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Enhanced antifungal bioactivities of traditional Chinese medicines prepared by ultrafining technology

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ABSTRACT

Ultrafining technology is the application of the controlling of matter on 100-1000 nanometer scale. The application of ultrafining or nanotechnology to herbal preparations is increased recently since harvesting of the large-scale raw plant materials usually induces various environmental problems, including the desertification and dust storms. Therefore, the utilization of ultrafining technology to the traditional Chinese medicine plant materials not only improve their bioactivity, but also reduce the raw materials required. It would effectively decrease the environmental degradation associated with the harvesting of the raw herbal products. In the present report, several commercially prepared traditional Chinese medicinal products by ultrafining technology were identified to exhibit enhanced antifungal bioactivities compared to the corresponding non-nanoscaled herbal medicines. These results would encourage the researchers to apply ultrafining technology in the preparation of herbal medicines to explore effective new pesticides.

Keywords: *ultrafining technology; herbal preparation; plant-derived pesticide; Coptis chinensis; Magnolia officinalis.*

INTRODUCTION

Nanotechnology is the science and technology related to particles ranging in dimensions from 1 to 100 nanometers, in which usually unique properties initiated novel applications. The ultrafining technology was the modification of nanotechnology since some biomaterials, such as traditional Chinese medicines, would absorb each other to form larger aggregates as they were grinding to nanoparticles. These techniques possess major potentials to generate new products with numerous benefits. Nanotechnology is a new and fundamental technique and considered as one of the major technologies of the 21st century (National Nanotechnology Initiative 2006). The principle of nanotechnology is that materials with known properties and functions at their normal sizes would usually exhibit different and profound properties and functions at their nanosizes. Recent advances in the fields of nanoscience and nanotechnology have created various possibilities in different applications as amendments, including industrial, medicines, cosmetics, synthetic textiles, agricultural, and foods. According to the market products analysis, sales of products incorporating emerging nanotechnology will rise from less than 0.1% of global manufacturing output today to 15% In 2014, totally 2.6 trillion US dollars. This value will approach the size of the information technology and telecom industries combined and will be 10 times larger than biotechnology revenues. Among these, the agricultural and food markets are expected to increase from 2.6 billion US dollars in 2003 to 20.4 billion US dollars in 2010 (Allianz and OECD 2005). The development of nanoscience, nanotechnology, and ultrafining application has led the advances of the nanosized inorganic and organic particles which exhibit increasing applications as amendments in industrial, medicine and therapeutics, and food packaging products. In the scientific fields such as foods and medicines, several reviews had already been published related to the application of nanoparticles or ultrafining materials. However, there are no systematic and comprehensive reviews regarding the application of nanotechnology in agricultural products and the advantages and risks inherited from this technique. The chapter will introduce the benefits of the plant-derived pesticides prepared by nanotechnology or ultrafining technology. Moreover, the potentials of herbal pesticides prepared by nanotechnology in reducing the global warming pressure will also be emphasized.

IMPORTANCE AND RISK OF NANOTECHNOLOGY

The chemical, physical, optical, electrical, catalytic, magnetic, mechanical, and most importantly the biological and medicinal properties of nanosized materials are fundamentally different from those of their macroscopic or bulk counterparts (Dresselhaus *et al.* 2004). Researchers usually prepare new nanosized materials in two ways. They can reduce the particles as small as a nanometer, or about one-hundred-thousandth the width of a human hair. In addition, researchers could manage individual atoms and molecules to form microscopic tubes, spheres, wires, and films for specific duties, such as generating electricity or transporting drugs in the human body. Nanotechnology has the potential to revolutionize fields as seemingly disparate as medicine, food science and technology, recreation and sport sciences, and civil engineering. In the fields of life sciences, a comprehensive series of nanosized clusters, rods, and tubes are being examined in laboratory animals to explore their ability to target and kill cancer cell lines. Scientists, futurists, and ethicists all agree that the nanotechnology would profoundly shape the human body and the physical and social environments in which humans live because of these extraordinary numbers of devices, processes, and applications that can be produced with nanotechnology.

In contrast, nanotoxicology is expected to emerge as a critical discipline in addressing the environmental, health, and occupational hazards of nanoscale substances over the course of their life cycle (Donaldson *et al.* 2004). Previous report on particle toxicology has focused on such materials as coal, asbestos, mineral fibers, and ambient particulate matter (Borm 2002). Nanomaterials are expected to exhibit different environmental transport behaviors, with colloidal aggregates expected to have the least mobility (Lecoanet *et al.* 2004; Lecoanet and Wiesner 2004). In general, nanoparticles are expected to be water insoluble, and the low aqueous solubility may generally improve the persistence of the chemical and absorption by biological systems. Therefore, if these nanoparticles are dispersed in the environment, there are both positive and negative effects; for examples, if they are able to remediate, or if they absorb contaminants and disperse them more widely. Given the lack of robust environmental fate and toxicity data for nanoparticles, it would be a risk to predict toxic potency and exposure levels. It will also be challenging to develop an environmental fate and transport model not just for the bulk material, but also for the potential aggregates and degradation products in environmental surroundings. For nanosized materials, qualitative risk comparisons of expected environmental concentrations to ecologically acceptable concentrations are also not well characterized. It is important to have detailed and reliable data on which to base life-cycle and risk assessments. Although adequate and representative data sets are critical, it is important to emphasize that these tools could be based on relatively incomplete data, for example, by comparison of their analogs that are similar in the size, chemical structure, and energy cost. Consequently, the decision made for nanomaterials should not only be risk or environment oriented, as other aspects like cost, energy performance, lifespan, and customers preferences are also equally significant (Cobb and Macoubrie 2004; Gaskell *et al.* 2004; Gaskell *et al.*

2005; Lee *et al.* 2005; Scheufele and Lewenstein 2005; Tenbült *et al.* 2005).

PLANT DERIVED PESTICIDES

Although the synthetic fungicides usually successfully controlled the plant diseases, it also contributed to enhance the population of fungicide-resistant pathogens (Brent *et al.* 1990). Among the usually-used methodology of controlling the plant diseases, natural herbal preparations or the commonly called plant derived pesticides or are generally considered as safe to the human even all the creatures since these natural secondary metabolites are easily decomposed in the ground and would not show negative or long-term effects to the environment (Huang and Chou 2005). Recently, more and more reports are focusing on the development of new plant derived pesticide preparations (Chung *et al.* 2002; Kim *et al.* 2003; Muto *et al.* 2005; Yen *et al.* 2008; Lin *et al.* 2010). These new preparations would suppress the development of plant diseases effectively and in the meanwhile reduce the damage caused by traditional synthetic fungicides hopefully.

Various research publications evidenced that the secondary metabolites of many plant species displayed antifungal bioactivity, such as compounds identified from the rhizomes of *Curcuma longa* (Kim *et al.* 2003); the seeds of *Cassia tora* (Kim *et al.* 2004); the stem, leaves, and flowers of *Lavandula stoechas* (Angioni *et al.* 2006), etc. The glucosinolates are rich in the seeds of mustard (*Brassica juncea* cv. Bau Sin) and enzymatic hydrolysis of these principles produced the allyl isothiocyanates which exhibited highly inhibitory effects against *Rhizoctonia solani* Kühn AG-4, causal agent of root rot of cabbage (Chung *et al.* 2002). Many species of natural plants possessed antifungal or antimicrobial bioactivity are usually used in clinically medical preparations. For example, extracts from the galls of *Melaphis chinensis* and the leaves of *Aloe vera* were purified and several antifungal substances against plant pathogenic fungi were characterized (Ahn *et al.* 2005; Ho *et al.* 2006; de Rodríguez *et al.* 2005). In addition, the polar extracts of *Coptis chinensis* (goldthread), *Polygonum cuspidatum* (Japanese knotweed), *Cinnamomum cassia* (cinnamon), *Rheum officinale* (Chinese rhubarb), *Polygonum multiflorum*, and *Eugenia caryophyllata* (clove) were reported to exhibit inhibitions of conidial germination of *Oidium murrayae* (Chu et al. 2006). Moreover, the water-soluble extracts of clove at the concentration of 1% (w/v) showed the inhibitory percentage of 100 % towards the conidial germination and mycelial growth of *Colletotrichum higginsianum*, which is one of the important fungal pathogens causing anthracnose disease of numerous economical crops, including legumes, ornamentals, vegetables, and fruit trees. Not only the polar constituents possessed the antifungal bioactivity, various essential oils also displayed significant antifungal activity such as clove oil and eugenol were equally effective in reducing disease severity of anthracnose caused by this pathogen in greenhouse (Lin *et al.* 2010).

Rhizoctonia solani Kühn which resulted in damping-off diseases of numerous crops in Taiwan is continuously a serious problem for the commercial production of vegetable seedlings grown in cell-plug systems (Huang and Yang 1992; Shiau *et al.* 1999). The chemical fungicides could control this pathogen effectively, however, the risk of development of resistance increases gradually due to the extensively utilization of these fungicides. Recently, the biological control methods such as use of natural secondary metabolites from the plants or microbes resources had performed extensive studies. In our lab the methanol extracts of Coptis rhizome and Magnolia stem barks were selected as the target aimed to discover new plant-derived fungicides for controlling *Rhizoctonia* damping-off in cabbage seedlings according to the preliminary antifungal bioactivity examinations. Herein we wish to illustrate the determination of the antifungal activities of the natural extracts. In addition, we also explore and compare the contents of bioactive compounds in the extracts prepared by ultrafining technology and traditional grinding methods. Although the utilization of synthetic chemical fungicides controlled the damping-off diseases more effectively, it also led to the development of resistance and pollution of environment. On the contrary, the herbal preparations would be safer and less dangerous to the ecosystem and all the human beings.

ULTRAFINING OF TRADITIONAL CHINESE MEDICINES AS PLANT DERIVED PESTICIDES

Recently, interests in the application of nanotechnology to preparation of traditional Chinese medicine are increased (Yang *et al.* 2003; Liu *et al.* 2008; Liu 2009; Musthaba *et al* 2009; Wu 2010; Tsai *et al* 2011; Hu and Jiang 2012). The ultrafining technology is the superfine grinding method regarding the use of mechanical or hydrodynamic process of biomaterial particles crushed to the size less than 1 μm. Several biomaterials would absorb each other to form larger aggregates as they were grinding to nanoparticles. Aggregation of metal particles, with a diameter size within the micrometer range, in the culture medium and internalization into human osteoblasts have been reported by Vallés *et al.* (2008) by reflection on a confocal microscope. In some research reports, the ultrafining technology is defined as producing particles with the size between 100 nm and 1 μm. However, in the food and drug systems, nanoparticles could also be known as being submicronic (< 1 μm) colloidal systems (Brigger *et al.* 2002). Various approaches of nanotechnology have been proposed, such as the use of polymeric micro- and nanoparticles, micro- and nanoemulsions, or liposomes (Singla *et al.* 2002). Also nanoparticles based on solid lipids demonstrated to be a promising alternative drug delivery system (Miglietti *et al.* 2000; Cavalli *et al.* 2001; Chen *et al.* 2001; Serpe *et al.* 2004). In addition, the nanospheres are the particles having a matrix type structure in which the active ingredient is dispersed throughout, whereas the nanocapsules have a polymeric membrane and an active ingredient core. Nanoparticle systems with average particle size slightly above the 100 nm border have also been reported in literature, including nanonized curcuminoids (Tiyaboonchai *et al.* 2007), paclitaxel (Arica Yegin *et al.* 2006) and praziquantel (Mainardes and Evangelista 2005) which have a mean particle size of 450, 148, and 200 nm, respectively.

The major raw plant materials commonly used in traditional Chinese medicines is cultivated in the grassland regions, such as the Inner Mongolian. The harvesting of the large amount plant materials results in more and more serious environmental problems, for examples, the desertification and dust storms. Therefore, the utilization of nanotechnology or ultrafining technology to the commonly used traditional Chinese medicine plant materials may enhance their bioactivity and also reduce the amount of the raw materials required. It would significantly decrease the environmental degradation associated with the harvesting of the large amount plant materials. Global warming is the increase in the average measured temperature of the Earth's near-surface air and oceans since the mid-20th century, and its projected continuation. Increasing global temperature is expected to raise the sea level, increase the intensity of extreme weather events, and significantly change the amount and pattern of precipitation, likely leading to an expanse of tropical areas and increase of desertification (Climate change 2007). Desertification is the degradation of land in arid, semi-arid and dry sub-humid areas resulting primarily from human activities and influenced by climatic variations. Current desertification is taking place much faster worldwide than historically and usually arises from the demands of increased populations that settle on the land in order to grow crops and graze animals. A major impact of desertification is loss of biodiversity and productive capacity, for example, by transition from land dominated by shrublands to non-native grasslands. In the semi-arid regions of southern California, many coastal sage scrub and chaparral ecosystems have been replaced by non-native, invasive grasses due to the shortening of fire return intervals. This can create a monoculture of annual grass that do not support the wide range of animals once found in the original ecosystem (Wilson 2001).

Desertification is widespread in many areas of the People's Republic of China. The populations of rural areas have increased since 1949 for political reasons as more people have settled there. While there has been an increase in livestock, the land available for grazing has decreased. Also the importing of European cattle such as Friesian and Simmental, which have higher food intakes, has made things worse (Brown 2006). Overpopulation of human beings has resulted in the destruction of the tropical wet forests and subtropical dry forests, mainly because the widening practices of slash-and-burn and other methods of subsistence farming necessitated by famines in developed countries. Follow-up the destruction of the forests is typically huge scale ground erosion, loss of the nutrients in the soil, and successively complete desertification. Since most of the important plant materials used in traditional Chinese medicine are cultivated in regions such as the Inner Mongolian grassland that attract so many environmental concerns, the large-scale harvesting of the raw plant materials induced more and more serious situations such as dust storms. Hence, application of nanotechnology or ultrafining technology to the plant materials commonly used in traditional Chinese medicines not only increase the contents of bioactive constituents in the plant extracts but also decrease the quantity of the pharmaceuticals required and, therefore, alleviate environmental degradation related to the large-scale harvesting of the raw plant materials. As reported in the previous literature, the nanoparticles counterpart would be 2-3 times more effective than the crude extract (Yen *et al.* 2008). That is, the required raw materials for nanoparticle preparation may only be one third of the medicinal plant materials harvesting now. It will decrease the cultivated areas significantly and therefore slow down the desertification process.

Nanonization possesses many advantages, such as increasing compound solubility, reducing medicinal doses, and improving the absorbency of herbal medicines compared with the respective crude drugs preparations (Brigger *et al.* 2002). The nanotechnology or ultrafining technology of drug formulation not only enhances the absorption of poor water soluble drugs but also improves the therapeutic effectiveness of drugs in pharmaceutical research. Nanoparticle formulation is one of the novel drug delivery systems which possesses various advantages, including increasing drug solubility, enhancing dissolution rate, improving the bioavailability, and decreasing the dosage required for the same effects, compared with the crude or micronized medicines (Liversidge and Cundy 1995; Müller and Peters 1998; Liversidge *et al.* 2003). There are various reports relating to the nanoparticle formulation of traditional Chinese medicine. Liu *et al.* reported the comparison of the efficacies of medicinal plant materials *Salvia miltiorrhiza* prepared using nanotechnology and traditional grinding method (Liu *et al.* 2008). Enhanced antioxidant bioactivities were observed for the extracts prepared using nanotechnology in all tested assays. These results suggest greater liberation of active components in the Danshen samples prepared using this novel technique. It could be further investigated that if the nanonization processes would improve the absorption of bioactive compounds in the gastrointestinal system by monitoring the constituents with the *in vitro* Caco-2 cell lines model (Liu *et al.* 2008). Another case is reported by Yen *et al.* and they utilized the nanosuspension method to develop ethanolic extract of *Cuscuta chinensis* loaded PF68 nanoparticles system (Yen *et al.* 2008). The results indicated that an 1/5 oral dose of *C. chinensis* nanoparticles could exhibit similar hepatoprotective and antioxidant effects as that of ethanolic extract of common *C. chinensis* powder. In addition, the experimental data also demonstrated that the hepatoprotective and antioxidant effects of 50 mg/kg *C. chinensis* nanoparticles are more effective than the ethanolic extract at a dose of 125 mg/kg. These results were evidenced that the traditional Chinese medicines prepared by nanotechnology or ultrafining technology could improve the bioactivity and also reduce the amount of the plant materials required.

Coptis rhizomes

Coptis genus (Ranunculaceae) includes 10-15 species of flowering plants native to Asia and

North America. *C. chinensis*, usually called as Chinese goldthread, is a species of goldthread native to China, and is one of the 50 fundamental herbs used in traditional Chinese medicine. The Coptis rhizomes are plentiful of isoquinoline alkaloids, such as berberine, palmatine, and coptisine (Miyazaki *et al.* 1981; Parmar *et al.* 1997; Kim *et al.* 2000). Various pharmacological effects of Coptis rhizomes extracts or its chemical components have been reported. Among these effects, the antimicrobial bioactivity of the extracts and berberine (Kumar *et al.* 2015; Dziedzic *et al.* 2015) demonstrates the potential of application as biopesticides. The stems and roots of *C. chinensis* were purchased in the herbal markets. The traditional grinding medicinal powders were prepared with a common pulverizer. The nanosized or sub-nanosized particles prepared by ultrafining nanotechnology were provided by Diamond Nano-Biochem Company (Figure 1). The dried plant materials were ground by the atomizer and further sprayed granulating with the aid of floating bed. The resulting materials were dried to form the nanoparticles under the dry processes. In our study, the Brunauer–Emmett– Teller (BET) specific surface area (S_{BET}) of the samples was evaluated on the basis of nitrogen adsorption isotherms. The surface morphologies and micro-structures of the samples were analyzed by scanning electron microscope (SEM). Dynamic light scattering method was used to determine the parameters of Coptis Rhizome powder processed by ultrafining technology and the resulted data including the particle size distribution and polydispersity were compared with those of traditional grinding medicinal powders. The particle size distribution was located in the region between 80 and 600 nm and the average size was 183.7 nm. The polydispersity of Coptis Rhizome powder processed by ultrafining technology was measured as 0.209. These data indicated that the samples used in this report could be regarded as nanoparticles or ultrafining particles. The antifungal bioactivity examinations were performed and the methanol extracts (1 %) of Coptis Rhizome powder displayed the inhibitory percentage of 56.35 % (at 84 hrs) against the growth of *R. solani* AG-4 RST-04. Therefore, the methanol extracts of Coptis Rhizome powder were subjected to the combinations of conventional column chromatography to explore the antifungal compounds. And the purified indicator compound berberine chloride, which was characterized with the aid of spectral and mass analysis, was successively examined for its antifungal activities.

Figure 1. Comparison of the Coptis rhizome powder prepared by ultrafining and traditional grinding (Left: ultrafining powder; Right: traditional grinding powder).

The sample solution of indicator compound berberine chloride (0.1 %) also inhibited the growth of the mycelial colony of *R. solani* AG-4 RST-04 with the percentage of 62.80 % (at 84 hrs). These experimental data suggested that the indicator compound berberine chloride should be involved in the antifungal effect of the methanol extract of Coptis Rhizome against the growth of the mycelial colony of *R. solani* AG-4 RST-04. In order to compare the antifungal bioactivities between the Coptis Rhizome powder prepared by traditional grinding and processed by ultrafining technology, the 2-10 % solutions of Coptis Rhizome powder prepared by traditional grinding or processed by ultrafining technology were performed the fungus inhibitory experiments and the inhibitory percentages were recorded as shown (Figure 2). The 2 % sample solution of Coptis Rhizome powder prepared by traditional grinding at 60 h almost did not show any inhibitory effects against the growth of the mycelial colony of *R. solani* AG-4 RST-04. However, 2 % sample solution of Coptis Rhizome powder processed by ultrafining technology at 60 h displayed the inhibitory percentage of 20.8 % against the growth of examined fungus. Comparatively, 5 %, 7 %, and 10 % sample solutions of Coptis Rhizome powder prepared by traditional grinding and processed by ultrafining technology also exhibited a very similar trend. All of the sample solutions (2 %, 5 %, 7 %, 10 %) of Coptis Rhizome powder processed by ultrafining technology displayed more significant inhibitory percentages against the fungus growth than the corresponding solutions (the same concentrations) of powder prepared by traditional grinding. These experimental data evidenced that the higher quantity of berberine chloride released from the extracts of ultrafining Coptis Rhizome powder significantly improved the inhibitory bioactivity against the growth of the mycelial colony of *R. solani* AG-4.

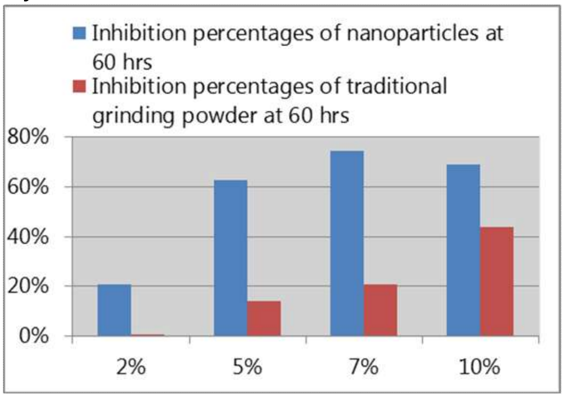

Figure 2. Comparison of antifungal activity of Coptis Rhizome powder prepared by ultrafining technology and traditional grinding method.

According to the experimental data in the present study (Kuo *et al.* 2014), the methanol extracts of Coptis Rhizome powder and the indicator compound berberine chloride are potential to explore as new plant derived pesticides to control *Rhizoctonia* damping-off in vegetable seedlings. In addition, application of ultrafining technology in Coptis Rhizome preparations may significantly increase the bioactive constituents and bioactivity. Hence it could reduce the amount of raw materials as well as contribute to decrease the environmental degradation. These results would encourage researchers to study the advances of bioactive constituents and related bioactivities as the ultrafining technology applied in the preparation of herbal medicines. Furthermore, the traditional Chinese medicines could be examined for their cytotoxicity, synergistic effects of other plants, and bioactivity of different combinations. Hopefully, the antifungal mechanism of these herbal preparations would be investigated in the near future for the purpose of developing new plant-derived pesticides.

Magnolia stem barks

In our continuous program aimed to search for the plant-derived pesticides, *Magnolia officinalis* was selected as the target since it is notorious for the inhibition of various bacteria and fungi. *M. officinalis* Rehd. et Wils. (Magnoliaceae) is called Hou-pu in Chinese, and is a rare and endangered species listed under Class II National Protection in China. The roots, stems, and branches of *M. officinalis* are extensively used in traditional Chinese medicine for the treatment of various disorders, such as depression coughing, asthma, liver disease, shoulder pain, urinary problems, and diarrhea (Nakazawa *et al.* 2003; Wang *et al.* 2005). Neolignans, sesquiterpenes, sesquiterpene neolignans (Konoshima *et al.* 1991; Yahara *et al.* 1991; Syu *et al.* 2004), and phenylpropanoids (Youn *et al.* 2007; Shen *et al.* 2009) have been identified from prior phytochemical studies of *M. officinalis* (Yahara et al. 1991). These constituents exhibit antimicrobial (Bae *et al.* 1998; Hu *et al.* 2011), anticancer (Yang *et al.* 2003; Shigemura *et al.* 2007; Vaid *et al.* 2010), anti-epileptic effects (Chen *et al.* 2011), antitumor (Konoshima *et al.* 1991), antibacterial (Ho *et al.* 2001), cytotoxic (Youn *et al.* 2007), and anti-inflammatory effects (Shen *et al.* 2009; Chao *et al.* 2010; Munroe *et al.* 2010). In this study, we wish to explore the bioactive principles and determined the antifungal activities and its mechanism of the naturally isolated compounds.

Dry powders of stem barks of *M. officinalis* prepared by either traditional grinding or ultrafining technology were generously provided by Diamond Nano-Biochem Company. Dynamic light scattering method was used to determine the parameters of *M. officinalis* stem bark powder processed by ultrafining technology and the resulted data including the particle size distribution and polydispersity were compared with those of traditional grinding medicinal powders. The distribution range of particle size of *M. officinalis* stem bark powder processed by ultrafining technology was between 80 and 500 nm and average particle size was 213.8 nm. The polydispersity of the sample powder processed by ultrafining technology was 0.341. These data indicated that the samples used in this report could be regarded as nanoparticles or ultrafining particles.

The stem bark samples of *M. officinalis* were powdered, extracted with methanol under reflux, and then concentrated in vacuum to yield crude extracts. The crude extracts were partitioned between ethyl acetate and water to afford ethyl acetate soluble fraction and water fraction, respectively. The antifungal bioactivity examinations of the methanol extract, ethyl acetate soluble fraction, and water fraction of the stem barks of *M. officinalis* were performed according to the published protocols (Kuo *et al.* 2010), respectively. The methanol extracts (1 %) of stem bark powder of *M. officinalis* displayed the inhibitory percentage of 92.7 % (at 84 hrs) against the growth of *R. solani* AG-4 RST-04. The partially purified ethyl acetate soluble fraction (1 %) was also examined for its antifungal activity and exhibited excellent antifungal effect on *R. solani* AG-4 RST-04 with the inhibitory percentage of 100.0 %. In contrast, the water soluble fraction (1 %) showed weaker antifungal activity only with the inhibition percentage of 40.3 %. These experimental data suggested that the bioactive constituents of the stem bark powder of *M. officinalis* against the growth of the mycelial colony of *R. solani* AG-4 were mainly distributed in the organic phase (ethyl acetate soluble fraction).

Therefore, the ethyl acetate soluble fraction of methanol extracts of the stem bark powder of *M. officinalis* were purified with the assistance of the combinations of conventional column chromatography to discover the bioactive compounds. And the purified indicator compounds honokiol and magnolol characterized with the spectral and mass analytical data, were successively examined for its antifungal activities. At the tested concentrations ranging from 100 to 3.125 ppm, they all exhibited significant inhibition effects against the growth of *R. solani* AG-4. The antifungal effects of both honokiol and magnolol also showed concentration-dependent tendency. Consequently, the purified honokiol and magnolol displayed antifungal activity with the IC_{50} of 6.09 and 8.97 ppm, respectively. These experimental data suggested that the indicator compounds honokiol and magnolol should be involved in the antifungal effect of the methanol extract of *M. officinalis* against the growth of the mycelial colony of *R. solani* AG-4 RST-04.

In order to compare the inhibitory bioactivities against the fungus *R. solani* AG-4 between the *M. officinalis* stem bark powder prepared by traditional grinding and processed by ultrafining technology, 1 %, 2 %, 3 %, and 5 % sample solutions of the *M. officinalis* stem bark powder prepared by traditional grinding or processed by ultrafining technology were performed the bioactivity examinations and the inhibitory percentages were recorded as shown (Figure 3). 1 % sample solution of *M. officinalis* stem bark prepared by traditional grinding at 84 h almost did not exhibit any inhibitory effects against the growth of the mycelial colony of *R. solani* AG-4. However, 1 % sample solution of *M. officinalis* stem bark processed by ultrafining technology at 84 h exhibited inhibition percentages of 17.23 ± 4.42 % against the growth of the mycelial colony of *R. solani* AG-4. Comparatively, 2 %, 3 %, and 5 % sample solutions of *M. officinalis* stem bark powder prepared by traditional grinding and processed by ultrafining technology also displayed very similar tendency.

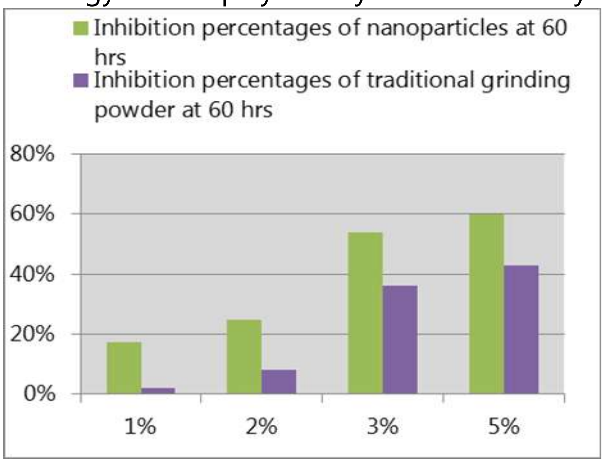

Figure 3. Comparison of antifungal activity of Magnolia stem barks powder prepared by ultrafining technology and traditional grinding method.

All the solutions (1 %, 2 %, 3 %, 5 %) of *M. officinalis* stem bark powder processed by ultrafining technology have more significant inhibitory activities against the growth of the mycelial colony of *R. solani* AG-4 than the corresponding solutions (the same concentrations) of powder prepared by traditional grinding. These inhibitory bioactivity data suggested that the higher quantity of indicator compounds released from the extracts of ultrafining *M. officinalis* stem bark powder may enhance the inhibition percentages against the growth of the mycelial colony of *R. solani* AG-4.

In this study, the bioactive principles in the methanol extracts of the stem barks of *M. officinalis* were explored according to the bioassay-guided purification concept. Repeated column chromatography isolation of the active ethyl acetate soluble fraction of the methanol extract of *M. officinalis* afforded two neolignans, honokiol and magnolol. The chemical structures of honokiol and magnolol were characterized by comparison of their physical and spectral data with those reported in the literature. And these two compounds really exhibited excellent antifungal bioactivity compared to other natural products. Therefore, the antifungal mechanism was merited to investigate. Since honokiol displayed more significant antifungal bioactivity, its effect on chitinase activity was studied to elucidate the antifungal mechanism. The results showed that the chitinase activity of *R. solani* AG-4 mycelia incubated with honokiol (0.1 %) was significantly induced. The chitinase activity of treated mycelia markedly increased and that of control mycelia stayed at a very low level. It indicated that the treatment with honokiol would induce the chitinase, and lead to the increase of the hydrolysis of chitin. Consequently, the cell membranes of fungi would be damaged. It had already been reported that the plasma membranes of *P. expansum* were markedly damaged and the leakage of protein and sugar was detected after the mycelia were treated with β-aminobutyric acid (Zhang *et al.* 2011). The fungal cell wall consists basically of a complex network of glucan, chitin and proteins, playing important roles in cell viability and pathogenicity (Peberdy 1990). The hydrolysis of glucan, chitin and protein can weaken the cell wall structure, then directly lead to the damage of cell wall. Our experimental data exhibited that the treatment with honokiol induced the increase of chitinase. The enhancement of chitinase activity led to the increase of the hydrolysis of chitin, thus cell membranes of mycelia were damaged. Therefore, the antifungal mechanism of honokiol against *R. solani* AG-4 mycelia might be that they induced the activities of chitinase and caused the hydrolysis of chitin, thus led to cell membrane disintegration, cytoplasm leakage and eventually the death of fungal cells. These results are promising and beneficial for further studies in developing new and more effective fungicides and environment-friendly fungicide additives in the agricultural chemistry field. In the comparison of the antifungal activity, all the solutions of *M. officinalis* stem bark processed by ultrafining technology exhibited more effective inhibitions against the *R. solani* AG-4 fungus growth than the same concentration solutions of powder processed by traditional grinding. In addition, the concentrations of honokiol and magnolol were as high as 74.96 and 287.43 μg/g, respectively, in the sample processed by ultrafining technology after extraction time of 10 min, and these values would not increase significantly with the extension of extraction time. On the contrary, the concentrations of honokiol and magnolol in *M. officinalis* stem bark sample powder prepared by the traditional grinding procedures significantly increased with the extension of extraction time. However, their contents are not more than those of the ultrafining samples. According to the above described experimental data, it is apparent that the release of the bioactive principles will be more rapid and more complete in the sample powder of traditional Chinese medicine processed by ultrafining technology as compared to those prepared using the traditional grinding methods. The above experimental data also evidenced that the higher quantities of honokiol and magnolol released from the *M. officinalis* stem bark powder samples processed by ultrafining technology could significantly enhance the inhibitory bioactivity of the plant pathogenic fungi *R. solani* AG-4.

Others

There are a lot of reports evidenced the metal based nanoparticles to constitute an effective antimicrobial agent against common pathogenic microorganisms. For examples, the nanoparticles such as gold, silver, magnesium oxide, titanium oxide, copper oxide, aluminum oxide, and zinc oxide are receiving considerable attention as antimicrobials or antifungals and additives in consumer, health-related and industrial products (Dibrov *et al.* 2002). However, these metal based nanoparticles were not used extensively in the agriculture due to lack of bioactivity and toxicology data. Nowadays several researches related to the utilization of nanoparticles as carriers of pesticides or as synergists to enhance the function of pesticides were reported. The emergence of nanotechnology and the development of new nanodevices and nanomaterials open up novel applications in agriculture and biotechnology (Scott and Chen 2003; Joseph and Morrison 2006). Recently, there are various reports of applying nanoparticles and nanocapsules to plants for agricultural uses (Pavel *et al.* 1999; Liu *et al.* 2002a–c; Cotae and Creanga, 2005; Pavel and Creanga, 2005; Joseph and Morrison 2006). Nanoparticles tagged to agrochemicals or other substances could significantly reduce the damage to other plant tissues and the amount of chemicals released into the environment.

NANOSIZED CALCIUM CARBONATE

Calcium carbonate is widely used as an inexpensive dietary calcium supplement in the human health and dietary applications. Calcium is also usually recognized for its important function in plants to increase nutrient uptake, build strong cell walls, and increase the vitality of the plants. It is critical to maintain high levels of bio-available calcium in achieving adequate levels of calcium uptake. Calcium plays a very important role in plant growth and nutrition, as well as in cell wall deposition. Chang et al. (2015) utilized nanosized calcium carbonate to increase the weight of the rice, seeds of the grass species *Oryza sativa*. In their experiment, the nanosized calcium carbonate could also effectively reduce the rice lodging. Moreover, the common diseases of rice, including sheath blight, brown spot, and lump smut, were prevented by spraying the nanosized calcium carbonate. The major pest on *O. sativa*, striped riec borer, was controlled effectively by the calcium nanoparticles.

SUMMARY

The nanotechnology or ultrafining technology had already been extensively applied in various scientific fields, however, the ultrafining of traditional Chinese medicines was still remained merited to investigate furthermore. In general, the amount of raw plant materials could be reduced and the bioactivity of the herbal preparations would be improved. Application of ultrafining technology in herbal preparations may significantly increase the bioactive constituents and improve the antifungal bioactivity. It could be addressed herein that the ultrafining technology applied in herbal preparations would be hopeful to discover new and effective plant derived pesticides. However, it should also be emphasized that nobody can promise that all the nanotechnology or ultrafining products would be completely safe and more bioactive. All the new products prepared by nanotechnology or ultrafining technology should be examined for its toxicity before it could be commercialized.

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Conflicts of Interest

The authors declare no conflict of interest.