin nanoemulsion injected intravenously (13 mg/kg) showed no significant difference in systemic exposure compared to free fisetin. However, when the fisetin nanoemulsion was administered intraperitoneally, a 24-fold increase in fisetin relative bioavailability was noted, compared to free fisetin. Additionally, the antitumour activity of the fisetin nanoemulsion in Lewis lung carcinoma bearing mice occurred at lower doses (36.6 mg/kg) compared to free fisetin (223 mg/kg). In conclusion, we have developed a stable nanoemulsion of fisetin and have shown that it could improve its relative bioavailability and antitumour activity.

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## 1. Introduction

Among the plant-derived compounds that have been linked to the chemoprevention and treatment of cancer, the flavonoids occupy a special place due to their abundance in human food and their relative non toxicity (Havsteen, 2002; Lopez-Lazaro, 2002; Middleton et al., 2000; Surh, 2003).

In a program aimed at finding new antiangiogenic agents in the flavonoid family, we have recently identified the natural flavonoid fisetin (3,3',4',7-tetrahydroxyflavone) as an interesting lead that can stabilize endothelial cells in vitro at non cytotoxic concentrations (Touil et al., 2009). Fisetin is found in several fruits, vegetables, nuts and wine (Arai et al., 2000; Kimira et al., 1998) and displays a variety of biological effects including antioxidant,

anti-inflammatory (Park et al., 2007; Woodman and Chan, 2004), anti-carcinogenic and in vitro anti-angiogenesis (Fotsis et al., 1997). Fisetin has been shown to inhibit several molecular targets, including cyclin-dependent kinases (Lu et al., 2005a,b; Sung et al., 2007), DNA topoisomerases I and II (Constantinou et al., 1995; Olaharski et al., 2005), urokinase (Jankun et al., 2006), actin polymerization (Böhl et al., 2007), and androgen receptor signalling (Khan et al., 2008).

In vivo, fisetin has recently been shown to possess interesting anticancer activity in mice bearing lung carcinoma (Touil et al., 2011), prostate tumours (Khan et al., 2008), and human embryonal carcinoma (Tripathi et al., 2011). Its in vivo mechanism of action appears rather complex and includes antiangiogenic, antiandrogenic and anti-metastatic activities (Chien et al., 2010; Khan et al., 2008; Touil et al., 2011; Tripathi et al., 2011).

Despite its highly interesting properties for cancer therapy, fisetin administration in vivo remains problematic partly due to its poor water solubility (Guzzo et al., 2006; Mignet et al., 2012). Fisetin bioavailability must therefore be significantly improved in order to optimize its delivery to tumours after in vivo administration. Although the design of suitable molecular carriers for flavonoids is an area of intense research, solutions are still far from being developed for therapy, and suitable molecular carriers for flavonoids have yet to be designed and tested (Leonarduzzi et al., 2010). To

**Abbreviations:** Fisetin, 3,3',4',7-tetrahydroxyflavone; EAhy 926, immortalized human umbilical vein endothelial cells; HLB, hydrophilic–lipophilic balance; HPLC, high performance liquid chromatography; PDI, polydispersity index.

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**Table 1**  
Fisetin solubility in various solvents.

Solvents	Solubility (mg/g)
Water	<1
Ethanol	<14
<i>Long chain triglycerides</i>	
Soybean oil	<1
Carthame oil	<1
<i>Medium chain mono- di-or triglycerides</i>	
Miglyol® 812 N (capric and caprylic acid triglycerides)	<1
Captex® 355 (capric and caprylic acid triglycerides)	<1
Labrafac Lipophile WL 1349® (capric and caprylic acid triglycerides)	<1
Imwitor® 742 (capric and caprylic mono- di- and triglycerides)	<4
<i>Short chain triglycerides: Triacetin (triester of glycerol and acetic acid)</i>	<6
<i>Fatty acid esters: Ethyl oleate</i>	<1
Capmul® PG8 (propylene glycol monocaprylate)	<5
Capric acid	<1

**Table 2**  
Fisetin solubility in various surfactants.

Surfactants	HLB <sup>a</sup>	Solubility (mg/g)
Span® 85 (sorbitan trioleate)	1.8	<2
Labrafil M 1944 CS® (glycerides and PEG 300 ester mixture)	4	<3
Capmul® MCM (mono diglyceride of capric and caprylic acids)	5	<7
Vitamin E TPGS (α tocopheryl acid succinate ester/PEG 1000)	13	<10
Cremophor EL® (polyethoxylated ricin oil)	13	<26
Myrj® 52 (polyoxyethylene glycol 200 monostearate)	16.9	<30
Tween® 80 (polysorbate 80)	15	<30
Labrasol® (caprylocaproyl polyoxyl-8 glycerides)	14	<54

<sup>a</sup> HLB, hydrophilic–lipophilic balance (a value <10 indicates a majority of lipophilic fractions and a value >10 indicates a majority of hydrophilic fractions).

do so, we therefore chose to formulate fisetin into nanoemulsion in order to hopefully achieve a better bioavailability.

Nanoemulsions represent good vehicles to formulate hydrophobic active molecules (Sarker, 2005). For example, nanoemulsions are widely used for parenteral administration of lipids, and have also been employed for intravenous administration of anticancer drugs like paclitaxel (Kan et al., 1999) and chlorambucil (Ganta et al., 2008). Also noteworthy, nanoemulsion has also been recently reported to contribute to the in vivo increase in efficacy of anticancer drugs, e.g., dacarbazine (Tagne et al., 2008) and camptothecin (Han et al., 2009).

The aim of the present study was therefore to design and characterize a nanoemulsion of fisetin that could be suitable for parenteral administration. We also evaluated the fisetin nanoemulsion pharmacokinetics after intravenous (i.v.) or intraperitoneal (i.p.) administration in mice, and determined its relative i.p. bioavailability compared to the i.p. administration of the free fisetin. Finally, the antitumour activity of the fisetin nanoemulsion was compared to the administration of free fisetin in Lewis lung carcinoma bearing mice.

## 2. Materials and methods

### 2.1. Materials

Fisetin (98% purity) was purchased from Shanghai FWD Chemicals Limited (Shanghai, China). The various purified oil phases were provided by the following companies: soybean oil (Société Industrielle des Oléagineux, Saint Laurent Blangy, France); carthame oil, ethyl oleate and n-capric acid (Sigma–Aldrich, Saint Quentin Fallavier, France); Miglyol® 812 N and Imwitor® 742 (Sasol Witten, Germany); Captex® 355 and Capmul® PG-8 (Abitec, Janesville, WI, USA); Labrafac lipophile WL 1349® (Gattefossé, Saint Priest, France); triacetin (VWR Fontenay-sous-Bois, France).

The surfactants were purchased from the following companies: egg lecithin containing 82.3% phosphatidylcholine (Lipoid E80®, Lipoid GmbH Ludwigshafen, Germany); polysorbate 80 (Tween® 80), sorbitan trioleate (Span 85®), polyoxyethylene glycol 200 monostearate (Myrj® 52) (Uniquema, Everberg, Belgium); polyoxyethylenated ricin oil (Cremophor EL®, BASF, Ludwigshafen, Germany); vitamin E TPGS (Eastman Chemical B.V., Paris, France); a mixture of glycerides and esters of PEG-8 (Labrasol®), a mixture of glycerides and esters of PEG 300 (Labrafil M 1944 CS®) (Gattefossé Saint Priest, France); glycerol monocaprylocaprate (Capmul MCM®, Abitec, Janesville, WI, USA). Glycerol was purchased from Labosi (Paris, France). Sterile water for injection was from Fresenius-Kabi (Sèvres, France). Sodium hydroxide 0.1 N was from Carlo Erba Reactif SDS (Peypin, France).

The other chemicals used for drug dissolution, plasma preparation and HPLC analysis were the following: methanol, acetonitrile, perchloric acid (Carlo Erba Reactif SDS, Peypin, France); DMSO, PEG 200, morin, phosphate buffer (pH 7.4) and mouse serum (Sigma–Aldrich, Saint Quentin Fallavier, France). All other

**Table 3**  
Fisetin solubility in various mixtures.

Mixtures (in percent)	Solubility (mg/g)
Ethanol/Tween® 80 (2%)	<21
Miglyol® 812N/Tween® 80 (2%)	<1
Soybean oil/Tween® 80 (15%)	<3
Soybean oil/Span 85® (10%)	<1
Miglyol® 812N/Span 85® (7.5%)	<3
Soybean oil/Labrasol® (50/50)	ND <sup>a</sup>
Miglyol® 812N/Labrasol® (50/50)	ND <sup>a</sup>
Capmul MCM®/Labrasol® (50/50)	<21
Tween® 80/Labrasol® (20/80)	<45
Tween® 80/Labrasol®/soybean oil (12/44/44)	ND <sup>a</sup>
Tween® 80/Labrasol®/Miglyol® 812 N (12/44/44)	<30
Mirj 52®/Solutol HS 15®/Capmul MCM® (57/29/14)	<24
Tween® 80/Labrasol®/Capmul MCM® (17/69/14)	<40

<sup>a</sup> ND, not determined because the phases were not miscible in these proportions.

chemicals were of pharmaceutical grade or of the highest analytical purity available.

## 2.2. Fisetin solubility studies

Fisetin solubility was assessed according to the approached solubility method (Mulak and Cotty, 1975). Fisetin solubility was first separately assessed in different oil phases and surfactants, and thereafter in various mixtures of oil/surfactant (Tables 1–3). Increasing fisetin concentrations were introduced in the various phases under agitation and heating at  $60 \pm 2^\circ\text{C}$  until a precipitate was observed. The solubility was determined after cooling at room temperature.

## 2.3. Nanoemulsion preparation

Fisetin was dissolved in several fractions in a mixture of Labrasol®/Tween® 80 heated at  $60 \pm 2^\circ\text{C}$  under sonication (Sonifier® 450, Branson, Danbury, CT, USA). This mixture was added to the oil phase (Miglyol® 812) in which lecithin (Lipoid E80®) has previously been dispersed by heating. Aqueous and oil phases have been heated at  $70 \pm 2^\circ\text{C}$  thereafter. Emulsification was accomplished by phase inversion (Becher, 1965), i.e., the aqueous phase was added to the oil phase. The mixture was then submitted to a high shear mixer (Ultraturrax® T25, Ika, Staufen, Germany) for 10 min at 21,500 rpm (set at 5) that allowed the formation of a crude emulsion. An additional 15 min sonication in the cold of the previous emulsion using a Sonifier® 450 set at 90% and output 3 allowed to obtain a submicron emulsion. For intravenous (i.v.) and intraperitoneal (i.p.) administration, sodium hydroxide 0.1 N was used to adjust pH to 7.0 and glycerol 2.25% was added to adjust tonicity. Two methods of sterilization were investigated: steam sterilization using  $120^\circ\text{C}$ , 15 min cycle and filtration through a  $0.22\ \mu\text{m}$  filter.

## 2.4. Nanoemulsion characterization and stability studies

Nanoemulsions were visually inspected for eventual creaming, coalescence, phase separation and/or precipitation. After dilution (1/1000) in water, the mean droplet size, size distribution, the zeta potential ( $\zeta$ ), and the polydispersity index (PDI) were determined using a Zetasizer Nano ZS (Malvern Instruments, Orsay, France). The PDI reflects the polydispersity of the emulsion ranging from 0 to 1, with lower values indicating a more monodispersed suspension. pH was determined using a pH meter 210 (MeterLab, Copenhagen, Denmark). A short term stability of the optimized formulations over a period of 30 days was accomplished at room temperature ( $+20 \pm 2^\circ\text{C}$ ) and at  $+4^\circ\text{C}$  by evaluating the above mentioned parameters. Measurements were performed in triplicate.

For the determination of the fisetin concentration, nanoemulsions were diluted (1/2000) in methanol, vortexed, and  $100\ \mu\text{L}$  were injected onto a reversed-phase HPLC system (Shimadzu CLASS-VP®, version 5.3), equipped with an octadecylsilane column (Beckman Ultrasphere ODS,  $5\ \mu\text{m}$ ;  $4.6\ \text{mm} \times 250\ \text{mm}$ ) thermostated at  $20^\circ\text{C}$ , and a UV detector set at 360 nm. The mobile phase was composed of 25% acetonitrile and 75% acidified water (2% (v/v) glacial acetic acid), at a flow rate of 1 mL/min. In these conditions the retention time of fisetin was 8 min. The area of the fisetin peak was reported to a calibration curve to determine the concentration of fisetin. Calibration curves were linear with correlation coefficients near unity.

## 2.5. Effect of fisetin nanoemulsion on endothelial cells

Immortalized human endothelial cells (EAhy 926) (Edgell et al., 1983) were grown in DMEM containing 2 mM L-glutamine, 10%

foetal bovine serum, 100 U/mL penicillin and  $100\ \mu\text{g/mL}$  streptomycin ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ). Cells were plated onto 96-well plates at 5000 cells per well in  $100\ \mu\text{L}$  of culture medium. The fisetin-free nanoemulsion or the fisetin nanoemulsion were added to the cells for a 2 h exposure time, and the cell morphology was assessed under microscopy at a magnification of X100 (Nikon Diaphot, Nikon Corp., Japan).

## 2.6. Fisetin pharmacokinetics in mice

### 2.6.1. Mice and treatments

Female 8 weeks old C57BL/6J mice (body weight 18–22 g), were purchased from Janvier (Le Genest-St-Isle, France). After an overnight fasting period, mice were administered the various treatments as described hereafter. For the intravenous (i.v.) administration into the tail vein, 21 mice received the free fisetin formulated in 20% DMSO, 20% PEG 200 and 60% saline at a final concentration of  $1.3\ \text{mg/mL}$  (hereafter referred as “free fisetin”). The total volume injected i.v. was  $200\ \mu\text{L}$  for a 20 g mouse, which corresponds to a volume of DMSO of  $40\ \mu\text{L}$ . It should be noted that an undiluted DMSO volume of  $50\ \mu\text{L}$  can be administered safely i.v. to mice without toxicity (Willson et al., 1965), and that in our studies, the final DMSO dose per mouse corresponds to  $40\ \mu\text{L}$  (for a 20 g mouse), which was further diluted in saline and injected slowly over 1 min. We did not encounter any acute mortality using this formulation in our studies.

A second group of 21 mice received the fisetin nanoemulsion in a final volume of  $50\ \mu\text{L}$ , previously sterilized by filtration through  $0.22\ \mu\text{m}$  filters. The fisetin dose was  $13\ \text{mg/kg}$  for the free fisetin or its nanoemulsion. Mice were sacrificed at 5, 10, 15, and 30 min, 1, 2, and 4 h, the blood was obtained by cardiac puncture onto heparinized syringes, centrifuged ( $10,000 \times g$ , 10 min), and the harvested plasma was kept frozen at  $-20^\circ\text{C}$  until HPLC analysis. For the intraperitoneal (i.p.) administration, the free fisetin (prepared as described above for the i.v. route) was injected at the maximum tolerated dose by this route ( $223\ \text{mg/kg}$ ). The fisetin nanoemulsion dose was  $112.5\ \text{mg/kg}$  corresponding to an injected volume of  $450\ \mu\text{L}$  i.p. Three mice per time point were sacrificed at 0, 0.25, 0.5, 1, 2, 4, 8, 15 and 24 h to harvest the blood by cardiac puncture (heparinized syringe). Plasma was obtained by centrifugation ( $10,000 \times g$ , 10 min), and frozen at  $-20^\circ\text{C}$  until HPLC analysis. All animal experiments have been carried out in accordance with institutional and French regulations concerning the protection of animals, and with the European Commission regulations.

### 2.6.2. Determination of fisetin concentration in plasma

Fisetin concentration in plasma was determined by HPLC as followed: to  $100\ \mu\text{L}$  of plasma was added  $60\ \mu\text{L}$  of a morin methanolic solution at  $0.5\ \text{mg/mL}$  (internal standard), and  $200\ \mu\text{L}$  of cold acidified methanol (methanol:perchloric acid 70%, 200:1, v:v) to precipitate proteins. After vortexing for 5 min the samples were kept on ice for 15 min, and centrifuged at  $10,000 \times g$  at  $4^\circ\text{C}$ . The supernatant ( $100\ \mu\text{L}$ ) was injected onto a reversed-phase HPLC system as described above with the UV detector set at 360 nm. The ratio of the area of the fisetin peak divided by the internal standard peak area was reported to a calibration curve to determine the concentration of fisetin. Calibration curves were linear with correlation coefficients near unity. The quantification limit of the system was  $0.1\ \mu\text{g/mL}$ .

### 2.6.3. Pharmacokinetic parameters determination

The following non compartmental pharmacokinetic parameters were calculated using standard methods (Gibaldi and Perrier, 1982): maximum concentration ( $C_{\text{max}}$ ) extrapolated to time zero for the i.v. route; area under the plasma concentration versus time curve from time zero to the time of the last measurable

concentration ( $AUC_{0-t}$ ) calculated by the trapezoidal method; terminal half-life =  $\ln 2/K_{el}$ , where  $K_{el}$  is the terminal elimination rate constant. The mean residence time (MRT) was calculated as the  $AUMC/AUC$ , where AUMC is the area under the first moment curve. Clearance was calculated as the dose/AUC, and the volume of distribution  $V_{ss}$  as the  $CL \times MRT$ . The mean absorption time (MAT) after i.p. administration was calculated as the  $MRT_{i.p.}$  minus  $MRT_{i.v.}$  (Gibaldi and Perrier, 1982). Relative bioavailability ( $F_{REL}$ ) comparing the free fisetin and its nanoemulsion for the same route of administration was determined by the following formula:  $F_{REL} = (AUC_{NE} \times dose_{FREE}) / (AUC_{FREE} \times dose_{NE})$ .

## 2.7. Evaluation of antitumour activity in mice

Female 8 weeks old C57BL/6J mice (body weight 18–22 g) (Janvier, Le Genest-St-Isle, France) were used for antitumour evaluation. Lewis lung tumour fragments (about 2 mm diameter) were implanted subcutaneously (s.c.) bilaterally into mouse flanks using a 12 gauge trocar. Four days after tumour implantation, the mice were submitted to the following i.p. treatments (4 mice per group) for 12 consecutive days (days 4–15): 4 control mice received a fisetin-free nanoemulsion; 4 mice received the fisetin nanoemulsion corresponding to 18.3 mg/kg of fisetin; and, 4 mice were injected the fisetin nanoemulsion at 36.6 mg/kg. Tumour growth was assessed using caliper bi-dimensional measurements (in mm) and the tumour volume ( $mm^3$ ) was calculated according to the following formula:  $(width^2 \times length)/2$ .

## 2.8. Statistical analysis

Data are presented as the mean  $\pm$  SEM. Comparison between tumour volumes was assessed by the Student *t* test.

# 3. Results

## 3.1. Fisetin solubility in various solvents

Table 1 presents the solubility of fisetin in various solvents. Fisetin was not soluble in water ( $<1$  mg/g) and was weakly soluble in ethanol ( $<14$  mg/g). Fisetin was also found weakly soluble for all the oil phases tested, with a maximum solubility value  $<6$  mg/g for triacetin and  $<5$  mg/g for propylene glycol monocaprylate.

Table 2 shows fisetin solubility in frequently used surfactants. In lipophilic surfactants (low HLB value), fisetin solubility was low, whereas its solubility was markedly increased in hydrophilic surfactants with high HLB values. The best surfactant was found to be Labrasol® which could solubilize up to 54 mg/g of fisetin.

Finally, the solubility values of fisetin using several combinations of oils and surfactants in various proportions are presented in Table 3. It was found that the mixture composed of Tween® 80 and Labrasol® (20/80) could solubilize fisetin most efficiently up to a maximal concentration of 45 mg/g.

## 3.2. Development of a fisetin nanoemulsion

### 3.2.1. Emulsification capacity

Several nanoemulsion formulations were thereafter tested for their emulsification capacity and 3 cases could be observed, as follows (Table 4): (a) no emulsification was obtained for certain formulations lacking Lipoid® E80 (e.g., formulations 3–7) or containing soybean oil and Capmul® MCM, as in formulation 2; (b) formation of a nanoemulsion was observed for formulations 1, 8 and 9; (c) and, formulation 10 allowed the formation of a clear solution.

### 3.2.2. Fisetin incorporation into nanoemulsion formulations

We have next evaluated the maximum quantity of fisetin that could be incorporated into each nanoemulsion (formulations 1, 8, 9), or solution (formulation 10). Table 4 shows that formulation 1 could incorporate only 1 mg/g of fisetin, whereas formulations 8 and 9 allowed the incorporation of 5 mg/g of the flavonoid. These formulations were found visually stable on day 1. Although formulation 10 allowed to solubilize up to 16 mg/g of fisetin due to its higher content in Labrasol® and Tween® 80, it was unfortunately found to precipitate on day 1 for concentrations of 8 and 16 mg/g, but was found stable for a fisetin concentration of 4 mg/g.

### 3.2.3. Choice of final fisetin nanoemulsion formulation

Considering the above results, formulations 8 and 9 were therefore considered a good compromise between fisetin content and nanoemulsion stability. These formulations were further tested for their particle diameter and polydispersity index (PDI) and it was observed that preparation 8 containing soybean oil yielded a nanoparticle diameter of  $323 \pm 2$  nm with a PDI of 0.153, whereas preparation 9 containing Miglyol® 812N showed a nanoparticles diameter of  $146 \pm 3$  nm and a PDI of 0.015. Formulation 9 was therefore chosen for further in vitro and in vivo testing because of its good fisetin content, its nanoparticle size and low PDI value. The final composition of nanoemulsion 9 containing 5 mg/mL of fisetin was therefore as follows: Miglyol® 812N (10%), Labrasol® (10%), Tween® 80 (2.5%), Lipoid E80® (1.2%), glycerol (2.25%), NaOH (0.1 N) to adjust to pH 7, water to 100%.

## 3.3. Stability of the fisetin nanoemulsion

We next performed short term stability studies of the fisetin nanoemulsion (preparation 9) over a period of 30 days at room temperature (20°C) and at 4°C (Table 5) by evaluating particle diameter, pH, zeta potential and the PDI.

Droplet size of nanoemulsion 9 stored at 20°C increased markedly as a function of time until phase separation occurred on day 30, whereas for the 4°C storage condition, particle diameter remained relatively stable for the 30 day examination period. A slight decrease in pH was noted at both storage temperatures. For the zeta potential, negative values were observed and remained stable over 30 days for the 4°C storage condition. The PDI presented an important increase over time at 20°C, whereas it was found stable and relatively low at 4°C. In order to be administered via parenteral routes, we also checked if the nanoemulsion could sustain standard steam sterilization conditions (121°C for 15 min) but this resulted in phase separation. However, sterilization of nanoemulsion 9 has been successfully carried out using a 0.22 µm filter with preservation of homogeneity and size characteristics.

## 3.4. Effect of fisetin nanoemulsion on endothelial cells

Free fisetin has been reported to exert a distinct morphological effect on endothelial cells that is characterized by the rapid development of pseudopods at non cytotoxic concentrations (Touil et al., 2009). We therefore tested if the fisetin nanoemulsion (preparation 9) could exert the same morphological effects on Eahy 926 endothelial cells to verify if the active principle is indeed released from the pharmaceutical preparation. Fig. 1A depicts control endothelial cells exposed to 1% DMSO which show typical cobblestone appearance, whereas exposure to free fisetin led to the expected pseudopods formation (Fig. 1B). Endothelial cells exposed to control nanoemulsion without fisetin resemble normal control endothelial cells (Fig. 1C), whereas the pseudopods are indeed observed in the cells exposed to the fisetin nanoemulsion (Fig. 1D). This observation indicates that fisetin is indeed released from the



**Table 4**  
Composition of the various emulsions.

Component	Formulation number									
	1	2	3	4	5	6	7	8	9	10
Soybean oil	10	10	20	–	–	–	–	10	–	–
Miglyol® 812N	–	–	–	10	10	15	20	–	10	–
Triacetin	10	–	–	–	–	–	–	–	–	–
Capmul MCM®	–	10	–	–	–	–	–	–	–	6
Labrasol®	–	–	20	10	9.6	14.4	19.2	10	10	27
Tween® 80	2.5	2.5	–	2.5	0.4	0.6	0.8	2.5	2.5	7
Lipoid E80®	1.2	1.2	–	–	–	–	–	1.2	1.2	–
Water	76.3	76.3	60	77.5	80	70	60	76.3	76.3	60
Observations	NE <sup>a</sup>	LOE <sup>b</sup>	LOE <sup>b</sup>	LOE <sup>b</sup>	LOE <sup>b</sup>	LOE <sup>b</sup>	LOE <sup>b</sup>	NE <sup>a</sup>	NE <sup>a</sup>	Sol. <sup>c</sup>
Fisetin incorporation on day 1 (mg/g)	1							5	5	4

<sup>a</sup> NE, nanoemulsion.

<sup>b</sup> LOE, lack of emulsification.

<sup>c</sup> Sol., clear solution.

**Table 5**  
Fisetin nanoemulsion (preparation 9) stability parameters.

Parameter	Day					
	0	1	4	7	15	30
Diameter (nm) 20 °C	153 ± 2 <sup>a</sup>	189 ± 1	331 ± 7	749 ± 54	2882 ± 87	Phase separation
Diameter (nm) 4 °C	138 ± 5	138 ± 0	152 ± 4	144 ± 2	147 ± 2	154 ± 2
pH at 20 °C	7.11 ± 0.01	6.80 ± 0.02	6.61 ± 0.02	6.51 ± 0.02	6.42 ± 0.01	–
pH at 4 °C	7.09 ± 0.01	6.79 ± 0.02	6.81 ± 0.02	6.65 ± 0.03	N.D.	6.53 ± 0.01
Zeta potential, $\zeta$ , 20 °C (mV)	–28.4 ± 0.6	–30.8 ± 0.9	–34.8 ± 1.4	–40.3 ± 0.4	–39.9 ± 0.5	–
Zeta potential, $\zeta$ , 4 °C (mV)	–32.7 ± 1.1	–26.7 ± 1.0	–33.5 ± 0.5	–34.7 ± 1.6	–32.5 ± 2.9	–34.1 ± 2.5
PDI <sup>b</sup> 20 °C	0.129	0.090	0.201	0.371	1.000	–
PDI <sup>b</sup> 4 °C	0.151	0.157	0.136	0.128	0.147	0.115

<sup>a</sup> Mean ± SEM of 3 determinations.

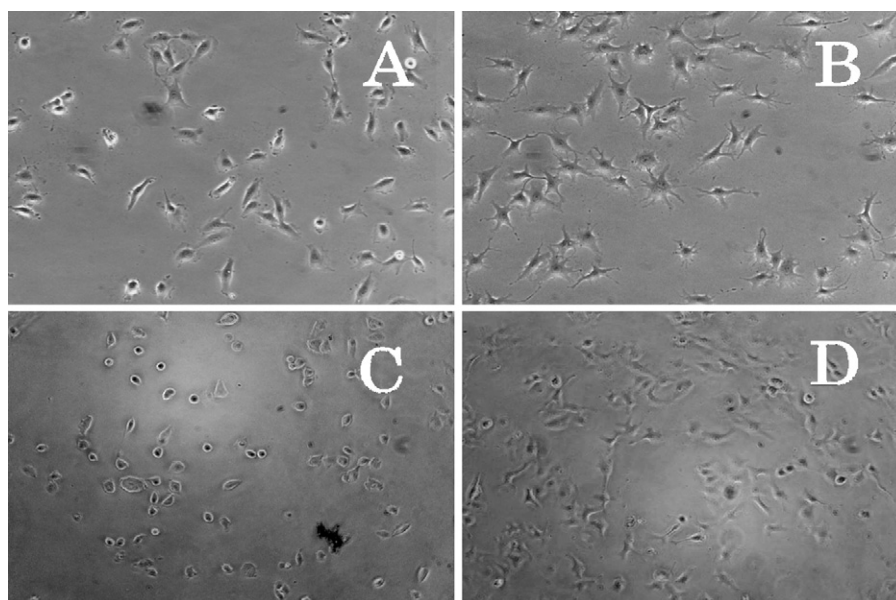
<sup>b</sup> PDI, polydispersity index.

nanoemulsion and can exert similar morphological effects as the free fisetin on endothelial cells.

### 3.5. Fisetin nanoemulsion pharmacokinetics in mice

The developed fisetin nanoemulsion (preparation 9) was next administered *in vivo* to evaluate its pharmacokinetics in mice. We

first examined the intravenous route (*i.v.*) by injecting the free fisetin formulation or its nanoemulsion at a dose of 13 mg/kg. We noted a very similar pharmacokinetic profile between the two formulations with plasma concentrations versus time curves almost superimposable (Fig. 2). Indeed, similar pharmacokinetic parameters in terms of  $C_{max}$ , AUC and terminal half-life were observed for both formulations (Table 6). The *i.v.* route administration of the



**Fig. 1.** Morphologic effects of fisetin on Eahy 926 endothelial cells after a 2 h exposure period. (A) Control with 1% DMSO; (B) free fisetin (25 µg/mL); (C) fisetin-free nanoemulsion (0.25%); (D) fisetin nanoemulsion 12.5 µg/mL. Original magnification, 100×.

**Table 6**

Fisetin pharmacokinetic parameters after intravenous or intraperitoneal administration of free fisetin and fisetin nanoemulsion in mice (preparation 9).

Parameter	Intravenous		Intraperitoneal	
	Free fisetin	Fisetin nanoemulsion	Free fisetin	Fisetin nanoemulsion
Dose (mg/kg)	13	13	223	112.5
$C_{max}$ ( $\mu\text{g/mL}$ )	6.0	5.3	2.53	22.96
Elimination constant, $K_{el}$ ( $\text{h}^{-1}$ )	1.136	1.072	0.165	0.226
Terminal half-life (h)	0.61	0.65	4.19	3.07
$AUC_{0 \rightarrow t}$ ( $\mu\text{g h/mL}$ )	1.12	1.13	4.07	48.53
AUMC ( $\mu\text{g h}^2/\text{mL}$ )	1.09	1.12	28.26	143.56
Mean residence time (MRT) (h)	0.97	0.99	6.95	2.96
Mean absorption time (MAT) (h)	–	–	5.98	1.97
Clearance (CL) ( $\text{L/kg/h}$ )	11.64	11.50	54.80	2.32
Volume of distribution, $V_{ss}$ ( $\text{L/kg}$ )	11.33	11.36	380.62	6.86
Relative bioavailability, <sup>a</sup> $F_{rel}$	–	–	1	24

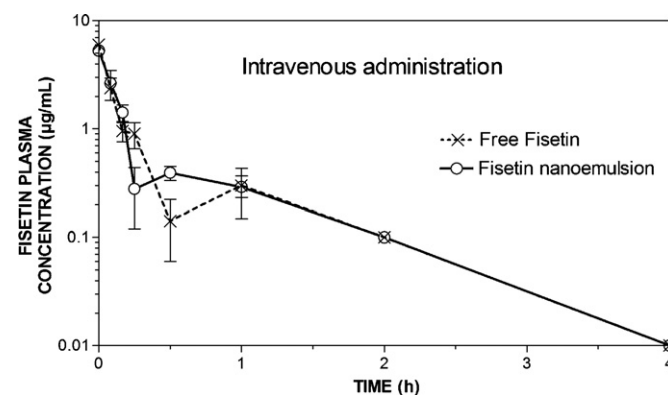
$$^a F_{rel} = (AUC_{NE} \times \text{dose}_{FREE}) / (AUC_{FREE} \times \text{dose}_{NE}).$$

fisetin nanoemulsion was however found relatively toxic, because we noted a mortality rate of 3/21 mice (14%), apparently due to the rapid administration.

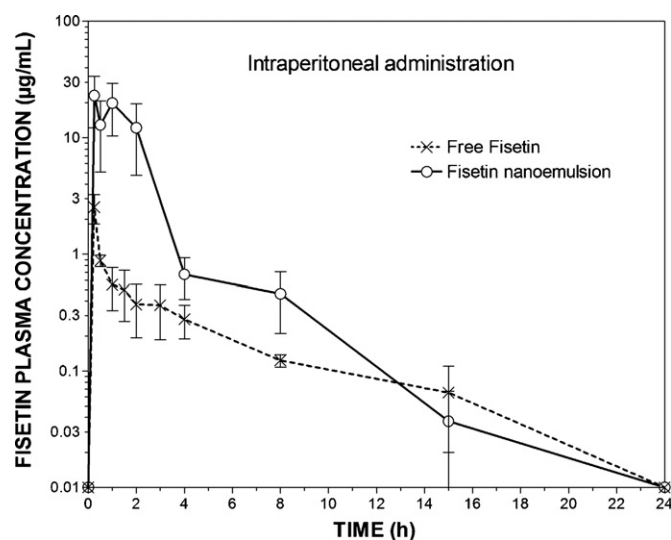
In order to avoid the acute toxicity of the i.v. administration, we next explored the intraperitoneal (i.p.) route. For the fisetin nanoemulsion, a comparison of its pharmacokinetics after i.p. administration with the free fisetin injected by the same route is presented in Fig. 3. Compared to the i.p. administration of the free fisetin, it can be seen that the injection of the fisetin nanoemulsion led to a significant increase in fisetin plasma concentrations, even at a fisetin nanoemulsion dose (112.5 mg/kg) half that of the free fisetin dose (223 mg/kg). The pharmacokinetic parameters presented in Table 6 indicate that not only the maximum plasma concentrations reached were higher for the nanoemulsion, but the relative bioavailability was 24-fold higher compared to the free fisetin. This increase in bioavailability with the nanoemulsion appears to be due to a faster absorption with this drug formulation as shown by a shorter mean absorption time (MAT) of 1.97 h compared to 5.98 h for the free fisetin. It is also noteworthy that following the i.p. administration of the fisetin nanoemulsion, no mortality was observed.

### 3.6. Fisetin nanoemulsion antitumour activity in mice

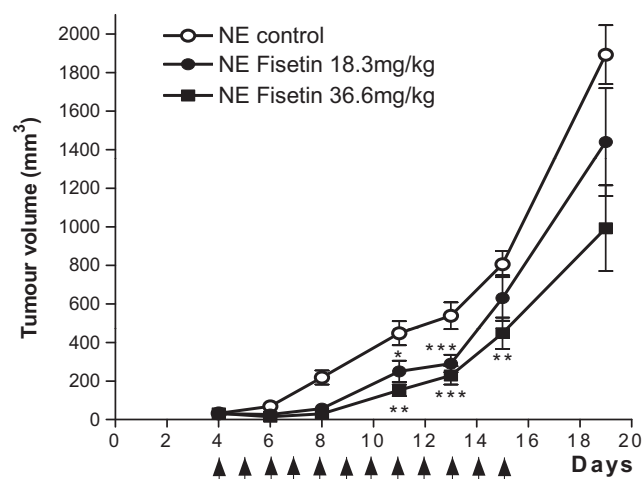
We were next interested to evaluate the antitumour activity of the developed fisetin nanoemulsion (preparation 9) in Lewis lung carcinoma bearing mice. A group of 4 control mice received the empty nanoemulsion and 2 other groups of 4 mice received the fisetin nanoemulsion at an equivalent dose of fisetin of 18.3 and 36.6 mg/kg administered i.p. for 12 days starting on day 4 post tumour implantation. Results depicted in Fig. 4 show that the



**Fig. 2.** Fisetin pharmacokinetics in mice after intravenous administration of free fisetin (dotted line) at 13 mg/kg, and after the intravenous administration of fisetin nanoemulsion at 13 mg/kg (solid line). Error bars, SEM.



**Fig. 3.** Fisetin pharmacokinetics in mice after intraperitoneal administration of free fisetin (dotted line) at 223 mg/kg, and after intraperitoneal administration of fisetin nanoemulsion at 112.5 mg/kg (solid line). Mean  $\pm$  SEM.



**Fig. 4.** Antitumour activity of the nanoemulsion of fisetin. Lewis carcinoma bearing mice received the fisetin nanoemulsion (NE Fisetin) by intraperitoneal injection at 18.3 and 36.6 mg/kg for 12 consecutive days (indicated by arrows). Control mice received the nanoemulsion without fisetin (NE control). The asterisks represent a  $P$  value significant at 0.05 (\*), 0.01 (\*\*) or 0.005 (\*\*\*) level. Mean  $\pm$  SEM of 8 tumours per time point.

fisetin nanoemulsion can significantly inhibit tumour growth in a dose-dependent manner, even at these relatively low dose levels of fisetin.

#### 4. Discussion

The main objectives of this study were to develop a nanoemulsion of the hydrophobic flavonoid fisetin, determine its pharmacokinetics in mice, and evaluate its anticancer activity *in vivo*. The first problems encountered were the low fisetin solubility in the classical oil phases usually employed in formulation, e.g., soybean oil or medium chain triglycerides (Date and Nagarsenker, 2008). The best solubility was observed with triacetin, a short chain triglyceride composed of triester of glycerol and acetic acid. However, triacetin does not exhibit remarkable emulsifying properties (Poullain-Termeau et al., 2008). Fisetin was also found weakly soluble in lipophilic surfactants, but was more soluble in hydrophilic surfactants. As a matter of fact, the maximum solubility was observed with Labrasol®, a mixture of triglycerides and polyethylene glycol esters possessing a hydrophilic–lipophilic balance of 14. We did not observe any synergistic effect between the oil phase and surfactant, be it hydrophilic or lipophilic. Hence, the fisetin low solubility in lipid phases leads to a weak association of the active principle in this phase and therefore requires more solubilising surfactant, as previously reported for a nanoemulsion of carbamazepine (Kelmann et al., 2007). We finally found that the best mixture was the one composed of Labrasol®/Tween® 80 which allowed to achieve an acceptable fisetin concentration of 5 mg/mL.

Although Labrasol® has already been employed in injectable preparations (Nornoo et al., 2008), no standard preparation has been developed so far, to our knowledge, with this proprietary formulation. Labrasol® is mostly used in auto-emulsifying systems for oral administration (Kommuru et al., 2001) and it has been shown to increase oral absorption of hydrophilic drugs, e.g., gentamicin (Hu et al., 2001). Available toxicity data on Labrasol® show that it is relatively non toxic when given orally to rats with a LD50 of 22 g/kg (Gad et al., 2006). However, acute toxicity data are not available for the intravenous or intraperitoneal routes. In our experiments, a mortality rate of 14% was noted for the i.v. route, whereas no mortality was observed for the i.p. route. The other components of the nanoemulsion are not likely to contribute to acute toxicity because medium chain triglycerides (e.g., Miglyol® 812N) and lecithins are already widely used in injectable preparations. Tween® 80 can also be ruled out in this toxicity because it is frequently used at high concentrations, e.g., 25% in docetaxel preparation without apparent toxicity (Strickley, 2004). Therefore, a compromise will have to be found between the toxicity of the excipient and the final concentration in fisetin.

With regard to the physico-chemical properties of the fisetin nanoemulsion, it was found that the preparation containing Miglyol® 812N (No. 9) showed acceptable fisetin content, nanoparticle size and PDI. This fisetin nanoemulsion was found to be unstable at room temperature with increasing diameter and PDI values over time, with phase separation occurring on day 30. This relatively slow process could be explained by the Ostwald ripening in which larger particles grow at the expense of smaller ones due to the higher solubility of the smaller particles and to molecular diffusion through the continuous phase (Capek, 2004). This phenomenon finally ends up in the coalescence of the emulsion (Tadros et al., 2004). Because this phenomenon is temperature sensitive, it was noted that nanoemulsions kept at 4 °C were remarkably stable with almost unchanged particle diameter and PDI over the 30 day examination period. PDI smaller than 0.250 are considered acceptable for parenteral preparations (Müller et al., 2004). However, it was also noted, as expected, that the

nanoemulsion was particularly unstable in steam sterilization conditions (121 °C, 15 min) probably due to the non-ionic surface active agents which are not stable at high temperatures (Nornoo et al., 2008). To overcome this problem, sterilization by filtration could therefore be employed for nanoemulsions (Floyd, 1999). The formulated fisetin nanoemulsion presented a negative zeta potential which is probably due to the anionic fractions of the employed lecithin (Wang et al., 2006).

We have observed that fisetin nanoemulsion could exert its distinct morphological effects on endothelial cells similar to the free fisetin (Touil et al., 2009) indicating that fisetin could be released from its nanoemulsion formulation. These morphological changes on endothelial cells are attributed to a stabilization of the cytoskeleton as previously shown by increased acetylated alpha tubulin (Touil et al., 2009).

Concerning the i.v. administration of the free fisetin and its nanoemulsion, we did not observe any pharmacokinetic difference, as expected for a classical emulsion, contrary to what is observed for a pegylated formulation which can increase the residence time (Reddy and Venkateswarlu, 2005). As a matter of fact, upon injection of a classical emulsion, the particles interact with the apolipoproteins and are captured by the reticulo-endothelial system leading to their rapid elimination from the blood compartment (Kawakami et al., 2000). One possibility to increase the residence time using the i.v. administration would be to pegylate the emulsion that could increase the surface hydrophilic properties by forming a steric barrier that could result in a longer retention time in plasma (Tamilvanan, 2004).

We demonstrated that the i.p. administration of the fisetin nanoemulsion led to a significant improvement in bioavailability compared to the i.p. administration of the free fisetin, with a 24-fold increase in relative bioavailability compared to the free fisetin administered via the same route. This could be due to a faster absorption phase from the peritoneal cavity with the nanoemulsion compared to free fisetin. As a matter of fact, the mean absorption time was shorter with the nanoemulsion (2 h) compared to the free fisetin (6 h). Similar results were reported for the hydrophobic taxoid paclitaxel where i.p. administration was leading to a significant improvement in bioavailability compared to the i.v. administration (Soma et al., 2009). In addition, this enhanced bioavailability of the fisetin nanoemulsion is also probably resulting from the unique lymphatic distribution after i.p. administration which is a favourable property, especially with anticancer drugs that must access lymph nodes which are frequently harbouring metastases (Nishioka and Yoshino, 2001).

We also demonstrated that the fisetin nanoemulsion could elicit a significant antitumour activity *in vivo* in Lewis lung tumour bearing mice. It is noteworthy that a relatively low dose of the fisetin nanoemulsion corresponding to 36.6 mg/kg of fisetin was able to reduce by 53% the tumour volume, whereas a 6-fold higher dose (223 mg/kg) was required to obtain a similar tumour growth inhibition with the free fisetin, as recently reported by Touil et al. (2011). This indicates that the nanoemulsion of fisetin is favourable to its anticancer action *in vivo* probably by increasing its bioavailability, as shown in this study.

#### 5. Conclusion

In conclusion, we have developed a nanoemulsion of fisetin that allowed to solubilize a relatively high concentration of fisetin (5 mg/mL), thanks to the use of two surface active agents, i.e., Tween® 80 and Labrasol®. However the latter agent appeared to be relatively toxic when using the i.v. route, but was not found toxic by the i.p. route. The developed fisetin nanoemulsion could also markedly increase the bioavailability of fisetin after i.p.

administration and was also found to significantly improve its anti-tumoral activity in tumour bearing mice compared to the free fisetin. The developed nanoemulsion of fisetin could therefore advantageously be employed to improve the antiangiogenic and anticancer activities of this flavonoid, as well as other flavonoids sharing the same problems of in vivo administration.

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## References

- Arai, Y., Watanabe, S., Kimira, M., Shimoi, K., Mochizuki, R., Kinae, N., 2000. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *J. Nutr.* 130, 2243–2250.
- Becher, P., 1965. Technique of emulsification. In: *Emulsions: Theory and Practice*. Reinhold Publishing Corp, New York, pp. 267–325.
- Böhl, M., Tietze, S., Sokoll, A., Madathil, S., Pfennig, F., Apostolakis, J., Fahmy, K., Gutzeit, H.O., 2007. Flavonoids affect actin functions in cytoplasm and nucleus. *Biophys. J.* 93, 2767–2780.
- Capek, I., 2004. Degradation of kinetically-stable o/w emulsions. *Adv. Colloid Interface Sci.* 107, 125–155.
- Chien, C.S., Shen, K.H., Huang, J.S., Ko, S.C., Shih, Y.W., 2010. Antimetastatic potential of fisetin involves inactivation of the PI3K/Akt and JNK signaling pathways with downregulation of MMP-2/9 expressions in prostate cancer PC-3 cells. *Mol. Cell. Biochem.* 333, 169–180.
- Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A., Moon, R., 1995. Flavonoids as DNA topoisomerase antagonists and poisons: structure–activity relationships. *J. Nat. Prod.* 58, 217–225.
- Date, A.A., Nagarsenker, M.S., 2008. Parenteral microemulsions: an overview. *Int. J. Pharm.* 355, 19–30.
- Edgell, C.J., McDonald, C.C., Graham, J.B., 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. U. S. A.* 80, 3734–3737.
- Floyd, A.G., 1999. Top ten considerations in the development of parenteral emulsions. *Pharm. Sci. Technol. Today* 4, 134–143.
- Fotsis, T., Pepper, M.S., Aktas, E., Breit, S., Rasku, S., Adlercreutz, H., Wahala, K., Montesano, R., Schweigerer, L., 1997. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Res.* 57, 2916–2921.
- Gad, S.C., Cassidy, C.D., Aubert, N., Spainhour, B., Robbe, H., 2006. Nonclinical vehicle use in studies by multiple routes in multiple species. *Int. J. Toxicol.* 25, 499–521.
- Ganta, S., Paxton, J.W., Baguley, B.C., Garg, S., 2008. Pharmacokinetics and pharmacodynamics of chlorambucil delivered in parenteral emulsion. *Int. J. Pharm.* 360, 115–121.
- Gibaldi, M., Perrier, D., 1982. Noncompartmental analysis based on statistical moment theory. In: Swarbrick, J. (Ed.), *Pharmacokinetics*. Marcel Dekker, Inc., New York and Basel, pp. 409–417.
- Guzzo, M.R., Uemi, M., Donate, P.M., Nikolaou, S., Machado, A.E., Okano, L.T., 2006. Study of the complexation of fisetin with cyclodextrins. *J. Phys. Chem. A* 110, 10545–10551.
- Han, M., He, C.X., Fang, Q.L., Yang, X.C., Diao, Y.Y., Xu, D.H., He, Q.J., Hu, Y.Z., Liang, W.Q., Yang, B., Gao, J.Q., 2009. A novel camptothecin derivative incorporated in nano-carrier induced distinguished improvement in solubility, stability and anti-tumor activity both in vitro and in vivo. *Pharm. Res.* 26, 926–935.
- Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* 96, 67–202.
- Hu, Z., Tawa, R., Konishi, T., Shibata, N., Takada, K., 2001. A novel emulsifier, labrasol, enhances gastrointestinal absorption of gentamicin. *Life Sci.* 69, 2899–2910.
- Jankun, J., Selman, S.H., Aniola, J., Skrzypczak-Jankun, E., 2006. Nutritional inhibitors of urokinase: potential applications in prostate cancer prevention and treatment. *Oncol. Rep.* 16, 341–346.
- Kan, P., Chen, Z.B., Lee, C.J., Chu, I.M., 1999. Development of nonionic surfactant/phospholipid o/w emulsion as a paclitaxel delivery system. *J. Control. Release* 58, 271–278.
- Kawakami, S., Yamashita, F., Hashida, M., 2000. Disposition characteristics of emulsions and incorporated drugs after systemic or local injection. *Adv. Drug Deliv. Rev.* 45, 77–88.
- Kelmann, R.G., Kuminek, G., Teixeira, H.F., Koester, L.S., 2007. Carbamazepine parenteral nanoemulsions prepared by spontaneous emulsification process. *Int. J. Pharm.* 342, 231–239.
- Khan, N., Asim, M., Afaq, F., Abu, Z.M., Mukhtar, H., 2008. A novel dietary flavonoid fisetin inhibits androgen receptor signaling and tumor growth in athymic nude mice. *Cancer Res.* 68, 8555–8563.
- Kimira, M., Arai, Y., Shimoi, K., Watanabe, S., 1998. Japanese intake of flavonoids and isoflavonoids from foods. *J. Epidemiol.* 8, 168–175.
- Kommuru, T.R., Gurley, B., Khan, M.A., Reddy, I.K., 2001. Self-emulsifying drug delivery systems (SEDDS) of coenzyme Q10: formulation development and bioavailability assessment. *Int. J. Pharm.* 212, 233–246.
- Leonarduzzi, G., Testa, G., Sottero, B., Gamba, P., Poli, G., 2010. Design and development of nanovehicle-based delivery systems for preventive or therapeutic supplementation with flavonoids. *Curr. Med. Chem.* 17, 74–95.
- Lopez-Lazaro, M., 2002. Flavonoids as anticancer agents: structure–activity relationship study. *Curr. Med. Chem. Anticancer Agents* 2, 691–714.
- Lu, H., Chang, D.J., Baratte, B., Meijer, L., Schulze-Gahmen, U., 2005a. Crystal structure of a human cyclin-dependent kinase 6 complex with a flavonol inhibitor, fisetin. *J. Med. Chem.* 48, 737–743.
- Lu, X., Jung, J., Cho, H.J., Lim, D.Y., Lee, H.S., Chun, H.S., Kwon, D.Y., Park, J.H., 2005b. Fisetin inhibits the activities of cyclin-dependent kinases leading to cell cycle arrest in HT-29 human colon cancer cells. *J. Nutr.* 135, 2884–2890.
- Middleton Jr., E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* 52, 673–751.
- Mignet, N., Seguin, J., Ramos Romano, M., Brullé, L., Touil, Y.S., Scherman, D., Bessodes, M., Chabot, G.G., 2012. Development of a liposomal formulation of the natural flavonoid fisetin. *Int. J. Pharm.* 423, 69–76.
- Mulak, G., Cotty, J., 1975. Solubilité et constante diélectrique. *Sci. Technol. Pharm.* 4, 281–288.
- Müller, R.H., Schmidt, S., Buttle, L., Akkar, A., Schmitt, J., Brömer, S., 2004. SolEmuls-novel technology for the formulation of i.v. emulsions with poorly soluble drugs. *Int. J. Pharm.* 269, 293–302.
- Nishioka, Y., Yoshino, H., 2001. Lymphatic targeting with nanoparticulate system. *Adv. Drug Deliv. Rev.* 47, 55–64.
- Nornoo, A.O., Osborne, D.W., Chow, D.S., 2008. Cremophor-free intravenous microemulsions for paclitaxel I: formulation, cytotoxicity and hemolysis. *Int. J. Pharm.* 349, 108–116.
- Olaharski, A.J., Mondrala, S.T., Eastmond, D.A., 2005. Chromosomal malsegregation and micronucleus induction in vitro by the DNA topoisomerase II inhibitor fisetin. *Mutat. Res.* 582, 79–86.
- Park, H.H., Lee, S., Oh, J.M., Lee, M.S., Yoon, K.H., Park, B.H., Kim, J.W., Song, H., Kim, S.H., 2007. Anti-inflammatory activity of fisetin in human mast cells (HMC-1). *Pharmacol. Res.* 55, 31–37.
- Poullain-Termieu, S., Crauste-Manciet, S., Brossard, D., Muhamed, S., Nicolaos, G., Farinotti, R., Barthelemy, C., Robert, H., Odou, P., 2008. Effect of oil-in-water submicron emulsion surface charge on oral absorption of a poorly water-soluble drug in rats. *Drug Deliv.* 15, 503–514.
- Reddy, P.R., Venkateswarlu, V., 2005. Pharmacokinetics and tissue distribution of etoposide delivered in long circulating parenteral emulsion. *J. Drug Target.* 13, 543–553.
- Sarker, D.K., 2005. Engineering of nanoemulsions for drug delivery. *Curr. Drug Deliv.* 2, 297–310.
- Soma, D., Kitayama, J., Ishigami, H., Kaisaki, S., Nagawa, H., 2009. Different tissue distribution of paclitaxel with intravenous and intraperitoneal administration. *J. Surg. Res.* 155, 142–146.
- Strickley, R.G., 2004. Solubilizing excipients in oral and injectable formulations. *Pharm. Res.* 21, 201–230.
- Sung, B., Pandey, M.K., Aggarwal, B.B., 2007. Fisetin, an inhibitor of cyclin-dependent kinase 6, down-regulates nuclear factor-kappaB-regulated cell proliferation, antiapoptotic and metastatic gene products through the suppression of TAK-1 and receptor-interacting protein-regulated IkkappaBalpha kinase activation. *Mol. Pharmacol.* 71, 1703–1714.
- Surh, Y.J., 2003. Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer* 3, 768–780.
- Tadros, T., Izquierdo, P., Esquena, J., Solans, C., 2004. Formation and stability of nano-emulsions. *Adv. Colloid Interface Sci.* 108–109, 303–318.
- Tagne, J.B., Kakumanu, S., Nicolosi, R.J., 2008. Nanoemulsion preparations of the anticancer drug dacarbazine significantly increase its efficacy in a xenograft mouse melanoma model. *Mol. Pharm.* 5, 1055–1063.
- Tamilvanan, S., 2004. Oil-in-water lipid emulsions: implications for parenteral and ocular delivering systems. *Prog. Lipid Res.* 43, 489–533.
- Touil, Y.S., Fellous, A., Scherman, D., Chabot, G.G., 2009. Flavonoid-induced morphological modifications of endothelial cells through microtubule stabilization. *Nutr. Cancer* 61, 310–321.
- Touil, Y.S., Seguin, J., Scherman, D., Chabot, G.G., 2011. Improved antiangiogenic and antitumour activity of the combination of the natural flavonoid fisetin and cyclophosphamide in Lewis lung carcinoma-bearing mice. *Cancer Chemother. Pharmacol.* 68, 445–455.
- Tripathi, R., Samadder, T., Gupta, S., Surolia, A., Shaha, C., 2011. Anticancer activity of a combination of cisplatin and fisetin in embryonal carcinoma cells and xenograft tumors. *Mol. Cancer Ther.* 10, 255–268.
- Wang, J.J., Sung, K.C., Hu, O.Y., Yeh, C.H., Fang, J.Y., 2006. Submicron lipid emulsion as a drug delivery system for nalbuphine and its prodrugs. *J. Control. Release* 115, 140–149.
- Willson, J.E., Brown, D.E., Timmens, E.K., 1965. A toxicologic study of dimethyl sulfoxide. *Toxicol. Appl. Pharmacol.* 7, 104–112.
- Woodman, O.L., Chan, E.C., 2004. Vascular and anti-oxidant actions of flavonols and flavones. *Clin. Exp. Pharmacol. Physiol.* 31, 786–790.