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Cytotoxic effect of novel *Flammulina velutipes* sterols and its oral bioavailability *via* mixed micellar nanoformulation

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ABSTRACT

The aim of this study was to investigate the anti-tumor effect of sterols initially separated from *Flammulina velutipes* and the pharmacokinetics and tissue distribution after oral administration of *F. velutipes* sterol nanomicelles (FVSNs). *F. velutipes* sterol (FVS) consisted of mainly ergosterol (54.78%), 22,23-dihydroergosterol (27.94%) and ergost-8(14)-ene-3β-ol (discovered for the first time in *F. velutipes*). *In vitro* cytotoxicity assay of FVS against U251 cells and HeLa cells showed that at 72 h treatment, the FVS (IC_{50} = 23.42 μg/mL) exhibited strong inhibitory effect against U251 cells, even overwhelmed the standard anti-tumor drug (5-fluorouracil) to an extent, while the HeLa cells were not significantly susceptible to the FVS. To improve the solubility and bioavailability of FVS, a model for insoluble anti-tumor drugs, FVSNs were prepared. *In vitro* characterization of FVSNs revealed satisfactory size distribution, loading capacity and encapsulation efficiency. Pharmacokinetic study in SD rats demonstrated that the mixed micellar nanoformulation significantly enhanced the bioavailability of FVS than free drug. Additionly, tissue distribution in mice manifested that the biodistribution of FVSNs as compared to the free FVS suspension were significantly improved. In conclusion, the nanomicelles developed in our study provided a promising delivery system for enhancing the oral bioavailability and selective biodistribution of FVS, a potential anti-tumor agent.

1. Introduction

Enormous efforts are ongoing to identify new anticancer compounds from plants, no wonder in eastern countries, China especially, Traditional Chinese medicine (TCM) occupied, and still occupies a significant position in primary health care in less developing rural areas and also greatly appreciated in well developed urban due to its thousands-year-old clinical tradition, or in the West, interest in TCM stems from the hope that it might complement Western medicine and bioactive natural products as chemical lead compounds for the generation of semi-synthetic derivatives. However, the unclear multi-components and therapeutic mechanism, limited water solubility and bioavailability as well as poor targeting specificity greatly hamper the drug ability of these promising antitumor candidates. Naturally, of particular interests are technologies for improving the bioavailability and targeted delivery with the administration of these compounds. Of further interest is the elucidation of the assessment of their kinetics, tissue distribution and elimination routes. These data have indeed become an important issue to link data from pharmacological assays and clinical values. Besides, a better understanding of the pharmacokinetics and bioavailability of phytopharmaceuticals can also aid in designing rational dosage regimens (Bhattaram et al., 2002).

Flammulina velutipes, belonging to *Basidiomycotina* of *Eumycota*, popularly known as golden-needle mushroom or winter mushroom, is a kind of nutritional and vegetable functional food with high edible value for its rich content in proteins and carbohydrates, high in fiber and low in fat (Ko et al., 2007; Yang et al., 2007). With the polysaccharides, proteins and glycoproteins as the important components isolated, *F. velutipes* have been correlated with multiple pharmacological activities such as immunomodulatory, antioxidant, cholesterol-lowering, hypoglycaemia-induction and anti-cancer activities (Ko et al., 1995; Leung et al., 1997; Wu et al., 2008; Yang et al., 2012). However, when it comes to small

Abbreviations: FVS, *Flammulina velutipes* sterol; FVSNs, *Flammulina velutipes* sterol nanomicelles.

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molecules, the information on biological activities is limited, which is well proven by the scarce studies on *F. velutipes* sterol (FVS). To our best knowledge, no research has been reported on the *in vitro* antineoplastic activity of FVS in the treatment of gliomas and cervical carcinoma up to now.

Given the chemical property, FVS can easily dissolve in ether, chloroform, hot ethanol as well as other organic solvents, but to a lesser extent in water, as a result its future utilization in the clinical area would be discounted extremely heavy. In recent years, advances in nanotechnology (polymeric nanoparticles or micelles, nanocapsules, liposomes, carbon nanotubes, phytosomes and microemulsion *etc.*) have shown promise in improving component solubility, enhancement of bioavailability, increasing absorbency of the organism, reducing medicinal herb doses, together with achieving steady-state therapeutic levels of drugs over an extended period compared with traditional Chinese herb drug preparations (Ajazuddin and Saraf, 2010; Bhadoriya et al., 2011; Yang et al., 2010). Among these nanocarriers, nanomicelles have emerged as one of the most useful modalities for suitable and versatile drug carriers. The polymeric micelles are self-assembling nanoscale systems and formed by microphase separation in selective solvents. Its unique features including hydrophilic outer shell and hydrophobic inner core impart its ability to dissolve in water, which leads to the spontaneous formation of nanomicelles with the competency of encapsulating and solubilizing compounds of different nature. Through a sterol-loaded microemulsion formulation, our previous study (Yi et al., 2012) succeeded in enhancing oral bioavailability of FVS. However, no significant improvement appeared in the data of elimination half-life ($t_{1/2}$) of sterol encapsulated in microemulsions compared with free sterol, suggesting its less-than-ideal duration of pharmacologic action. Comparatively, self-assembled nanomicelles, composed of polymeric amphiphiles, have a potential to bring several advantages to therapeutic systems because of the long circulation time, high drug loading capacity and low dose of formulation needed (Ajazuddin and Saraf, 2010; Gong et al., 2012; Jiang et al., 2011; Muthu et al., 2009). Besides, compared with long-circulating liposomes, nanomicelles are of some unique merits in controlled release profiles, cell permeability as well as fewer adverse effects (such as hand - foot syndrome and hypersensitivity reaction) (Nishiyama and Kataoka, 2006). Furthermore, Polymeric nanomicelles possess several prominent advantages over conventional surfactant micelles in that they have better thermodynamic stability in physiological solution, as indicated by their low critical micellar concentration (CMC), which makes the nanomicellar formulation stable and prevents their rapid dissociation *in vivo* (Muthu et al., 2009).

Currently, several passive targeted nanomicelles containing anticancer drugs, such as paclitaxel (extracted from the needles of yew trees *Taxus baccata* L.), doxorubicin and cisplatin, are already under preclinical and clinical invetigations (Nishiyama and Kataoka, 2009). Pharmacokinetic and bioavailability studies that have been conducted for these pharmaceuticals of great practical importance are critically evaluated. Xiao and co-workers labeled a micelle system covalently with ^{125}I and loaded the nanomicelles with ^{14}C-paclitaxel. The liquid scintillation counting confirmed that ^{14}C-labeled paclitaxel sequestered in nanomicelles had increased uptake by tumor tissue and slower pharmacokinetics than Taxol® and may be clinically useful for both tumor imaging and improved chemotherapy applications (Xiao et al., 2012). Generally speaking, nanomicelles, from a pharmacokinetic point of view, could be exploited as promising highly efficient drug delivery vehicles for anti-tumor drugs.

Till date, neither extensive *in vivo* nor *in vitro* investigations have been reported by any other researchers on the effectiveness of a mixed micellar nanoformulation as a delivery system to enhance oral bioavailability of FVS. In this study, anti-tumor components were initially screened from *F. velutipes* using lipid rafts chromatography based on tyrosine kinase receptor (unpublished results). This was followed by methyl thiazolyl tetrazolium (MTT) bioassay of the isolated compounds and then the preparation of the nanomicelles formulation (a polyphase dispersed system containing PVP-phospholipid-sodium cholate loaded with FVS). The *F. velutipes* sterol nanomicelles (FVSNs) were finally evaluated for its bioavailability and selective tissue distribution as against the free FVS using rats and mice respectively.

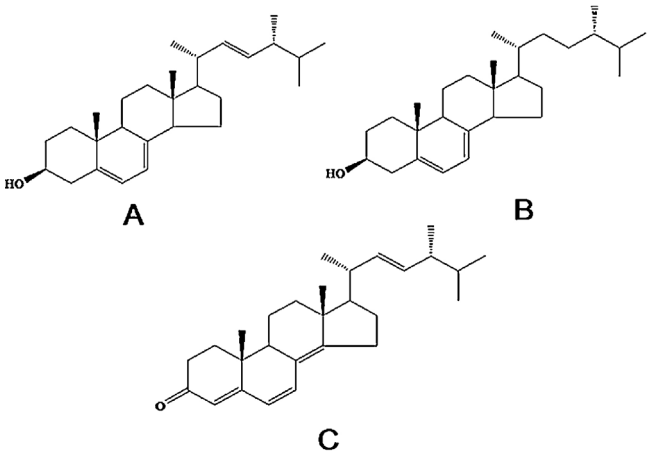

Fig. 1. Chemical structures of ergosterol (A), 22,23-dihydroergosterol (B) and ergine (C).

2. Materials and methods

2.1. Materials

2.1.1. Substances

F. velutipes was kindly supplied by Zhengdong Ecological Agriculture Development Center (Jiangsu, China) and air blown to dryness at 55 °C before use. 5-fluorouracil was supplied by Jin Yao Amino Acid Co., Ltd. (Tianjin, China). Reference A – ergosterol (Fig. 1A, purity ≥98%) was obtained from Acros Organics (Geel, Belgium). 22,23-dihydroergosterol (Fig. 1B, reference B) with the purity ≥98% was prepared in our laboratory. Ergine (ergosta-4,6,8(14),22-tetraen-3-one, Fig. 1C) of 98% purity was purchased from BioBioPha Co., Ltd. (Yunnan, China). PVP-K30 (pharmaceutical grade, molecular weight of 40,000, polydispersity of 2.5) was produced by Sunpower New Material Co., Ltd (Shanghai, China). Phospholipid (soybean lecithin, for injection, with phosphatidylcholine content of 70%) was purchased from Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Sodium cholate and absolute ethanol were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ethanol of 95% purity was purchased from Guangyuan Co., Ltd. (Shandong, China). Pure chromatographic methanol was obtained from Hanbon Sci. &Tech. (Jiangsu, China). Analytically pure ethyl ether was purchased from Kelong Chemical Reagent Factory (Sichuan, China). All the other chemicals used in the study were of analytical grade and obtained commercially.

2.1.2. Cell line

Human glioma cell lines (U251) and human cervical carcinoma cell lines (HeLa) were obtained from Cell Bank of Academy of Science (Shanghai, China). They were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum in an atmosphere of humidified air containing 5% CO_2 at 37 °C in culture flasks.