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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 *Citrus 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 glandular 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 antimicrotubular effects have been reported, and pharmaceutical potential clinical applications have been discussed (Sun 2007; Chaimovitch *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-

scribed by Altshuler *et al.* (2013). Briefly, unlabeled porcine brain tubulin and Atto 488-labeled tubulin were polymerized as following: 12 μL of 10 mg mL^{-1} tubulin and 4 μL of 10 mg mL^{-1} of fluorescent tubulin were mixed with 0.8 μL of 100 mmol L^{-1} guanosine-5'-triphosphate (GTP) and 40 μL PME buffer (100 mmol L^{-1} piperazine-N,N'-bis (2-ethanesulfonic acid (Pipes)/KOH (pH 6.9), 1 mmol L^{-1} MgSO_4 , 1 mmol L^{-1} EGTA). And then the tubulin mixture was incubated with crude fruit extract (stock concentration 5 mg mL^{-1}) to a final concentration of tubulin 50 $\mu\text{g mL}^{-1}$ and *Citrus* extracts 250 $\mu\text{g mL}^{-1}$ at 37°C for 1 h.

2.5. Microscopy and quantification of MT polymerization

The MT/*Citrus* extracts (water as the control) mixtures were diluted 50-fold with PME buffer, and aliquots of 20 μL were mounted on a prewarmed objective slide. The response of MTs to crude fruit extracts was recorded by an AxioImage Z.1/ApoTome microscope using GFP filter set (excitation at 470 nm, beamsplitter at 495 nm, and emission at 525 nm) (Zeiss, Jena, Germany). MT responses (density, length, and thickness) were quantified from digital images recorded at constant exposure time by ImageJ software (<http://rsb.info.nih.gov/ij/>). The images were first transformed into binary images by thresholding using default parameters, and then inverted, such that MTs appeared black on a white background. MTs were then automatically selected using the “analyse particle” tool with a detection threshold of 10^2 pixels to eliminate background noise. The recognised particles were then fitted using the “fit ellipse” tool to get estimates for the major axis as a measure for MT length, and the minor axis as a measure for MT thickness.

3. Results

3.1. Two subgenera of citrus differ in their metabolic profile

Although many of the classical MT drugs are from alkaloids, recently also flavonoids, phenolics, and terpenoids with microtubular efficacy have been described (Touil *et al.* 2009; Chaimovitch *et al.* 2010; Xue *et al.* 2012). For *Citrus*, at least two subgenera, *Sinocitrus* Tseng and *Cephalocitrus* Tanaka were different in the profile of flavonoids (Xi *et al.* 2014a, b; Zhang *et al.* 2014) (Appendix B). In fact, extracts from the peel of the two subgenera show distinct and specific differences. For instance, among the flavanones, hesperidin dominated in subgenus *Sinocitrus* Tseng, accompanied by narirutin, eriocitrin, and neohesperidin, whereas naringin was the leading flavanone, followed by neohesperidin in subgenus *Cephalocitrus* Tanaka.

3.2. Effects of citrus fruit extracts on polymerization of MTs

The differences in the metabolic profile between *Sinocitrus* Tseng and *Cephalocitrus* Tanaka provided the motivation to compare MT-related activities among different subgenera of *Citrus*. In addition to the accessions from subgenera *Sinocitrus* Tseng and *Cephalocitrus* Tanaka, we included other accession from *Citrophorum* Tanaka, *Papedocitrus* Swingle, as well as the sister genus *Fortunella* (Appendix A). The *in vitro* effect of *Citrus* fruit extracts from peels and pulps of different genotypes on the number of polymerized MTs is presented in Fig. 1-A. Treatment by peels of *Citrus limonia* Osbeck and *Citrus ichangensis* Swing., pulp of *C. ichangensis* Swing. increased the number of polymerized MTs; whereas peels of *Citrus limon* (L.) Burm.f. and *Citrus sinensis* (L.) Osbeck had negative effects. Since the initial concentration of tubulin dimers was identical in experiments, these changes of numbers reported on differences in MT nucleation. We also determined average length of MTs as measure for elongation activity. MTs were prolonged by treatment of fruit extract from pulp of *C. limonia* Osbeck, and *F. margarita* (Lour.) Swing. (Fig. 1-B); whereas they were shorter when assembled in presence of fruit extract from peel of *C. limonia* Osbeck, and pulp of *C. limon* (L.) Burm.f. and *C. sinensis* (L.) Osbeck (Fig. 1-B). The microscopy images of *in vitro* effects on number and length of polymerized MTs are presented in Appendix C. To detect possible differences in MT-crosslinking or bundling, the thickness of MTs was measured as shown in Fig. 1-C. However, here none of the tested compounds produced any significant differences. To detect potential non-linearities of elongation, we also plotted frequency distributions over the lengths of individual MTs as shown in Fig. 2. A narrow poisson-type distribution was observed for some treatments (*C. limonia* Osbeck_peel, *C. sinensis* (L.) Osbeck_peel), while a significantly broader distribution emerged in some cases (*Fortunella margarita* (Lour.) Swing.). And some of the distributions exhibited even a small second additional peak (*Citrus limonia* Osbeck_pulp). These findings indicated that these treatments produced two populations of MTs—a majority of relatively short MTs, and a minority of much longer MTs. As for the peel extracts of the same accession (*C. limonia* Osbeck), the decrease of MTs length is correlated with an increase of MT number; whereas, a pronounced elongation and reduced in number induced by pulp extracts indicate that this elongation is not limited by the concentration of free tubulin dimers.

4. Discussion

Plant secondary compounds have mainly evolved as tools to interact with other organisms. Therefore, many of these

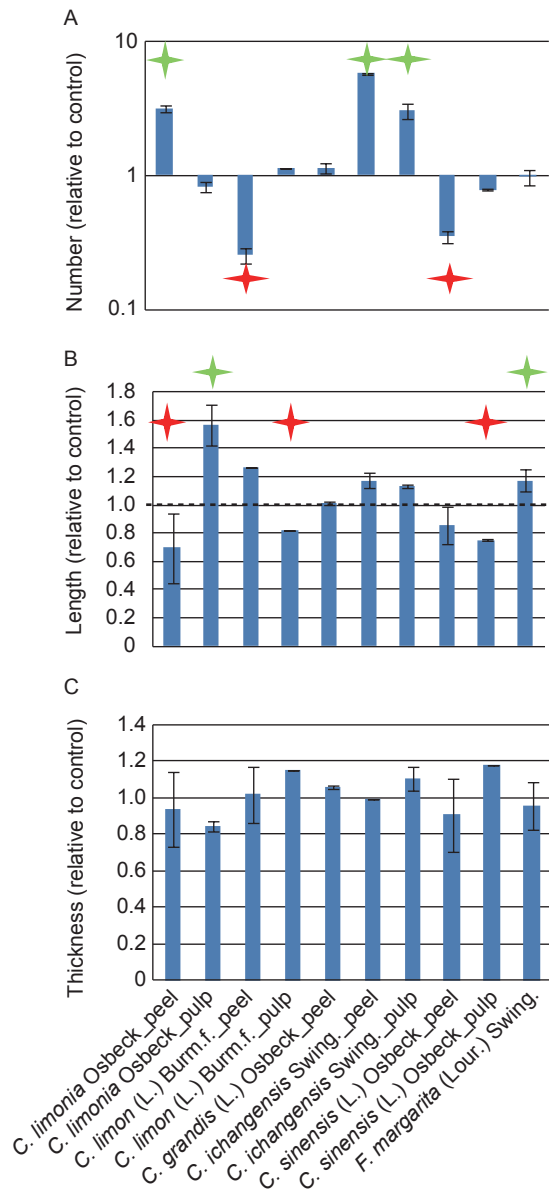


Fig. 1 *In vitro* microtubule (MT) responses to extracts of *Citrus limonia* Osbeck, *Citrus limon* (L.) Burm.f., *Citrus grandis* (L.) Osbeck, *Citrus ichangensis* Swing., *Citrus sinensis* (L.) Osbeck, *Fortunella margarita* (Lour.) Swing. A, effect on number of MTs. B, effect on length of MTs. C, effect on thickness of MTs. Interesting targets are marked with stars (green, positive effect; red, negative effect). Values represent means and standard errors from a population of at least 2000 individual MTs in each data point for two biological replicates.

compounds exert specific effects in animals including humans. The medical potential of more than 10^6 plant-specific secondary compounds is far from being exploited, or even only described (Saito and Matsuda 2010). The cytoskeleton as central component of cell division and morphology offers numerous targets for manipulation by chemical genetics. Due to its nonlinear dynamics and the complexity of acces-

sory proteins, it is possible to design approaches requiring low concentrations of the compounds and enabling specificity with suppression of undesired side effects. To screen natural products for cytoskeletal activity has therefore great potential (Sadot 2014). One strategy to identify candidate compounds is to search plants that are used by traditional systems of medicine. Traditional Chinese Medicine (TCM) as mainly phytotherapeutic system of healing with the longest written record provides ample resources for this approach. For instance, the harringtonines, one of the classes of alkaloids, with great potential for tumour therapy, have been recovered from the endemic conifer genus *Cephalotaxus*. The first transcriptome of a species from this taxon allows to conclude on pharmaceutically valuable pathways already (Qiao *et al.* 2014). In the current work, we have extended this approach to screen Chinese *Citrus* for the bioactivities targeted to MTs. By comparison of extracts originating from different subgenera that differ with respect to their profiles of flavones and flavanones, we observed specific effects upon MT assembly *in vitro*.

The number of polymerized MTs depends on the number of nucleation events. In the cell, where the concentration of free tubulin heterodimers is limiting, nucleation is under control of accessory proteins, such as γ -tubulin (Kollman *et al.* 2011), or the TCP-1 ring complex (Brown *et al.* 1996; Himmelspach *et al.* 1997). However, under the conditions of our *in vitro* assay, the concentration of free heterodimers exceeded the critical concentration (Mitchison and Kirschner 1984), therefore the observed effects of the extracts were unlikely to be caused by altered activity of the γ -tubulin ring complex. Moreover, although the accessory proteins were not removed deliberately from our neurotubulin preparation by ion-exchange chromatography, the spontaneous constitution of a γ -ring complex *in vitro* was very unlikely. The time consuming step of nucleation is the formation of oligomeric chains of ab-heterodimers that subsequently align to form a two dimensional sheet (Job *et al.* 2003). We therefore assume that the increased number of nucleation events must be caused by promotion of these oligomeric tubulin sheets that precede the formation of tubular stumps acting as nucleation centers. Compounds that modulate, for example, the GTPase function of tubulin would either increase or decrease the number of MTs. Alternatively, a modulation of MT-associated proteins that are copurified would give a similar result — a good candidate would be microtubule associated protein tau (MAP τ) which is abundant in axons and is copurified with tubulin. The affinity of this MAP to MTs is regulated by phosphorylation and its hyperphosphorylation is discussed as one possible cause for Alzheimer disease (del Alonso *et al.* 1997).

The effect of compounds on length of MTs may reflect the stability of the plus-end (Howard and Hyman 2003).

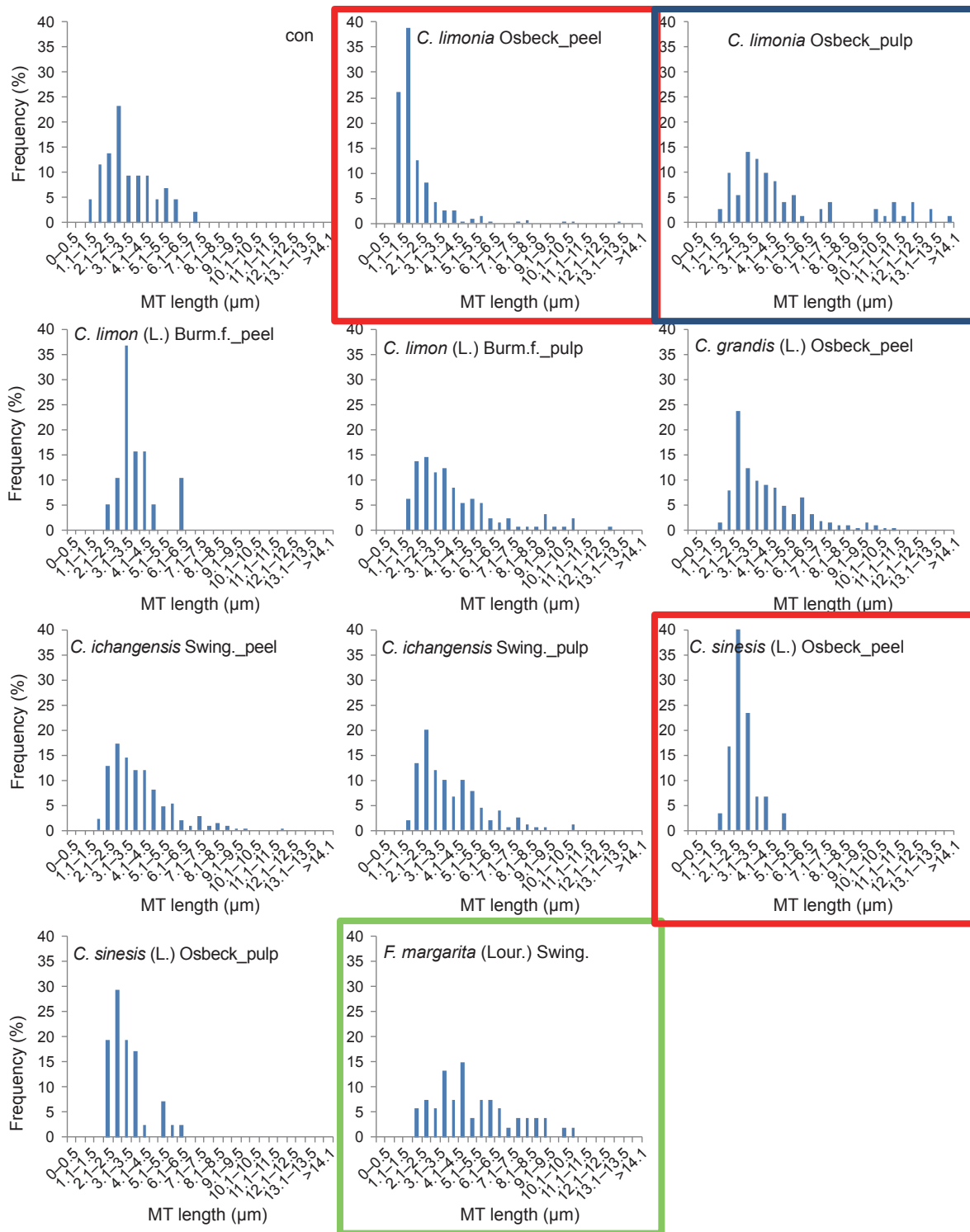


Fig. 2 Effect of *Citrus* (*Citrus limonia* Osbeck, *Citrus limon* (L.) Burm.f., *Citrus grandis* (L.) Osbeck, *Citrus ichangensis* Swing., *Citrus sinensis* (L.) Osbeck, *Fortunella margarita* (Lour.) Swing.) extracts on frequency distribution of lengths of individual MTs: broader (green frame), narrower (red frames), double peaks (blue frame). Solvent control (con) is water. Values are from a population of at least 2000 individual microtubules (MTs) for each treatment in two biological replicates. Frequency distributions of lengths are plotted, where the occurrences of values within an interval of 0.5 μm in length is counted as in one group, in total of 29 groups.

Again, this might be dependent on the activity of the GTPase function of tubulin. For instance, the length distribution of MTs

polymerized *in vitro* can be controlled when GTPase activity is modulated through changing the concentration of Mg²⁺ ions

(Martin *et al.* 1987). Also, the extent of the GTP-cap at the plus end defines the innate stability of the MTs, because the structure of the dimer changes from straight to kinked when the GTP is cleaved to GDP (Akhmanova and Steinmetz 2008). Alternative mechanisms might target to plus-end binding proteins that prevent catastrophic decay by complexing the divergent protofilaments at the plus-end of MTs. Again, it is not very likely that these complexes would form spontaneously *in vitro*, even if these proteins were copurified in sufficient amounts.

The thickness of MTs was examined to detect possible cross-linking or bundling of MTs (Subramanian *et al.* 2010). However, there is no evidence for such activities for the extracts tested here.

Interestingly, the frequency distribution over MT length turned out to be complex for some of the tested extracts. Theoretically, the frequency distribution of lengths follows a skewed Poisson distribution due to the limited number of nucleation sites over the pool of available tubulin dimers. Fragmentation of this distribution locating at broader or even two-peaked areas have been classically interpreted as manifestation of dynamic instability (Mitchison and Kirschner 1984), indicating that some extracts contain compounds interfering with the stability of the MT plus-end.

5. Conclusion

The differences in the secondary metabolite profiles between different *Citrus* subgenera and the difference in their effect on MT polymerization *in vitro*, can now be used to identify active compounds that are responsible for this effect. In order to find out the major secondary metabolites in these *Citrus* extracts, the analysis by quadrupole time-of-flight mass spectrometry is performed in our lab. Meanwhile, we are currently testing molecular candidates identified in the extracts for their activities in the MT *in vitro* assay. This will then be followed by studies on their cellular effect in mammalian cancer cell line to identify their cellular and molecular mode of action.

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Appendix associated with this paper can be available on <http://www.ChinaAgriSci.com/V2/En/appendix.htm>

References

- Akhmanova A, Steinmetz M O. 2008. Tracking the ends: A dynamic protein network controls the fate of microtubule tips. *Nature Reviews Molecular Cell Biology*, **9**, 309–322.
- del Alonso A C, Grundke-Iqbal I, Barra H S, Iqbal K. 1997. Neurobiology abnormal phosphorylation of tau and the mechanism of alzheimer neurofibrillary degeneration: Sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 298–303.
- Altshuler O, Abu-Abied M, Chaimovitch D, Shechter A, Frucht H, Dudai N, Sadot E. 2013. Enantioselective effects of (+)- and (-)-citronellal on animal and plant microtubules. *Journal of Natural Products*, **76**, 1598–1604.
- Bloomquist J R. 1996. Ion channels as targets for insecticides. *Annual Review of Entomology*, **41**, 163–190.
- Brown C R, Doxsey S J, Hongbrown L O, Martin R L, Welch W J. 1996. Molecular chaperones and the centrosome — a role for TCP-1 in microtubule nucleation. *Journal of Biological Chemistry*, **271**, 824–832.
- Chaimovitch D, Abu-Abied M, Belausov E, Rubin B, Dudai N, Sadot E. 2010. Microtubules are an intracellular target of the plant terpene citral. *Plant Journal*, **61**, 399–408.
- Ding X, Guo L, Zhang Y, Fan S, Gu M, Lu Y, Jiang D, Li Y, Huang C, Zhou Z. 2013. Extracts of pomelo peels prevent high-fat diet-induced metabolic disorders in C57BL/6 mice through activating the PPAR α and GLUT4 pathway. *PLOS ONE*, **8**, e77915.
- Dostál V, Libusová L. 2014. Microtubule drugs: action, selectivity, and resistance across the kingdoms of life. *Protoplasma*, **251**, 991–1005.
- Gmitter Jr F G, Hu X. 1990. The possible role of Yunnan, China, in the origin of contemporary *Citrus* species (Rutaceae): *Economic Botany*, **44**, 267–277.
- Goodsell D S. 2000. The molecular perspective: Microtubules and the taxanes. *The Oncologist*, **5**, 345–346.
- Himmelspach R, Nick P, Schäfer E, Ehmann B. 1997. Developmental and light-dependent changes of the cytosolic chaperonin containing TCP-1 (CCT) subunits in maize seedlings, and the localization in coleoptiles. *The Plant Journal*, **12**, 1299–1310.
- Howard J, Hyman A A. 2003. Dynamics and mechanics of the microtubule plus end. *Nature*, **422**, 753–758.
- Jia M, Li X. 2005. *Chinese Traditional Medicine Records of Ethnic Minorities*. China Medical Science Press, Beijing. (in Chinese)
- Job D, Valiron O, Oakley B. 2003. Microtubule nucleation.

- Current Opinion in Cell Biology*, **15**, 111–117.
- Kollman J M, Merdes A, Mourey L, Agard D A. 2011. Microtubule nucleation by γ -tubulin complexes. *Nature Reviews Molecular Cell Biology*, **12**, 709–721.
- Li J W H, Vederas J C. 2009. Drug discovery and natural products: end of an era or an endless frontier? *Science*, **325**, 161–165.
- Lodish H, Berk A, Zipursky S L, Matsudaira P, Baltimore D, James D. 2000. Microtubule dynamics and motor proteins during mitosis. In: *Molecular Cell Biology*. Section 19.5. 4th ed. H W Freeman and Company, New York. [2016-8-12]. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK21537/>
- Lu Y, Xi W, Ding X, Fan S, Zhang Y, Jiang D, Li Y, Huang C, Zhou Z. 2013. Citrange fruit extracts alleviate obesity-associated metabolic disorder in high-fat diet-induced obese C57BL/6 mouse. *International Journal of Molecular Sciences*, **14**, 23736–23750.
- Margolis R L, Wilson L. 1977. Addition of colchicine-tubulin complex to microtubule ends: The mechanism of substoichiometric colchicine poisoning. *Proceedings of the National Academy of Sciences of the United States of America*, **74**, 3466–3470.
- Martin S R, Butler F M M, Clark D C, Zhou J M, Bayley P M. 1987. Magnesium ion effects on microtubule nucleation *in vitro*. *Biochimica et Biophysica Acta*, **914**, 96–100.
- Mitchison T, Kirschner M. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature*, **312**, 232–242.
- Owells R J, Hartke C A, Dickerson R M, Hains F O. 1976. Inhibition of tubulin-microtubule polymerization by drugs of the Vinca alkaloid class. *Cancer Research*, **36**, 1499–1502.
- Pasqua G, Monacelli B, Valletta A. 2004. Cellular localisation of the anti-cancer drug camptothecin in *Camptotheca acuminata* Decne (Nyssaceae). *European Journal of Histochemistry*, **48**, 321–328.
- Popov N, Schmitt S, Matthies H. 1975. Eine störungsfreie mikromethode zur bestimmung des proteingehaltes in gewebehomogenaten. *Acta Biologica et Medica Germanica*, **34**, 1441–1446. (in Germany)
- Portran D, Gaillard J, Vantard M, They M. 2013. Quantification of MAP and molecular motor activities on geometrically controlled microtubule networks. *Cytoskeleton*, **70**, 12–23.
- Qiao F, Chong H, Wang R, Yin J, Qian D, Yang X, Jiang X, Nick P. 2014. *De-novo* characterization of a *Cephalotaxus hainanensis* transcriptome and genes related to paclitaxel biosynthesis. *PLOS ONE*, **9**, e106900.
- Rattan R S. 2010. Mechanism of action of insecticidal secondary metabolites of plant origin. *Crop Protection*, **29**, 913–920.
- Sadot E. 2014. Plant Compounds acting on the cytoskeleton. In: Nick P, Opatrny Z, eds., *Applied Plant Cell Biology*. Springer, Berlin Heidelberg. pp. 301–323.
- Saito K, Matsuda F. 2010. Metabolomics for functional genomics, systems biology, and biotechnology. *Annual Review of Plant Biology*, **61**, 463–489.
- Shelanski M L, Gaskin F, Cantor C R. 1973. Microtubule assembly in the absence of added nucleotides. *Proceedings of the National Academy of Sciences of the United States of America*, **70**, 765–767.
- Subramanian R, Wilson-Kubalek E M, Arthur C P, Bick M J, Campbell E A, Darst S A, Milligan R A, Kapoor T M. 2010. Insights into antiparallel microtubule crosslinking by PRC1, a conserved nonmotor microtubule binding protein. *Cell*, **142**, 433–443.
- Sun J. 2007. D-Limonene: Safety and clinical applications. *Alternative Medicine Review*, **12**, 259–264.
- Tan S, Li M, Ding X, Fan S, Guo L, Gu M, Zhang Y, Feng L, Jiang D, Li Y, Xi W, Huang C, Zhou Z. 2014. Effects of fortunella margarita fruit extract on metabolic disorders in high-fat diet-induced Obese C57BL/6 mice. *PLOS ONE*, **9**, e93510.
- Touil Y S, Fellous A, Scherman D, Chabot G G. 2009. Flavonoid-induced morphological modifications of endothelial cells through microtubule stabilization. *Nutrition and Cancer*, **61**, 310–321.
- Wink M. 2000. Interference of alkaloids with neuroreceptors and ion channels. *Studies in Natural Products Chemistry*, **21**, 3–122.
- Xi W, Fang B, Zhao Q, Jiao B, Zhou Z. 2014a. Flavonoid composition and antioxidant activities of Chinese local pummelo (*Citrus grandis* Osbeck.) varieties. *Food Chemistry*, **161**, 230–238.
- Xi W, Zhang Y, Sun Y, Shen Y, Ye X, Zhou Z. 2014b. Phenolic composition of Chinese wild mandarin (*Citrus reticulata* Balnco.) pulps and their antioxidant properties. *Industrial Crops and Products*, **52**, 466–474.
- Xue X Y, Liao M J, Lin L F, Zhang Z, Zhou X W, Zhou X, Luo H M. 2012. Phosphorylation of Akt is involved in protocathechuic acid-induced neurotrophic activity. *Neurological Research*, **34**, 901–907.
- Zhang Y, Sun Y, Xi W, Shen Y, Qiao L, Zhong L, Ye X, Zhou Z. 2014. Phenolic compositions and antioxidant capacities of Chinese wild mandarin (*Citrus reticulata* Blanco) fruits. *Food Chemistry*, **145**, 674–680.
- Zhou K, Ye M. 2010. *China Fruit Records: Citrus Volume*. China Forestry Press, Beijing. pp. 62–130. (in Chinese)

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