# Exploring natural products: from herbal resources, microbial synthesis to animal models

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# Chapter 1

**Introduction and scope of this thesis**

**Abstract**

The impact of nature on human health is significant, particularly in the field of medicine. In this chapter, a selective overview is provided on the contributions of plant-derived sources, microbial biosynthesis sources, and animal models to the field of medicine, which have been made by advancements in science and technology. Furthermore, this chapter describes the relevant research conducted within this thesis and its potential significance, approached from these three perspectives.

## Introduction

Nature is a treasure for mankind. We utilize natural resources to eat, to cure, to wear, to exchange, to study, to work, to play, and to live. As a medical scientists, almost everything from nature in our labs can be used as a cure or tool to serve human health. Whereas the resources on the earth are limited, and their attributes usually are not as perfect as we might hope, scientists dedicate their lives to further exploring nature, to reveal its mystery. This helps to promote the sustainable development and deployment of natural resources for making products that promote our health and well-being.

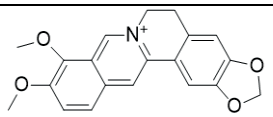
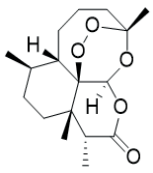
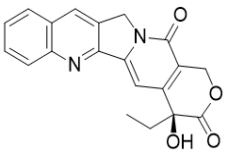
In this thesis, novel tools for natural product discovery and development are being explored and exemplified with some handy species: a herb, *Perilla frutescens*; a bacterium, *Bacillus subtilis*; and an animal, *Meriones unguiculatus*.

### Herbal resources

For the past millenniums in human history, natural products (NPs) have dominantly occupied the medical area as the "traditional medicine[1]" until the recent centuries. Among all traditional medicines, the herb is the main source of the NPs, for instance, in "The Grand Compendium of Materia Medica (Běn Cǎo Gāng Mù, 本草纲目)" about 60% of traditional Chinese medicines are herbs[2].

It is not difficult to find out the landmarks of the drugs derived from herbs in human history (see Table 1).

**Table 1 Herb-derived drugs.**

| No. | Drug | Source | Formula | Structure | Function |
|---|---|---|---|---|---|
| 1 | Berberine[3–5] | *Coptis chinensis* Franch. | $C_{20}H_{18}NO_4^+$ |  | Anti-bacterial diarrhea |
| 2 | Artemisinin[6,7] | *Artemisia annua* | $C_{15}H_{22}O_5$ |  | Anti-malarial, anti-infective |
| 3 | Camptothecin[8,9] | *Camptotheca acuminata* | $C_{20}H_{16}N_2O_4$ |  | Antineoplastic, topoisomerase-I inhibitor |

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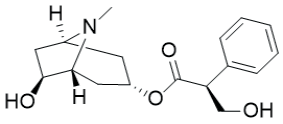
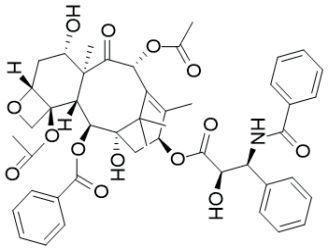
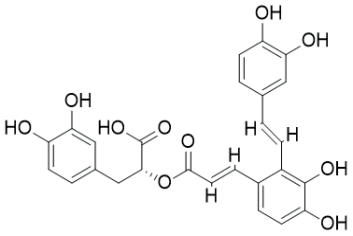
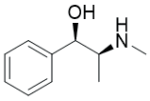
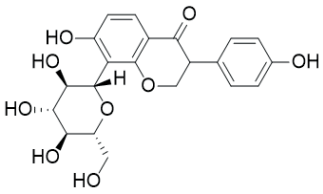
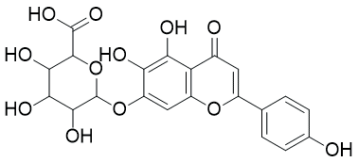
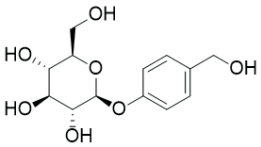
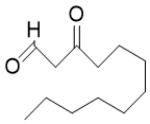
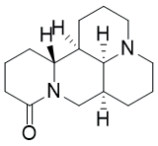
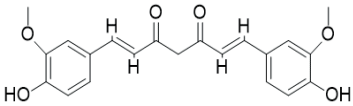
|---|---|---|---|---|---|
| 4 | Anisodamine[10,11] | *Anisodus tanguticus* (Maxim.) Pascher | $C_{17}H_{23}NO_4$ |  | Spasmolysis, anticholine |
| 5 | Paclitaxel[12,13] | *Taxus chinensis* | $C_{47}H_{51}NO_{14}$ |  | Antineoplastic |
| 6 | Salvianolic acid A[14,15] | *Salvia miltiorrhiza* Bge. | $C_{26}H_{22}O_{10}$ |  | Anti-myocardial infarction, protects cerebrovascular |
| 7 | Ephedrine[16,17] | *Ephedra sinica* Stapf | $C_{10}H_{15}NO$ |  | Relieving asthma, and spasmolysis |
| 8 | Puerarin[18,19] | *Puerarialobata* (Willd.) Ohwi | $C_{21}H_{20}O_9$ |  | Protecting cardiovascular |
| 9 | Breviscapine[20,21] | *Erigeron breviscapus* (Vant.) Hand. -Mazz. | $C_{21}H_{18}O_{12}$ |  | Treating ischemic cerebrovascular disease |
| 10 | Gastrodin[22,23] | *Gastrodia elata* Bl. | $C_{13}H_{18}O_7$ |  | Tranquilizing, and hypnotic |
| 11 | Decanoyl acetaldehyde[24,25] | *Houttuynia cordata* Thunb. | $C_{12}H_{22}O_2$ |  | Antibacterial |
| 12 | Matrine[26,27] | *Sophora flavescens* Alt. | $C_{15}H_{24}N_2O$ |  | Anti-hepatitis B |
| 13 | Curcumin[28,29] | *Curcuma Longa* L. | $C_{21}H_{20}O_6$ |  | Anti-hepatitis B |

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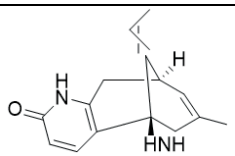
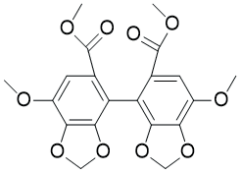
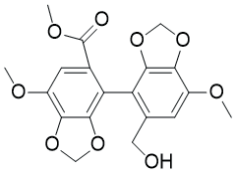
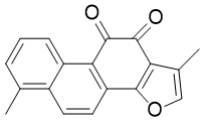
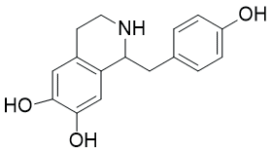
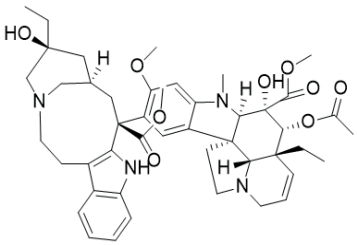
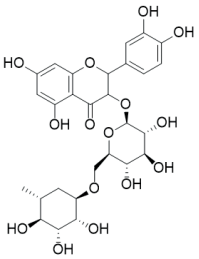
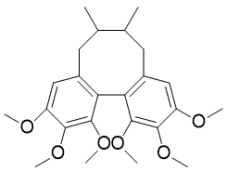
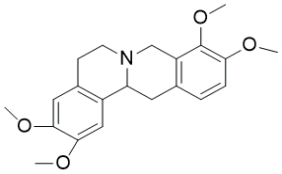
| 14 | Selagine[30,31] | *Selaginella tamariscina* (Beauv.) Spring | $C_{15}H_{18}N_2O$ |  | Brain protection |
| 15 | Bifendate[32] | *Schisandra chinensis* (Turcz.) Baill. | $C_{20}H_{18}O_{10}$ |  | Liver protection |
| 16 | Bicyclol[33,34] | *Schisandra chinensis* (Turcz.) Baill. | $C_{19}H_{18}O_9$ |  | Liver protection & anti-hepatitis |
| 17 | Tanshinone[35,36] | *Salvia miltiorrhiza* Bge. | $C_{18}H_{12}O_3$ |  | Cardio-cerebrovascular protection |
| 18 | Higenamine[37,38] | *Aconitum carmichaelii* Debeaux. | $C_{16}H_{17}NO_3$ |  | Cardiac function test drug |
| 19 | Vinblastine[39] | *Catharanthus roseus* (L.) G. Don | $C_{46}H_{58}N_4O_9$ |  | Antineoplastic |
| 20 | Rutin[40,41] | *Ruta graveolens* L. | $C_{27}H_{30}O_{16}$ |  | Vascular protection |
| 21 | Deoxyschizandrin[42,43] | *Schisandra chinensis* (Turcz.) Baill. | $C_{24}H_{32}O_6$ |  | Liver protection & anti-hepatitis |
| 22 | Tetrahydropalmatine[44,45] | *Corydalis yanhusuo* W.T.Wang | $C_{21}H_{25}NO_4$ |  | Non-narcotic analgesic, adrenergic agent |

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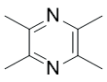
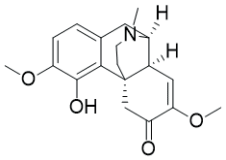
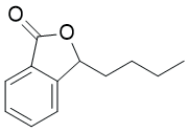
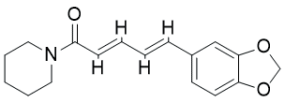
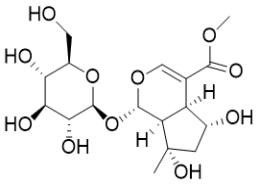
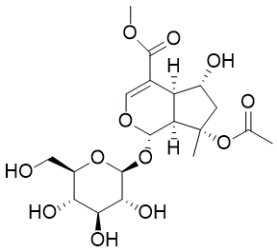
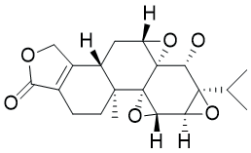
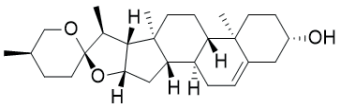
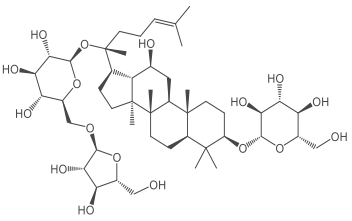
| 23 | Tetramethylpyrazine[46] | *Ligusticum chuanxiong* Hort. | $C_8H_{12}N_2$ |  | Vasodilator |
| 24 | Sinomenine[47] | *Sinomenium acutum* | $C_{19}H_{23}NO_4$ |  | Antirheumatic |
| 25 | Butylphthalide[48,49] | *Apium graveolens* | $C_{12}H_{14}O_2$ |  | Neuroprotective, platelet aggregation inhibitor |
| 26 | Piperine[50,51] | *Piper nigrum* L. | $C_{17}H_{19}NO_3$ |  | Cytochrome P-450 enzyme inhibitor |
| 27 | Shanzhiside methyl ester[52,53] | *Lamiophlomis rotata* (Benth.) Kudo | $C_{17}H_{26}O_{11}$ |  | Anti-rheumatic diseases |
| 28 | 8-O-Acetyl shanzhiside methyl ester[52,54] | *Lamiophlomis rotata* (Benth.) Kudo | $C_{19}H_{28}O_{12}$ |  | Analgesic |
| 29 | Triptolide[55,56] | *Tripterygium wilfordii* Hook. f | $C_{20}H_{24}O_6$ |  | Antineoplastic |
| 30 | Diosgenin[57,58] | *Dioscorea polystachya* Turczaninow | $C_{27}H_{42}O_3$ |  | Anti-cholestasis, anti-lipemic |
| 31 | Notoginsenoside Fe[59] | *Panax notoginseng* (Burk.) F. H. Chen ex C. Y. Wu et K.m. Feng | $C_{47}H_{80}O_{17}$ |  | Hemostasis |

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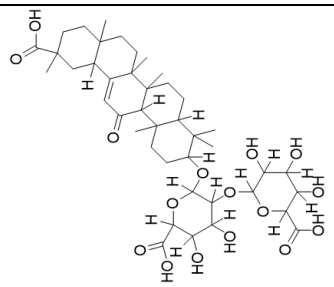
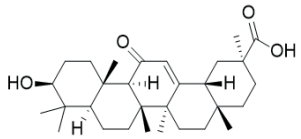
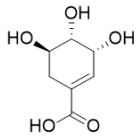
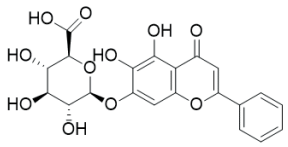
| 32 | Glycyrrhizic acid[60,61] | *Glycyrrhiza uralensis* Fisch. | $C_{42}H_{62}O_{16}$ |  | Anti-inflammatory |
| 33 | Glycyrrhetic Acid[60,62] | *Glycyrrhiza uralensis* Fisch. | $C_{30}H_{46}O_4$ |  | Anti-inflammatory |
| 34 | Shikimic acid[63,64] | *Illicium verum* Hook. f. | $C_7H_{10}O_5$ |  | Antibacterial |
| 35 | Baicalin[65–67] | *Scutellaria baicalensis* Georgi | $C_{21}H_{18}O_{11}$ |  | Anti-asthmatic, anti-inflammatory, anti-infective |

As far as we know, all the mentioned herbal drugs in Table 1 are still playing an important role in the clinic. Unfortunately, many of the valuable herbs, are also rare leading to shortages and destruction of natural habitats. Therefore, scientists endeavored to find replacements for medical manufacturing, e.g. same family substitute, recycled herb residues, replacement by using different plant parts, and, finally, biosynthesis. Below are some examples of the alternative resources developed for high-value medicinal plants (see Table 2).

**Table 2 Alternative resources of herbs.**

| Herb | Medicinal part | Efficacy[68] | Substitute |
|---|---|---|---|
| *Panax ginseng* C. A. Meyer | Root, rhizome | To tonify the original qi greatly, resume pulse, secure collapse, tonify spleen, replenish kidney, engender fluid, nourish the blood, tranquilize the mind, and replenish wisdom. | Stem and leaf of *Panax ginseng*; root and rhizome of *Codonopsis pilosula* (Franch.) Nannf.[69–71] |
| *Cephalotaxus fortune* Hook. f. | Seed, root, stem, leaf | To expel parasite, moisten lung for arresting cough, and antineoplastic. | Endophytic fungus of *Cephalotaxus fortunei*[72] |
| *Dendrobium nobile* Lindl | Stem | To boost the stomach, engender fluids, nourish yin, and clear heat. | The whole plant of *Pholidota yunnanensis* Rolfe[73] |
| *Cistanche* | Stem | To tonify the kidney yang, replenish essence and blood, moisten the intestines, | Stem of *Cistanche salsa* |

| | | | |
|---|---|---|---|
| *deserticola* Ma | | and open the bowels. | (C.A.Mey.) Beck[74] |
| *Daemonorops draco* Bl. | Resin | To activate blood, relieve pain, resolve stasis, stanch bleeding, promote tissue regeneration, and promote wound healing. | Artificially induced Resina Draconis[75] |
| *Taxus brevifolia* | Bark | To relieve pain and inflammation, anti-hypertensive and antineoplastic. | Taxus cell culture or semi-synthetic (baccatin III) from leafs[76] |
| *Cordyceps sinensis* (Berk.) Sacc. | Stroma | To tonify the kidney, replenish lung, stanch bleeding, and resolve phlegm. | Stroma of *Cordyceps militaris* (L.ex Fr.) Link. [77] |

**Microbial synthesis**

Whereas some scholars have devoted themselves to searching the herbal families and testing the medicinal parts, some others go the other way: biosynthesis. People have engineered bacteria to synthesize herb-derived compounds for the pharmaceutical industry, e.g. Taxol[78] and artemisinin[79]. Among engineered bacteria, *E. coli* and *B. subtilis* are the most frequently used species. Interestingly, *B. subtilis* is more suitable to synthesize medical compounds, since it has the GRAS status (generally regarded as safe).

Actually, *Bacillus subtilis* has many advantages for being used in biosynthesis. It can not only form protective endospores, which permit it to withstand some extreme environment[80,81], but also possesses a fast-growth rate in simple media, high protein- secretion capacity, and excellent fermentation properties[82,83]. In industries, people can find *B. subtilis* in various fields, e.g. food enzymes, feed additives in agriculture, aquaculture, food and beverage processing, and pharmaceuticals[84,85].

Although the main use of *B. subtilis* is in producing secreted enzymes, such as proteases and α-amylases, it is also being used for industrial production of natural products such as riboflavin[86].

In the past decades, scientists have discovered that *B. subtilis* is a promising host for terpenoid production[87]. Terpenoids, which are composed of isoprene units, are also known as isoprenoids. They form a large group of natural chemicals, comprising around 25% of small natural compounds[88]. Structurally, according to the number of isoprene units, they can be divided into hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25), triterpenoids (C30), tetraterpenoids (C40) and

polyterpenoids[89]. They are everywhere in our daily lives and have been broadly used in numerous fields, including energy, cosmetics, food, and pharmaceuticals.

In the medical area, terpenoids have been used to treat virus and bacterial infection, inflammation, and carcinoma, and many of them have been reported to act as antioxidant[90]. Some examples are artemisinin (antimalarial), paclitaxel (anticancer), ginsenosides (nutrient and anticancer), and carotenoids (anti-oxidant).[91–95] These functional terpenoids can be extracted from plants, however, the concentrations usually are very low. Extracting them, commonly, is not cost-efficient. Moreover, harvesting from the wild will endanger the natural spread of the plants in the environment. Although chemical synthesis can solve part of the issue, normally those compounds have a complex structure and show stereoselectivity. Thus, biosynthesis becomes essential to durably produce those promising natural products.

As a natural isoprene producer, *B. subtilis* was reported as a propitious potential platform for terpenoid biosynthesis. It has an endogenous 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprene precursor biosynthesis[96]. The pathway starts with the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (Dxs), which catalyzes the condensation of pyruvate and glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose-5-phosphate (DXP)[97]. Then the 1-deoxy-D-xylulose-5-phosphate reductoiso-merase IspC reduces and isomerizes the DXP to produce MEP[98]. After these two recognized rate-limiting steps[99], 4-diphosphocytidyl-2-C-methylerythritol synthase (IspD) introduces the cytidine monophosphate (CMP) moiety to MEP and brings out the 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME). Subsequently, IspE adds the phosphate groups to the CDP-ME, which generates 4-diphosphocytidyl-2-C-methyl-d-erythritol-2-phosphate (CDP-ME2P). In the following step, the IspF catalyzed the removal of the cytidyl moiety of CDP-MEP2P and its cyclization, which results in methyl erythritol cyclic diphosphate (MEcDP)[100]. The last reductive steps rely on by IspG and IspH. These two enzymes convert the MEcDP to 4-hydroxy-3-methylbut-2-enyl-diphosphate (HMBDP) and dihydroxylation/isomerization of HMBDP to either isopentenyl pyrophosphate (IPP) or dimethylallyl Diphosphate (DMAPP), respectively[101]. Therefore, numerous terpenoids including those for medical purposes can be produced in *B. subtilis* by building a downstream biosynthesis pathway.

As indicated above, in recent years scientists have made remarkable progress in terpenoid synthesis in *B. subtilis*, such as isoprene, amorphadiene, taxadiene, squalene, menaquinone-7

and carotenoids[87,88,102–105]. However, only a few studies have addressed the biosynthesis of polycyclic triterpenoids, and none of them discusses methods to improve their final production levels, which leaves us with an interesting challenge.

**Animal models**

Whereas herbs and bacteria have compounds with interesting pharmaceutical properties, before they can be used in practice they should be tested in disease models. Animals are most often used to establish disease models in medical areas. For example, in the current novel coronavirus disease (COVID-19) outbreak, as early as in 2020, the World Health Organization (WHO) has already assembled an international panel to develop animal models for COVID-19 and has developed models in more than 11 animal species to accelerate the testing of vaccines and therapeutic agents[106]. The importance of these animal models is apparent. In contrast to this speed and abundance in establishing models for acute diseases, for chronic diseases the choice is much more limited. Therefore, much effort has to be dedicated to developing chronic disease animal models.

A classic example is obesity and diabetes mellitus. As it is well known, more than one-third of the global population is overweight and hence at risk of developing type 2 diabetes mellitus (T2DM). This situation has caused the need to use and improve animal models to discover, optimize and validate novel therapeutics for more effective and safe use in humans. According to Matthias's research[107], people have already developed obesity and diabetes animal models in more than 13 species (including more than 50 models), which has greatly benefited the discovery and development of safer and more potent therapeutics for obesity and T2DM pandemics. However, for some other chronic medical complications, the development of predictive animal models has turned out more difficult. Among these complications, non-alcoholic fatty liver disease (NAFLD) caused liver fibrosis and cirrhosis is a typical case.

Fibrosis and cirrhosis are severe stages of liver dysfunction. They are the third and fourth stages of the natural history of NAFLD, and usually take decades to form clinical symptoms. The long disease progress is difficult to imitate in animal models. This issue seriously hindered drug development and research on disease prevention. To better mimic the pathological process of NAFLD-caused cirrhosis, researchers have developed two types of animal models, one is the genetic model, and the other one is a drug and/or diet-induced model. The genetic models normally relate to lipid metabolism genes, such as db/db mouse, ob/ob mouse, PPARα-/- mouse, PNPLA3 transgenic mouse, and (fa/fa) Zucker rats. On the

other hand, the drug and/or diet-induced models are more often used including methionine choline-deficient (MCD) diet[108,109], high-carbohydrate diet (HCD), and high-fat diet (HFD, including high-fat and high-fat, high-cholesterol diets)[110]. However, each of these animal models has deficiencies that cannot be ignored. Firstly, monogenic models cannot represent the real pathological changes of human diseases. Secondly, the drug and/or diet-induced models can rarely reach the "stable" fibrosis stage, not to mention cirrhosis[111,112].



Therefore, the Mongolian gerbil (*Meriones unguiculatus*) appears to attract researcher's attention, since it is not only a fat-sensitive animal[113–118], but also a species that is more suitable for carotenoid conversion and metabolism, metabolic disease, and cardiovascular disease studies[119]. Compared to humans, it was indicated that the gerbil's lipemic responses to dietary fat and cholesterol were more sensitive, and the responses can be detected in gerbils even without feeding dietary cholesterol[113–118,120,121]. Moreover, the gerbil also tends to acquire diabetes spontaneously[122], and has clearly reached the fibrosis stage of the nonalcoholic steatohepatitis (NASH) after diet-induction[123,124], which makes it into an ideal potential model for NAFLD-caused cirrhosis.

**Scope of this thesis**

The aim of this thesis contains three main aspects. The first part involves the comprehensive utilization of the pomace of an herb, perilla seed, for extracting its phenolic compounds. The second part focuses on the biosynthesis of terpenoids in the GRAS (generally regarded as safe) bacterium, *B. subtilis*. The third part employed the gerbil and established a nonalcoholic fatty liver disease cirrhosis model to be used for drug discovery and testing of natural products.

In **Chapter 1**, we describe the sketch and the main object of this thesis. Specifically, the role of natural products in drug discovery is discussed with an emphasis on functional phenolic compounds and terpenoids from herbs. Subsequently, we discuss the sustainable utilization of herbal resources and the improvements in nature products' biosynthesis, e.g. the terpenoid biosynthesis methods, and the pathways in *B. subtilis.* Subsequently, this thesis explores the utilization of animal models as a valuable tool for drug evaluation, with specific emphasis placed on the nonalcoholic fatty liver disease (NAFLD) cirrhosis model. The inclusion of the NAFLD model is justified due to perilla seeds' lipid-lowering and anti-inflammatory effects, as well as the critical role played by squalene as an essential step within the cholesterol biosynthesis pathway.

In **Chapter 2**, we identify and quantify the phenolic compounds from the seed and pomace of *Perilla frutescens* using HPLC/PDA and HPLC-ESI/QTOF/MS/MS. The purpose of the study is to investigate major phenolic compounds in perilla seeds and pomaces, to check if the pomace could be an alternative resource to the seed for nutritional and medical purposes. In this investigation, herb markers selected by principal components analysis (PCA) are then quantified in both seeds and pomaces. Moreover, fingerprinting approach and multiple discriminant analysis are applied to screen the phenolic markers in 22 batches of samples. Ten phenols are tentatively identified, among which four (rosmarinic acid, luteolin, apigenin, and rosmarinic acid-3-*O*-glucoside) are selected as herb markers. Perilla seeds and pomaces have shown similar phenol profiles, however, the pomaces contained almost two times more of the four herb markers compared to the seeds, which indicates perilla pomace is a promising alternative source of phenolic compounds.

In **Chapter 3**, we discuss the metabolic engineering of *B. subtilis* for terpenoid production and the encountered challenges. Firstly, the inherent terpenoid biosynthetic pathways of *B. subtilis* are summarised, including the inherent terpenoid biosynthesis enzymes of *B. subtilis.* Secondly, we describe the research progress of genetic engineering of MEP pathway enzymes in *B. subtilis.* Thirdly, the detection and metabolomics methods for engineering terpenoid pathways are also outlined, as these are important for setting up assays to evaluate biosynthetic efficiency. We focus especially on reported metabolites and the inherent terpenoid pathway intermediates in *B. subtilis.*

In **Chapter 4**, we employ *B. subtilis* to produce squalene, the common precursor of triterpenoids, by introducing multiples squalene synthases (SQSs) from bacteria, fungi, and plants into *B. subtilis*. Furthermore, the expression vector, cultivation temperature, and rate-limiting enzymes within the MEP pathway were systematically studied to enhance squalene production. Finally, a 29-fold increase in squalene titer is achieved by overexpressing SQS from *Bacillus megaterium* (BmSQS) and MEP pathway enzymes compared with the original strain. This represents the first trial of squalene synthesis and improvement in *B. subtilis*.

In **Chapter** 5, based on an HPLC-Q-Orbitrap-MS/MS study, we investigated the expression of dehydrosqualene synthase (CrtM) in *Bacillus subtilis* 168. The results have shown that *crtM* from *Staphylococcus aureus* can not only produce dehydrosqualene but also squalene and phytoene. More interestingly, compared to squalene synthase from *Bacillus megaterium*

expressed in *B. subtilis*, the CrtM from *S. aureus* can produce up to 2.4 times the amount of squalene. Besides, by adjusting the medium to a nutrition-rich medium or inserting *dxs* to upregulate the upstream donors, the CrtM was able to greatly increase the yield of squalene, which provides evidence for its potential in squalene biosynthesis. Moreover, these three products were also observed in *B. subtilis* strains that only contain SQS, indicating the promiscuity of the squalene synthase-like (SSL) enzymes. Looking into the sterostructures of presqualene diphosphate in CrtM and SQS enzymes highlights the resilience of their active sites and lends additional support to our hypothesis that the squalene synthase-like enzymes are promiscuous and the CrtM could be further remolded to produce more squalene.

In **Chapter 6**, we focus on developing a NAFLD-caused cirrhosis model in gerbil, to mimic the chronic progress characteristics of NAFLD. The dynamic relationship between hepatic lipid metabolism and cirrhosis was examined. The model's pathological process, lipid metabolism, oxidative stress, liver collagen deposition, and presence of relevant cytokines were tested and evaluated during the full-time frame of disease onset. The gerbil model can start non-alcoholic steatohepatitis within 9 weeks and can develop cirrhosis after 21 weeks of induction. The model's lipids metabolism disorder is accompanied by liver damage development. During the NAFLD progression, triglycerides (TG) and free fatty acids (FFA) have shown a distinct rise and fall tendency, and the turning points are at the fibrosis stage. Besides that, the ratios of total cholesterol (CHO) to high-density lipoprotein cholesterol (HDL-C) exhibited constant growth tendency, and have a good linear relationship with hepatic stellate cells (HSC) ($R^2 = 0.802, P < 0.001$). The model possesses a positive correlation between lipids metabolism and cirrhosis. The compelling rise and fall tendency of TG and FFA indicated that the fibrosis progression could lead to impairment in lipoprotein synthesis and engender decreased TG levels. CHO/HDL-C ratios can indicate fibrosis progress and be used as a blood indicator for disease prediction and prevention.

**Chapter 7,** summarizes the investigations in this thesis, and describes some future perspectives.

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