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POTENTIATION OF THE CYTOTOXIC ACTIVITY OF NUTRACEUTICAL PHLORETIN AGAINST CERVICAL CANCER BY FORMULATION INTO MICROEMULSION

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The present study aimed to investigate the cytotoxic effect of phloretin–a nutraceutical found in apples and its nanoparticulated form–on cervical cancer. Phloretin surfactant-based nanoparticles (microemulsions) were prepared and characterized for particle size, charge, polydispersity, stability under refrigeration conditions and morphology. The cytotoxic activity of phloretin and its microemulsion was tested in cervical cancer HeLa cell line using tetrazolium dye colorimetric assay. Results revealed that phloretin microemulsion exhibited suitable particle size (10.65 ± 0.10 nm), homogenous dispersion (PDI = 0.29 ± 0.05), and negative charge of –9.99 ± 0.81. The microemulsion was stable on storage for 3 months and displayed spherical particle morphology. Both phloretin and its microemulsion exhibited cytotoxic activity against HeLa cells, with IC50 values of 15.3 ± 0.8 and 2.94 ± 0.12 μg/mL, respectively, suggesting more than 5-fold potentiation of the cytotoxic effect of phloretin against cervical cancer by nanoencapsulation. Phloretin microemulsion is concluded to be a stable product of enhanced cytotoxic activity, and findings might be useful for research involving other nutraceuticals.

Keywords: cervical cancer; HeLa cells; phloretin; nutraceutical; nanoparticles; microemulsion.

1. INTRODUCTION

Throughout the years, nature has provided us with solutions for treatment of diseases, even though these might be difficult to treat. Finding new dietary anti-cancer active agents and verification of their anticancer mechanism has emerged as a current branch of new drug discovery research. The group of nutraceuticals that have recently gained interest includes phloretin, which is a dihydrochalcone molecule found in apples, pear and cherry (Fig. 1). Phloretin is considered a multi-task therapeutic molecule, which has been reported to exhibit several activities such as antioxidant and anticancer [1]. Till current date, phloretin has proven anticancer activity in several types of cancer including oral cancer [1, 2], lung cancer [3], colon cancer [4, 5], breast cancer [6], liver cancer [7], skin cancer and leukemia [8]. The cancer types not tested against phloretin include cervical cancer, which is among the most common cancers in women, with more than half million new cases arising each year and accounting for about 7.5% of cancer deaths in females [9]. Causes of cervical cancer include genetic factors, environmental factors such as stress, and viral infections such as human papilloma virus [9, 10].

Despite the promising features of phloretin, its bioavailability is hampered by low water solubility (logP 3.9), and hence, a recently adopted strategy to solubilize it in sufficient amount consists in encapsulating it within nanoparticles [1, 2]. Moreover, the encapsulation of active agents in nanoparticulated forms was reported to particularly potentiate the anticancer effect of compounds [11 – 13], especially if they are nutraceuticals [14 – 16]. Among the different types of nanoparticles, microemulsions present a very useful form for encapsulation of therapeutics, owing to the formation of aqueous and oily domains in the presence of surfactants and cosurfactants [17 – 19] causing solubilization of the poorly water-soluble drugs. Polysorbate 20 (Tween 20) is a polysorbate-type nonionic surfactant formed by the ethoxylation of sorbitan before the addition of lauric acid. It is a stable and safe detergent, and hence it was previously reported in the formulation of nanoparticles intended for pharmacological applications [20, 21].

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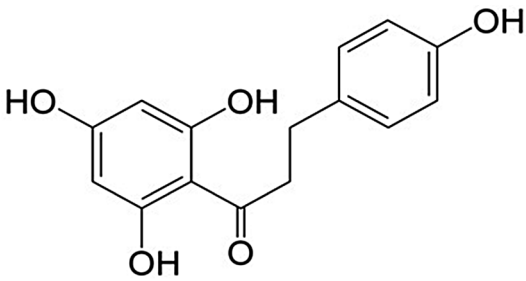

Fig. 1. Chemical structure of phloretin.

The novelty of this work consists in that, till current date, phloretin has only been encapsulated in chitosan nanoparticles [1, 2] and in the related microemulsion form only its therapeutic action for the treatment of vaginitis was reported [20]. The activity of phloretin against cervical cancer has not been tested until now. Therefore, the aim of this work was to prepare and characterize a surfactant-based nanosystem for the encapsulation of phloretin and test the efficacy of both the drug and nanosystem in treating cervical cancer.

2. MATERIALS AND METHODS

2.1. Materials

Phloretin was purchased from SkinActives Co., USA. The cell culture chemicals were purchased from Lonza, Belgium. All other chemicals were purchased from Sigma Aldrich Co., USA. HeLa cancer cells were obtained from the American Type Culture Collection (ATCC, USA).

2.2. Preparation and Characterization of Phloretin Microemulsion

A microemulsion form of phloretin was prepared by aqueous titration [21, 22], for which 100 mg of phloretin was dispersed in a mixture of 4.1 mL Tween 20, 0.28 mL oleic acid and 0.32 mL ethanol, followed by mixing on a magnetic stirrer. The mixture was titrated to a total weight of 10 gram with water in a dropwise manner to form oil in water microemulsion loaded with phloretin.

The particle size, zeta potential and polydispersity index (PDI) of phloretin microemulsion were measured using the Zetasizer device (Model ZS3600, Malvern, UK) [23]. The as-prepared phloretin microemulsion morphology was examined using transmission electron microscopy (TEM) without staining, after being dried on a carbon-coated grid (VERSA 3D, USA [24, 25].

The properties of phloretin microemulsion (particle size, PDI and zeta potential) were re-measured after 3 months storage at room temperature, to verify stability of the prepared formulation [26].

2.3. Evaluation of the Cytotoxicity of Phloretin and Its Microemulsion on Cervical Cancer HeLa Cells

The cytotoxic activity of phloretin and its microemulsion form on HeLa cells was assessed using the MTT assay as described elsewhere [27, 28], and the 50% inhibitory concentration (IC_{50}) was contrasted.

2.4. Statistical Analysis

All measurements were done in triplicate and reported as mean ± S. D. T-test was performed using Graphpad Instat, at significance of $P \leq 0.05$. The IC_{50} values were calculated using Graphpad Prism software (San Diego, CA. USA).

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Phloretin Microemulsion

Phloretin microemulsion was successfully prepared using the water dilution method, which is suitable for the large scale production of drug delivery systems. Despite its promising therapeutic activities, phloretin nanoparticles were not fully explored till current date, and only few reports were attempted by some researchers [1, 2, 29]. Therefore in the current manuscript, a water dilutable microemulsion is reported for loading a high dose of phloretin adn exploring its cytotoxic effect on cervical cancer.

The obtained phloretin microemulsion had a small particle size of 10.65 ± 0.1 nm, PDI value of 0.29 ± 0.05, and zeta potential of –9.99 ± 0.81 (Table 1). The small particle size in the microemulsion could be ascribed to their surfactant and cosurfactant content [21] and was further confirmed by TEM examonation. The low polydispersity of the microemulsion (less than 0.4) suggests the existance of homogenous nanoparticle formulation, and the effective loading of the high dose of phloretin within the microemulsion domains. The negative charge on the particles of the microemulsion could be attributed to the presence of ethanol among the constituents [30], and its relative low value might be assigned to

TABLE 1. Physicochemical Properties (Particle Size, PDI, and Zeta Potential) of Phloretin Microemulsion Tested as Freshly Prepared and After Storage

Particle size (nm) (Mean ± S.D.)		Zeta potential (mV) (Mean ± S.D.)		PDI (Mean ± S.D.)	
Freshly prepared	After storage	Freshly prepared	After storage	Freshly prepared	After storage
10.65 ± 0.1	11 ± 0.29	-9.99 ± 0.81	-10.11 ± 0.19	0.29 ± 0.05	0.31 ± 0.09

the fact that the majority of the formulation was dominated by the surfactant which is non-ionic in nature. The negative charge on the particles of the microemulsion could also be ascribed to the presence of oleic acid, which bears a net negative charge under physiological conditions.

TEM micrograph presented in Fig. 2 shows that the phloretin microemulsion consisted of homogenous non-aggregated spherical droplets, with particle size complying with those obtained with the Zetasizer device in the previous section. As can be seen from data in Table 1, no statistically significant changes occurred to the particle size, zeta potential or PDI of phloretin microemulsion after storage for 3 months ($P > 0.05$). The negative charge imparted significant physical stability to the microemulsion, in which none of its physicochemical properties (particle size, zeta potential and PDI) was changed after 3 months-storage, suggesting the stable nature of the microemulsion when stored under ambient conditions.

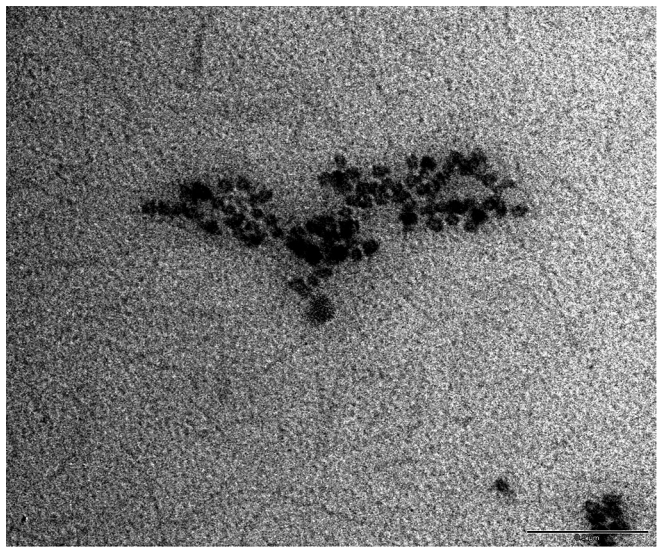

Fig. 2. Transmission electron microscopy image of phloretin microemulsion.

3.2. Cytotoxicity of Phloretin and Its Microemulsion on Cervical Cancer HeLa Cells

As can be seen from data in Table 2, both phloretin and its microemulsion exhibited cytotoxic activity against cervical cancer. Phloretin started to exhibit significant cytotoxic effect on HeLa cells starting from concentration 2 μg/mL, while its microemulsion exhibited cytotoxicity starting from 0.25 μg/mL. The calculated IC_{50} values were 15.3 ± 0.8 and 2.94 ± 0.12 μg/mL for phloretin and its microemulsion, respectively, suggesting significantly higher cytotoxic effect of the latter ($P < 0.05$).

As observed from the cytotoxicity testing results of phloretin and its microemulsion counterpart, it can be inferred that both phloretin on its own and in microemulsion form exhibited cytotoxic effect on HeLa cells. This could be ascribed to the ability of phloretin to inhibit cell growth, induce apoptosis and regulate the cell cycle [2, 6]. A prominent reason behind the apoptotic action of phloretin is the inhibition of glucose II transporter mechanism [7]. This glucose deprivation causes the proliferating cancer cells to lose their major energy source, and hence start to deteriorate [6]. Another reason is the ability of phloretin to activate the mitochondrial mediated cell death and to modulate cyclins [31]. The antioxidant nature of phloretin further augments its cytotoxic effect, since it can scavenge free radicals and inhibit lipid peroxidation [30]. All of the aforementioned therapeutic properties could be ascribed to the unique structure of phloretin; being an unsaturated polyphenolic compound, and hence it can reverse abnormal signaling and cellular transformation [31].

The superior cytotoxic effect of the microemulsion compared to phloretin alone (more than 5 folds higher inhibition for the former) could be ascribed to the better ability of the microemulsion particles to be internalized within the cancer cells owing to their small size [16]. The most reported chemotherapeutics for treatment of cervical cancer are cisplatin, carboplatin and paclitaxel [32]. Interestigly, phloretin microemulsion exhibited superior cytotoxic effect and significantly lower IC_{50} values compared to the values reported in the literature for cisplatin (5.4 μg/ml), carboplatin (323.5 μg/ml) and paclitaxel (112.5 μg/ml) on HeLa cells after incubation for 24 h as in our case [33, 34]. This result delineates phloretin as a potent antineoplatic drug in cervical cancer, and suggests that a microemulsion form of this nutraceutical can be a powerful therapeutic tool that combats

TABLE 2. Hela Cancer Cell Percentage Viability as Function of Concentration for Phloretin and Its Microemulsion

Sample conc. (μg/mL)	Cell % viability for phloretin	Cell % viability for phloretin microemulsion
500	3.91 ± 0.65	1.78 ± 0.42
250	5.86 ± 1.23	3.59 ± 0.65
125	9.72 ± 0.54	6.21 ± 1.37
62.5	17.54 ± 1.89	10.96 ± 0.58
31.25	38.15 ± 2.73	18.40 ± 0.84
15.6	49.23 ± 3.41	25.82 ± 0.96
7.8	70.46 ± 2.82	32.74 ± 1.42
3.9	86.92 ± 1.53	41.83 ± 2.91
2	93.04 ± 0.96	57.29 ± 2.73
1	99.73 ± 0.21	71.42 ± 2.54
0.5	100	82.97 ± 1.39
0.25	100	91.38 ± 0.46
0	100	100

cervical cancer while avoiding the possible side effects resulting from treatment with conventional chemotherapeutics.

Thus, in the present work, phloretin proved itself as a powerful cytotoxic agent against cervical cancer with comparable effect to cisplatin, and even more powerful than carboplatin and paclitaxel. Moreover, the nanoencapsulation of phloretin in microemulsion form resulted in the potentiation of its therapeutic effect in the treatment of cervical cancer. Futuristic studies will include testing both phloretin and its microemulsion form on resistant HeLa cells, as well as conduction of preclinical studies in animal models.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest

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