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RESEARCH ARTICLE

A method to evaluate the bioactive function of fruit extracts of Chinese wild *Citrus* with microtubular activity

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Abstract

China is one of the most important centers of origin for *Citrus* genetic resources. Due to the high content of secondary metabolites, mining wild Chinese *Citrus* for novel medical applications is promising. In this study, extracts of Chinese wild species from different taxonomical groups were screened for potential effects on microtubules (MTs) *in vitro*. MT density as a readout for nucleation, and frequency distribution over MT lengths as a readout for elongation and decay were determined by quantitative image analysis *via* a standardized coverslip assay using fluorescently labelled neurotubulin. Extract from peels of *Citrus ichangensis* Swing. strongly increased the density of MTs; whereas, extract from peels of *Citurs limon* (L.) Burm.f. exerted the opposite effect. Extract from pulp of *Citrus limonia* Osbeck promoted MT elongation, and in addition induced a small population of very long MTs. These data suggest that wild Chinese *Citrus* harbour compounds that act specifically on different aspects of MT nucleation, elongation, and decay.

Keywords: fruit extract, natural product-based pharmaceuticals, Chinese wild *Citrus*, microtubules secondary plant metabolites

1. Introduction

Plants generate secondary metabolites whose complex chemical structures have evolved for specific interaction with other molecules or cellular components (Li and Vederas 2009; Sadot 2014), or to specifically manipulate the biology of other organisms as so called allelochemicals (Rattan 2010). To ward off herbivorous insects is an important strategy for plant survival. Metabolites acting as insecticides often interfere with cellular or neural signaling (Wink 2000). For instance, the target for several alkaloids, one of the classes of natural products, are the acetylcholine receptors at the neuromuscular end plate (Bloomquist 1996; Goodsell 2000). However, for most secondary metabolites, the specific mode of action in the feeding insects is far from understood. In addition to alkaloids, phenolics and terpenoids have been reported as insecticides or pharmaceutical application (Pasqua *et al.* 2004; Rattan 2010).

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A very efficient way to block the development of a feeding animal is to inhibit cell division. Cell division depends on the microtubular division spindle, therefore inhibitors of microtubule (MT) assembly are powerful blockers of cell division and widely used, such as arresting tumour growth (Lodish *et al.* 2000). Since MTs are essential but different between plants and animals with respect to the molecular details of tubulin and associated proteins, they offer sufficient specificity for drug targets in medical applications. In the natural functional context (warding off insects), this specificity is further enhanced by the fact that the producer plant can sequester these toxic compounds by secretion in glandulair hairs or by storage in lysogenic oil bodies, which ensures that such compounds are blocking the feeding insect but do not impair the normal development of the host plant. Targets for such compounds can be the (relatively conserved) α- and β-tubulins themselves, but also their diverse specific posttranslational modifications, MT-associated proteins (MAPs), signalling pathways regulating tubulin isotype expression, or MT-dependent apoptotic pathways (Dostál and Libusová 2014).

Among the plant secondary metabolites with antimicrotubular effect, the taxanes (originally from *Taxus brevifolia*), the *Vinca* alkaloids (from *Catharanthus roseus*), and colchicine (from *Colchicum autumnale*) have been widely utilized for the treatment of human cancers (Owellen *et al.* 1976; Margolis and Wilson 1977; Goodsell 2000). For some components identified from *Citrus*, including quercetin, ferulic acid, p-coumaric acid, limonene, citronellal, and citral, their antimirotubular effects have been reported, and pharmaceutical potential clinical applications have been discussed (Sun 2007; Chaimovitsh *et al.* 2010; Altshuler *et al.* 2013).

China is one of the most important centers of *Citrus* origin (Gmitter and Hu 1990). The *Citrus* in China harbours wild genetic resources, which provide promising prospects to mine novel compounds for medical applications. Indigenous and traditional medical knowledge has been well established in China, where natural resource-derived compounds play an important role (Jia and Li 2005; Zhou and Ye 2010). There are records on traditional medical application of *Citrus* peels documented by more than 10 Chinese ethnic minorities. The clinical applications include remedies for diarrhea and bloating, relieving of cough and mucus (Jia and Li 2005). Some recent studies proposed that fruit extracts of some of the Chinese wild *Citrus* are candidates for preventing and ameliorating obesity and obesity-related metabolic disturbances (Lu *et al.* 2013; Tan *et al.* 2014). In the current study, we show that representatives of Chinese wild *Citrus*, located in different taxonomic clades contain secondary metabolites exert specific and differential effects on *in vitro* polymerization of MTs isolated from porcine brain.

2. Materials and methods

2.1. Plant materials

In 2013, during commercial maturity stage, the *Citrus* fruits (Appendix A) were collected from the field-grown *Citrus* trees in the National Citrus Germplasm Repository, Citrus Research Institute of Chinese Academy of Agricultural Sciences (CAAS), Chongqing, China.

2.2. Preparation of *Citrus* fruit extracts

The *Citrus* fruit extracts were prepared according to Ding *et al.* (2013). Briefly, peels and pulps were separated from the fruits manually, dried at 50°C and powdered by mechanical grinder. Then, the dried peel powder was extracted with 90% ethanol (5%, w/v) at 80°C for 2 h. The extract was filtered through Whatman No. 1 filter paper (Sigma-Aldrich, USA). Subsequently, the filtered solution was concentrated at 40°C with a rotary evaporator under reduced pressure and then freeze-dried. The resulting powder was stored at –20°C until use.

2.3. Tubulin isolation and labeling

To test the effect of different plant compounds upon MTs, *in vitro* experiments are essential. Since the current study was motivated by the search for pharmacologically active candidate compounds, these experiments should be done in animals. Moreover, it is experimentally extremely difficult to purify sufficient amounts of plant tubulin in the concentrations needed to cross the critical concentration (around 1 mg mL^{-1}). We therefore followed the strategy used by the majority of the field, to purify tubulin from porcine brain as most common source of vertebrate tubulin. Tubulin was purified from fresh porcine brains following the classical protocol Shelanski *et al.* (1973) by two cycles of temperature-dependent assembly/disassembly. The protein concentration of the final preparation was determined by the method of Popov *et al.* (1975) with bovine serum albumin as standard and adjusted to 10 mg mL^{-1}. The tubulin preparations were then subjected to SDS-PAGE on 10% polyacrylamide gels to evaluate their purity. Then the tubulin was fluorescently labelled by Atto 488 (Sigma-Aldrich, Germany) according to Portran *et al.* (2013). It should be noted that this neurotubulin was not subjected to ion-exchange chromatography and therefore, in addition to tubulin itself, was still containing MT-associated proteins.

2.4. Tubulin polymerization of MTs *in vitro*

The tubulin was polymerized according to the method de-