Insights Into the Mechanism of Rhizosphere Microorganisms Effecting on Quality Formation of Authentic *Angelica Sinensis* Under Different Soil Microenvironments

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1 Insights into the mechanism of rhizosphere microorganisms effecting
2 on quality formation of authentic *Angelica sinensis* under different
3 soil microenvironments.

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6 **Abstract**

7 **Background:** *Angelica sinensis* (Oliv.) Diels (*A. sinensis*), the commonly used famous-
8 region Chinese herbs, grew in different geographical locations which were crucial to
9 active components accumulation in medicinal plants. Rhizosphere microbiome played
10 a critical role in plant performance, including nutrient acquisition, growth and
11 development and plant diseases resistance etc., there were still few studies on how
12 microbiome effecting on accumulation of active components of *A. sinensis*. This study
13 aim to illustrate whether the variations of rhizosphere microbial communities and

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metabolites profilings of *A. sinensis* were dependant on soil microenvironments. Soils from the two main *A. sinensis* producing areas, Gansu and Yunnan Province, were selected for a pot experiment, and then divided into two parts, one part was sterilized and the other did not, planting with Gansu danggui 90-01, allowed to grow for 180 days. After the trial radix *A. sinensis* were collected for analysis of growth targets and chemical compositions, rhizosphere soils for microbial analyses.

Results: The metabolic profiles and rhizosphere microbial communities of *A. sinensis* grown under different soil microenvironments showed similar variation. The GN (Gansu non-sterilized), YN (Yunnan non-sterilized), GS (Gansu sterilized), and YS (Yunnan sterilized) group were significantly separated. Specifically, compared with Yunnan soil, antagonistic bacteria such as *Sphingomonas*, *Pseudomonas*, *Lysobacter*, *Pseudoxanthomonas*, etc. were significantly ($p < 0.05$) enriched in Gansu soil. The characteristic constituents of senkyunolide I and ligustilide dimers enriched in GS group has an extremely positive correlation with *Pseudomonas parafulva*; organic acids (including chlorogenic acid, dicaffeoylquinic acid and 5-feruloylquinic acid) and their ester coniferyl ferulate enriched in YS Group were significantly positively relevant with *Gemmatimonadetes* bacterium WY71 and *Mucilaginibacter* sp., respectively.

Conclusions: Given that soil microenvironments may contribute to growth and active components accumulation of *A. sinensis*. Further study aims at exploring the functional characteristics of quality-related bacterias, and finding the main response genes and the interactions between genes and the environments, to elucidate the main

mechanism of genuine *A. sinensis* quality formation.

Key words: *Angelica sinensis*; rhizosphere microorganism; metabolomics; quality; correlation

Background

Geo-authentic Chinese medicinal materials refer to traditional Chinese medicine (TCM) that have been selected through long-term TCM clinical practice and are produced in a specific region, with satisfactory curative effect, stable quality and high profile. The radix *A. sinensis* possessed traditional efficacies including nourishing blood, promoting blood circulation, regulating menstruation and relieving pain, was first recommended in therapy of gynecological diseases [1]. Beyond that, it was generally utilized for protecting the heart, boosting immune system, anti-arrhythmia, and anti-atherosclerosis, etc [2]. *A. sinensis* was mostly produced in Gansu, Yunnan, Qinghai and other places in China. Min County of Gansu Province has long been acknowledged as its traditional geoherb regions, cool and humid here, abundant rainfall, with mostly fertile cinnamon soil and black soil, the most appropriate material *A. sinensis* to grow. Land plants and soil microorganisms achieved mutual benefit through strong interactions. Plants provided habitats and reliable source of carbon and energy for soil microorganisms which could directly or indirectly facilitate the yield and quality of the host plants and improve the hosts' resistance through increasing effectiveness of soil mineral nutrition, production of phytohormones, degrading phytotoxic compounds, inhibiting soil-borne pathogens, etc [3-4]. Many studies

showed that plant species, growth and development, host genotypes, geographical location, etc. were the main drivers of rhizosphere microbial diversity [5-7]. Geographical position and cultivation practice were factors determine microbiome variation in field conditions; while under controlled greenhouse conditions, microbial composition varied with soil source and genotype [6]. A very large-scale longitudinal field study comparing changes in microbial communities among 27 maize inbred lines planted in three fields had yielded similar results [7]. The difference was that they found plant genetic effects had a marked impact on the rhizosphere microbiome, surpassing geographic location, plant age and climate events.

Increasing evidences demonstrated that plants-microbe interactions was vital for plant growth and health [8]. At present, the key research for study on genuine TCMs was mainly focused on soil properties and rhizosphere microorganism, important breakthroughs were made in the growth and yield improvement of famous medicinal materials, such as *Panax ginseng*, *Rehmannia glutinosa* and *P. notoginseng*, etc. Changes in rhizosphere microbial communities and accumulation of toxic substance played driving roles in replanting failure of TCMs. For example, soilborne pathogens *Fusarium* always flourished as plants grew, while antagonistic bacterium *Pseudomonas* and *Bacillus* showed opposite trend [9-10]. Although *Ilyonectria* was generally accepted as the major trigger of ginseng rusty root disease, but recent study found that the oxidation and deposition of rhizosphere Fe, Al and Mn driven by nitrate-dependent Fe (II)-oxidizing bacteria, resulting in rusty root disease [11]. The build-up of *Fusarium*

77 genus and accumulation of toxic diisobutyl phthalate (DiBP), inversely associated with
78 population of *Pseudomonas*, *Bacillus* and *Burkholderia* etc., contributed to negative
79 feedback between the soil and ginseng [9,12]. Similarly, phenolic acids accumulated in
80 the rhizosphere of *R. glutinosa* could restrain the growth of *Pseudomonas* spp., but had
81 positive effects on mycelial growth, sporulation and toxin production of *F. oxysporum*
82 [13]. Further research demonstrated that quorum quenching (QQ) bacteria that
83 participated in disruption of beneficial (*Pseudomonas* spp.) and harmful (*Enterobacter*
84 spp.) short-chain AHL-mediated QS (quorum sensing) bacteria balance in the
85 rhizosphere, accounting for a key factor of *R. glutinosa* replant diseases [14].

86 *A. sinensis* was a typical alpine plant with high requirements for temperature, light
87 intensity and altitude, suitable for growing in high elevation zones (2000~2500 m).
88 With the decrease of elevation, the radix *A. sinensis* production and quality were
89 reduced, and more vulnerable to early bolting, resulted in decrease in drug efficacy. In
90 the long-term cultivation practices, the traditional seedling raising manners, wasteland
91 seedling raising, has been retained. Due to the policy of Grain for Green and ecological
92 protection, rotating land seedling and cultivated wasteland seedling have become the
93 main choices, but they are immature to guarantee the quality of seedlings. Little
94 investigation was reported about soil microbial communities of authentic *A. sinensis*,
95 the unique living environments needs makes it hard on researching ways. Our previous
96 studies had stated that the number of dominant microbial species and the population
97 diversities of bacteria, actinomyces and fungi in the rhizosphere of Gansu province

were greater than that of other non-geoherb regions, such as Yunnan and Sichuan province. *Proteobacteria* and *bacteroidetes* were bacterial dominant phyla throughout the growing season of *A. sinensis*, while fungal dominant phyla varied with growth periods; the plant weight, root length, root diameter, soil pH, rainfall, and climate temperature were the main factors contribute to microbial community composition of *A. sinensis* [15]. The current study on plant-microbe interactions mainly focused on succession cropping obstacle of medicinal plants, responses of plant-microbe to environmental stress, changes in rhizosphere nutrition conditions, root system biomass and growth; but little support for accumulation of active constituents under different soil microenvironments [16]. There is a pressing need for clarify the mechanism of quality formation of authentic *A. sinensis*, to acquire *A. sinensis* materials in stable quality while enlarging its production.

In the present study, a pot experiment was carried out, consisting of four treatments (i) GN, which represent as the soil in pot taken from Gansu province, non-sterilized, (ii) GS, soil in pot taken from Gansu province, sterilized, (iii) YN, soil in pot taken from Yunnan province, non-sterilized, (iiii) YS, soil in pot taken from Yunnan province, sterilized, and four controls (without implants). Using the combination of metabolomics and 16s rRNA pyrosequencing technology, we aimed to address the following questions: (i) Are the rhizosphere microorganisms, and the growth and quality of *A. sinensis* vary in different soil microenvironments? (ii) Whether the growth and quality of *A. sinensis* has a positive response to changes of rhizosphere microorganisms.

Results

The morphology and quality of *A. sinensis* under different soil conditions

Regardless of whether the soil was sterilized or not, *A. sinensis* grown in Gansu soils (including GN and GS group) was significantly ($P < 0.05$) stronger than that of Yunnan soils (including YN and YS group), with more branches and larger diameters (Figure. 1A and B). Compared with non-sterilized soils, plants grown in sterilized soils displayed worse growth as illustrated by the thinner rhizome, reduced number of branches and decreased diameter (Figure. 1C and D). The surviving rate of plants from non-sterilized soils (GN, 0.77 ± 0.09 ; YN, 0.63 ± 0.18 ; $n = 6$) was remarkably ($p < 0.05$) higher than that of sterilized soils (GS, 0.48 ± 0.15 ; YS, 0.46 ± 0.19 ; $n=6$). In addition, we measured the dry weight, diameter, rootlet number and root length of all samples. The results showed that the average dry weight, root diameter, rootlet number, and root length of plants in GN group were significantly ($p < 0.05$) larger than that of YN group. Compared with GN group, plants in GS group showed noticeably ($p < 0.05$) reduced average dry weight, root diameter and root length. The root diameter of plants in YN group were significantly ($p < 0.05$) higher than in YS group, higher in GS group than YS group (Table. 1).

The major medicinal components (phthalides and organic acids) and nutrients (amino acids, nucleosides and nucleobases) were compressively analyzed to further explain the inherent differences in quality of *A. sinensis* among groups . The results showed that the contents of medicinal components tested were remarkably ($p < 0.05$)

different among groups. Specifically, the contents of chlorogenic acid and Z-ligustilide of *A. sinensis* grown in Yunnan soil were significantly ($p < 0.05$) higher than that of Gansu soil. Compared to the other three groups, the GN group *A. sinensis* possessed definitely ($p < 0.05$) higher contents of coniferyl ferulate and butylidenephthalide, and lower content of ferulic acid. The contents of senkyunolide A and butylphthalide in YN group *A. sinensis* were noticeably ($p < 0.05$) greater than other groups. The contents of senkyunolide I and senkyunolide H in YS group *A. sinensis* were significantly ($p < 0.05$) lower than other groups. However, both senkyunolide I and senkyunolide H showed no significant difference in the *A. sinensis* samples from GN, YN and GS groups (Table. 2).

Our previous publications indicated that *A. sinensis* samples have high contents of nutritive component including nucleosides, nucleobases and free amino acids [17]. Additionally, our previous reports also showed that the contents of nucleosides and nucleobases were no significant difference between different groups, while the contents of amino acids had certain differences. The mean content of arginine accounted for 86.02% of the total amino acids, much higher than other relatively abundant ones, such as γ -aminobutyric acid (4.78%), glutamine (2.18%) and proline (1.07%). Compared with the GN or GS group, the concentrations of arginine, γ -aminobutyric acid, glutamine and proline in YN or YS group displayed a decreasing tendency. The changes of chemical components among groups might be explained by differences in soil microenvironments.

Changes of *A. sinensis*'s metabolite profiling under different soil microenvironments

The quality of medicinal plants was not focused on a single compound, but involved in a special mixture of diverse, biochemistry related and unrelated compound groups [18]. UPLC with high-resolution mass spectrometry provided powerful functions for isolating and profiling a large amount of many kinds of metabolites in different matrices. In this study, a simple, rapid and sensitive UPLC-UV-QTOF-MS/MS method was developed to evaluate the variation of the metabolites in *A. sinensis* samples under different soil conditions. Principal components analysis (PCA) showed that metabolic profiles among groups were significantly ($P < 0.05$) separated, all samples clustered into four groups, GN, YN, GS and YS groups. Moreover, the GS and YS samples were found to be gathered close to GN samples, especially GS samples, but far away from YN samples (Figure. 2A). The R^2X , R^2Y and Q^2Y of the OPLS-DA model were 0.667, 0.966 and 0.851, respectively. The permutation results verified the stability and reliability of this OPLS-DA model. The hierarchical clustering heatmap showed similar gathering patterns of molecular features detected within groups and differences in molecular features detected between groups, which was consistent with the PCA clustering pattern (Figure. 3A).

In the UPLC-QTOF-MS/MS analysis, both positive and negative ion modes were employed for qualitative identification. A total of 44 metabolites were identified by chemical standards or tentatively identified by comparing their quasi-molecular ions,

empirical molecular formulas, and/or fragment ions with those of known chemicals (Supplementary Table S1) [19]. It was workable to select markers that contributed greatly to the grouping by setting the Variable Importance for Projection (VIP) value greater than 1.5 and the *p*-value less than 0.05 in the Moderated t-Test. In general, compared to YN and YS groups, the contents of ligustilide dimers increased in plants from GN and GS groups, while the content of chlorogenic acid was opposite. More concretely, compared with YN group, the plants of GN group was higher in ligustilide dimers, while the contents of unknown [19], senkyunolide A and chlorogenic acid declined. Secondly, compared with YS group, the plants of GS group displayed higher contents of ligustilide dimers, Z-6,7-epoxyligustilide and senkyunolide I, while the contents of both chlorogenic acid and dicaffeoylquinic acid showed decreasing trend. It was noticed that the plants in non-sterilized groups had higher levels of ligustilide dimers than that of the sterilized groups (Supplementary Fig. S1, Supplementary Table S2).

Annotation and community composition of rhizosphere bacteria under different soil microenvironments

The V4 region of 16S rRNA gene of total 36 samples were amplified using PCR and sequenced using the IonS5TMXL platform. An average of 80,118 clean reads per sample was obtained, the high-quality reads were clustered using > 97% sequence identity into 10,583 OTUs, and then annotated using Silva132 database. A total of 65 kinds of the OTUs were annotated at the phylum level, accounting for 87.60% of all

OTUs; and 721 kinds of the OTUs were annotated at the genus level . In the phylum level, we found that *Proteobacteria* (relative abundance of 55.38%), *Bacteroidetes* (10.65%) and *Acidobacteria* (7.26%) were the dominant species; in the genus level, the roots were dominated by *Stenotrophomonas* (9.97%), *Rhodanobacter* (4.19%) and *Lactobacillus* (1.48%).

α -diversity refers to the microbial diversity of each sample, similar conclusions were drawn by calculating the Chao1, Shannon's diversity index and Simpson diversity index, etc (Figure. 4A and B). The results showed that the community diversity of the non-sterilized groups were higher than that of the sterilized groups, and the community diversity in the Gansu groups were higher than that in Yunnan groups, but there was little difference between the planting groups and the corresponding non-planting groups. ANOSIM showed that the difference between groups were significantly ($p < 0.05$) greater than that of within groups, indicating that the results were reliable. Therefore, these results indicated that the diversity of the bacterial communities of *A. sinensis* was determined by the soil microenvironments, and the plant effect had little effect.

PCoA based on weighted UniFrac (WUF) distance metric showed that the community compositions of the planting groups and corresponding non-planting groups were highly similar. The sterilized and non-sterilized samples separated along the first axis, PC1 explained 38.65% of the total variation, indicating that indigenous bacteria were the most important factor affecting the rhizosphere bacterial community. The Gansu and Yunnan samples could be clearly distinguished on the second axis, PC2

explained 23.35% of the total variation, indicating that geographical location were the second largest factor accounting for the changes of rhizosphere bacterial community. However, when using an unweighted UniFrac (UUF) distance, geographic location had the largest source of variation in the tested factors, and indigenous bacteria described the second largest source of variation (Figure. 2B).

The results of the PCoA was consistent with unweighted pair-group method with arithmetic mean (UPGMA) clustering (Figure. 2C). The hierarchical clustering based on a UUF distance metric showed complete clustering of samples in GN, GS, YN, and YS groups. But the planting groups and the corresponding non-planting groups gathered together, indicating that plant effect made little difference to the overall community composition. The GS and the YS samples first gathered into a branch, then the YN samples, and finally the GN samples. It showed that GN group differed greatly with the other three groups. In order to support the above analysis statistically, we used ADONIS and MRPP, etc. to perform statistical tests on samples from different groups, similar conclusion was derived.

Comparison of rhizosphere bacteria composition between different groups

In order to fully understand the OTU distribution among groups, we constructed Venn diagrams, and then calculated the proportion of uniquely identified OTUs in each group and OTUs shared between different groups. Among them, the proportion of OTUs shared between the GN and GNck group, YN and YNck group, GN and YN group, GS and YS group, GN and GS group, YN and YS group accounted for 48.74%,

42.17%, 38.17%, 30.10%, 35.88%, and 32.50% of all OTUs, respectively. This result indicated the high similarity of the microbial community structure between the planting groups and corresponding non-planting groups. We then drew a Venn diagram for all planting groups, including GN, GS, YN, and YS group, it was shown that the number of unique OTUs belong to the GN group were the largest, with 1692 species; and the YS group was the least, with 226 species. The same phenomena were recorded in all non-planting groups.

At the phylum level, we selected the top 12 most abundant phylum for each sample or group to generate an accumulative column diagram of the relative abundance of all species. It could be seen that the microbial community was dominated by *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria*. Compared to the non-sterilized groups, a notable increase was observed in the relative abundance of *Proteobacteria* and *Bacteroidetes* of all sterilized groups, while the relative abundance of *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* decreased significantly ($p < 0.05$). Compared with the GN group, the relative abundance of *Chloroflexi* and *Planctomycetes* was significantly ($p < 0.05$) higher in YN group, while the relative abundance of *Acidobacteria* displayed significant ($p < 0.05$) reduction; Compared with the YS group, the GS group significantly ($p < 0.05$) increased the relative abundance of *Gemmatimonadetes*, *Actinobacteria*, and *Chloroflexi*, while the relative abundance of *Proteobacteria* was opposite. The discrepancy of microbial community between planting groups and corresponding non-planting groups was insignificant ($p > 0.05$).

Figure. 4C shows the distribution of phylum level.

At the genus level, a total of 721 taxa were identified from all 36 samples. The roots of *A. sinensis* were preferentially colonized by *Stenotrophomonas*, *Rhodanobacter* and *Lactobacillus*. The heatmap visualization with hierarchical clustering of top 35 most abundant genera showed (Figure. 4D) that there were distinct differences in microbial community structure and relative abundance of most predominant genera between different groups. Compared with the Yunnan group, the relative abundance of *Lysobacter* and *Brevundimonas* in Gansu group increased significantly ($p < 0.05$), while the relative abundance of *Rhodanobacter* and *Gemmatimonas* decreased significantly ($p < 0.05$). In addition, the relative abundance of *Sphingomonas* in the GN group was higher than that of YN group, while the opposite was true for relative abundance of *Mucilaginibacter*. Secondly, the relative abundance of *Pseudomonas* in GS group was higher than that of YS group; while the relative abundance of *Ramlibacter* and *Sphingomonas* were opposite. It was remarkable that the relative abundance of *Sphingomonas* in all non-sterilized groups was significantly ($p < 0.05$) higher than the corresponding sterilized groups.

Putative mechanistic associations between microbes and quality-linked metabolites.

The secondary metabolites of *A. sinensis* were mainly composed of phthalides, organic acids, and polysaccharides, among which phthalides were the main bioactive ingredients, with a wealth of structural diversity [2,20]. In the previous study, we found

that compared with the YS group, *Pseudomonas*, *Lysobacter*, *Pseudoxanthomonas*, and *Brevundimonas* were significantly ($p < 0.05$) enriched in GS group. The contents of ligustilide dimer and senkyunolide I in GS group were significantly ($p < 0.05$) higher than that in YS group, and were positively correlated with *Pseudomonas parafulva*, *Pseudoxanthomonas maxicana*, and *Brevundimonas bullata* (average Spearman $r > 0.5$) (Figure. 3B). Among them, ligustilide dimer was extremely significantly ($p < 0.05$) positively correlated with *P. parafulva* (Spearman $r = 0.82$) (Supplementary Table S3). It was speculated that the high abundance of *P. parafulva* in the GS group contribute to the accumulation of phthalides and was beneficial to the growth and quality formation of *A. sinensis*.

Some ferulic acid derivatives such as ferulic acid ester have stronger biological activity and lower toxicity. Organic acids and their esters were significantly ($p < 0.05$) enriched in YS group. It showed that chlorogenic acid, dicaffeoylquinic acid and 5-feruloylquinic acid were positively correlated with *Gemmatimonadetes bacterium* WY71 (average Spearman $r > 0.8$); coniferyl ferulate was positively correlated with *Mucilaginibacter* sp. (Spearman $r = 0.85$) (Figure. 3B) (Supplementary Table S3) [21]. *Mucilaginibacter* was widely present in the rhizosphere soil of *Angelica* medicinal plants. For example, *M. herbaticus* sp nov. [22], *M. polysacchareus* sp nov. [23], and *M. angelicae* sp nov. [24] were isolated respectively from the rhizosphere, root surface of *A. sinensis*, and rhizosphere of *A. polymorpha* Maxim. It was speculated that the high abundance of *Mucilaginibacter* in YS soil might be related to the accumulation of

ferulic acid derivatives.

Discussion

Driving factors of *A. sinensis*'s quality formation under different soil conditions

Considerable researches have indicated that the quality of Chinese drugs showed a significantly geographical differences, this might due to variation of altitude, climate, soil properties and rhizospheric microorganisms, etc. Our study showed that soil microenvironments was a significant factor contribute to the quality formation of *A. sinensis*. The tradition holds that the higher the contents of ferulic acid and Z-ligustilide, the better the quality of the radix *A. Sinensis*. Compared with the YN and GS group, GN group displayed significantly ($p < 0.05$) larger dry weight, root diameter, and root length (Table. 1); and the contents of chlorogenic acid and Z-ligustilide in Yunnan groups were significantly ($p < 0.05$) higher than that of Gansu groups (Table. 2). Compared with Yunnan groups, the content of senkyunolide I, butylidenephthalide, ligustilide dimers increased in Gansu groups, while ferulic acid displayed decreasing trend (Table. 1, Supplementary Table S2). Zhang et al found that the contents of ferulic acid and levistolide A in non-geoherb regions were higher than that of geoherb regions, while the opposite was true for senkyunolide I and butylidenephthalide [19], which supported our conclusions. Similar results had uncovered through a large-scale field investigation, the difference was that the root length and head diameter showed negative correlation to ferulic acid, as well as root length to Z-ligustilide [25]. Therefore, it was inaccurate to estimate the quality of radix *A. sinensis* from their appearance traits.

Phenolic acids were widely reported as key allelochemicals of medicinal plants, coumalic acid, ferulic acid, vanillic acid, 4-hydroxybenzoic acid, and syringic acid are found in root exudates of *P. ginseng*, *R. glutinosa*, and *Pinellia ternata* [26]. Imperatorin, vanillin, and ferulic acid were reported as possible allelopathic autotoxic substances in rhizosphere soil of *A. sinensis*, which could obviously inhibit the growth of its seedlings [27]. The increased content of ferulic acid in plants of Yunnan groups, indicating the larger accumulation of allelochemicals in the Yunnan soils, which led to the deteriorated growth of *A. sinensis*. Similarly, higher content of ferulic acid in sterilized groups than in non-sterilized groups may also be the reason for the weak growth of plants in the sterilized groups.

Effect of soil type on the composition of rhizosphere bacteria of *A. sinensis*

A continental-scale studies of soil bacterial populations showed that the richness and diversity of soil bacterial communities differed by ecosystem type, among which soil pH explained the largest source of variation [28]. For example, compared to loessial soil and continuous cropping soil, polysaccharides and glucose increased significantly in *Dioscorea opposita* Thunb. growing in sandy soil, moreover, the decreased soil pH might contribute to the high level of galacturonic acid from continuous cropping soil [29]. The soil of Gansu was slightly alkaline, while the soil of Yunnan was weakly acidic, different soil pH caused significant differences in the rhizosphere and root-associated microbiomes of plant. It was shown that the relative abundance of *Sphingomonas*, *Lysobacter* and *Pseudoxanthomonas* in the GN group was higher than

that of YN group; while the relative abundance of *Rhodanobacter*, *Gemmatimonas* and *Mucilaginibacter* decreased significantly ($p < 0.05$) (Figure. 4D). Compared with the YS group, the relative abundance of *Pseudomonas*, *Lysobacter* and *Pseudoxanthomonas* increased in GS group; while *Rhodanobacter*, *Ramlibacter*, *Dyella* were opposite (Figure. 4D). Wu et al found that the relative abundance of *Lysobacter*, *Pseudoxanthomonas*, *Pseudomonas* and *Burkholderia* were significantly ($p < 0.05$) increased in the newly cultivated soil of *R. glutinosa* compared to continuous monocropping soil [30]. Similarly, the abundance of beneficial bacteria such as *Arthrobacter*, *Burkholderia*, *Rhodanobacter*, and *Sphingobacterium* displayed a negatively correlation to the accumulation of toxic diisobutyl phthalate (DiBP) in monocropping soil of *P. ginseng* [12]. We speculated that genus *Lysobacter*, *Pseudomonas*, and *Burkholderia* etc. might be core microbes for radix or rhizoma medicinal herbs.

Previous studies demonstrated that *Sphingomonas* [31], *Lysobacter* [32-33] and *Pseudoxanthomonas* [34-35] had functions of enhancing resistance to diseases and insect pests, and degrading harmful substances in the soil. *Sphingomonas* could also promote plant absorption by secreting carbohydrate into the rhizosphere [31]. *Lysobacter* strains had strong antagonistic activity against a series of pathogens (including *R. solani*, *F. oxysporum* and *X. carmpestris*, etc.) [32]. Genus *Pseudomonas* was proved to be a group of plant growth promoting rhizobacteria (PGPR) [36-37]. For instance, *Pseudomonas* was found to be a key PGPR in the rhizosphere of garlic

regardless of growth periods, soil types and agricultural practices, the constructed six *Pseudomonas* strains displayed strong plant growth promoting effects [38]. These results indicated that the stronger growth characteristics of *A. sinensis* in Gansu group may be related to the increased abundance of these antagonistic microorganisms in the rhizosphere.

Effects of indigenous microorganisms on rhizosphere bacterial communities and growth of *A. sinensis*

Many years of planting sole crop, such as grain crops, vegetable crops, medicinal plants, etc., often bring about widespread continuous cropping problems. Speculation had it that soil sterilization could change the physical structure and microbiological properties of soil, which resulted in increased nutrient release, and inhibition of soil pathogens, thus promote plant growth. For example, *A. sinensis* from sterilized replant soil exhibited higher plant performance and enhanced activities of active oxygen scavenging enzymes, which might cause by the increased number and diversity of culturable microbial populations and bacterial functional groups [39]. While soil sterilization could relieve the soil succession cropping failure, it not only removed indigenous microbes, including some pathogens, but could also destroy the possible beneficial effects of soil microbes to host plants [40]. Some researches indicated that indigenous microorganisms were vital on plant growth and agricultural productivity, and it's possible effects on plant secondary metabolism [41]. For example, inoculation with PGPR *B. flexus* could alleviate host salinity stress damage by modifying its

physiological and biochemical state, such as enhancement of photosynthesis, osmotic regulator contents, antioxidant enzymes activities, and regulation of Na^+/K^+ homeostasis [42].

Our study showed that plants grown in sterilized soil displayed worse growth and decreased surviving rate, it might because the soil we used were newly cropped (Table. 1), which confirmed the prediction that indigenous microorganisms were essential to satisfy the plant growth and development. Likewise, *Glycyrrhiza uralensis* grown in sterilized soil displayed poorer plant growth and photosynthesis, inoculation with AM fungi in sterilized soil compensated for the absence of indigenous microbial communities, leading to increased plant growth and glycyrrhizin and liquiritin contents [40]. We also found that compared with the GS group, the relative abundance of *Sphingomonas* increased significantly ($p < 0.05$) in the GN group; while *Ramlibacter*, *Gemmatimonas* and *Brevundimonas* were opposite (Figure. 4D). The relative abundance of *Sphingomonas* and *Bradyrhizobium* in YN group were higher than that of YS group; while *Rhodanobacter*, *Ramlibacter* and *Dyella* decreased significantly ($p < 0.05$) (Figure. 4D). *Bradyrhizobium* was also a known PGPR that can produce plant hormones and QS metabolites to regulate the symbiotic relationship with the host and adapt to environmental changes [43]. QS-deficient (unable to produce cinnamoyl-HSL) mutant strains exhibited significant decreased colonization ability and reduced plant growth compared to wildtype strain [44]. The high abundance of beneficial bacteria, such as *Sphingomonas* and *Bradyrhizobium*, in the rhizosphere of non-sterilized groups

accounted for better growth and quality of *A. sinensis* in the unsterilized groups.

Potentially mechanistic associations between rhizosphere microbes and metabolites

Many plant endophytes are able to synthesize a variety of secondary metabolites, same or similar to their host, as well as some new compounds, some of them show good biological activities, which help to quality improvement of medicinal plants [45]. For example, inoculation with endophytes isolated from the alkaloid-rich genotype of *Catharanthus roseus*, can remarkably improve ajmalicine and serpentine contents in roots of low alkaloid yielding genotype [46]. The small moleculars secreted by roots such as amino acids, organic acids, sugars and secondary metabolites can specifically promote the growth of some soil microorganisms, causing the migration of soil microbial communities [47]. For instance, *Falciphora oryzae* colonized *Arabidopsis* roots by sensing signalling molecules from its roots, and then promoted the lateral root growth of *Arabidopsis* through biosynthesis of indole derivatives [48]. We speculated that rhizosphere microbes can also directly or indirectly involved in and influence the growth and metabolism process of hosts. For example, PGPR, mycorrhizal fungi and Actinomycetes from *Platycodon grandiflorum* [49], *P. ginseng* [50], and *Curcuma longa* [51] can significantly promote plant growth and resistance. We observed that the metabolite profiling of *A. sinensis* and microbial community composition in the rhizosphere soil had similar variation patterns among groups. Therefore, it was concluded that there existed a certain relationship between the secondary metabolites

of *A. sinensis* and its rhizosphere microorganisms.

Our research demonstrated that the higher contents of ligustilide dimers and senkyunolide I in GS group was positively correlated with *P. parafulva*, *P. maxicana*, and *B. bullata* (average Spearman $r > 0.5$), and the higher contents of chlorogenic acid, dicaffeoylquinic acid, 5-feruloylquinic acid in YS group were positively correlated with *Gemmatimonadetes bacterium* WY71 (average Spearman $r > 0.8$); similarly, coniferyl ferulate to *Mucilaginibacter* sp. (Spearman $r = 0.85$) (Supplementary Table S3). A positive association between a metabolite and species may indicate that the metabolite promotes the growth of the that species, or that the species produces that metabolite [52]. Studies have shown that certain microorganisms can promote the accumulation of metabolites through up-regulating the expression of genes involved in the synthesis of secondary metabolites, activating the host's defense responses, and synthesis of key enzymes that convert precursors into effective constituents [53]. For example, *Pseudomonas* sp. and *Pantoea* sp. in the rhizosphere of *Salvia miltiorrhiza* could stimulate the plant growth and synthesize of major phenolic acids, which due to its production of the abundant types and contents of phytohormones [54]. Inoculation of *Trichoderma asperellum* significantly increased the artemisinin concentration and dry weight of *Artemisia annua* L. leaves by up-regulating the expression of artemisinin biosynthesis key enzyme genes *HMGR1*, *FPS*, *ADS*, *CYP71AV1*, *CPR*, *DBR*, *DXS1* and *DXR1* [55]. Dark Septate Endophytes (DSE) could establish symbiosis with liquorice, promoted its plant N and P absorption, biomass, as well as accumulations of

glycyrrhizic acid and glycyrrhizin [56]. The genome of *P. parafulva* encoded a series of antibacterial secondary metabolites such as lipopeptides, pyridine, benazine and hydrogen cyanide, and exhibited an excellent antagonism to plant pathogens of rice and soybeans (including *Rhizoctonia solani*, *Xanthomonas axonopodis* and *Burkholderia glumae*.) [57-58]. A next step is to further identify whether the observed population structure was stable over time or correlated with changes in metabolomic or microbiome composition in *A. sinensis*, and to distinguish the plant genes that associate with these taxa and to better characterize their functions [7].

Conclusion

In summary, this study analysed changes of metabolite profilings and rhizosphere microbial communities of *A. sinensis* under different soil microenvironments, and provides some opinions into mechanism of rhizosphere microorganisms effecting on quality formation of authentic *A. sinensis*. The results proved that the surviving rate and growth status of *A. sinensis* in Gansu soils was stronger than that of Yunnan soils, higher in unsterilized groups than sterilized groups. High abundance of beneficial bacteria *Sphingomonas*, *Pseudomonas*, *Lysobacter*, *Pseudoxanthomonas* in Gansu soils and the enhanced growth characteristics of *A. sinensis* from Gansu soils revealed that the rhizosphere microbiome was necessarily required by plants for normal growth and development. Further more, ligustilide dimers enriched in GS group presented a notable positive correlation with the relative abundance of *P. parafulva*; organic acids enriched in YS group were positively correlated with the relative abundance of

476 *Gemmatimonadetes bacterium* WY71 and *Mucilaginibater* sp. Our results provided
477 foundation for further research of the relationship between rhizosphere microbes and
478 growth and quality formation of *A. sinensis* as well as its mechanism in order to
479 essentially elucidate the mechanism of the connection of microorganism and growth of
480 medicinal plants.

481 **Materials and Methods:**

482 **Pot experiment site and design**

483 The tested soils were taken from the experimental base of the Min Conty Medcinal
484 Plants Growing Technology Extension Centre (34°22'30" N, 104°53'20" E; black soil,
485 pH≈8.0) and the Medcinal Plants Research Institute Yunnan Academy of Agricultural
486 Sciences (26°28 '42" N, 100°4'34" E; red soil, pH≈6.5), where *Vicia faba* L. was the
487 previous crop. The soil samples then transported into Shili Town of Min County, Gansu
488 Province, where the average annual rainfall was 635.0 ± 17.4 mm, the annual average
489 temperature was 5.43 ± 0.71 °C, and the yearly mean sunshine duration was $2154.3 \pm$
490 68.7 h. After sieving (2 mm), the soil samples were then divided into two parts, one part
491 was sterilized by autoclaving at 121 °C for 4 hours; the other part did not. The *A.*
492 *sinensis* seedlings (Gansu danggui 90-01) were provided by Min County Xizhai town
493 Zhucan herbs Planting Professional Cooperative, and were transplanted during the local
494 traditional planting period. The experiment set up eight groups, including four
495 experimental groups (GN, GS, YN, YS) and four control groups (GN-ck, GS-ck, YN-
496 ck, YS-ck). The soil without implants served as a control group, six repeats of each

497 group.

498 The soil was put into a large plastic box and mixed thoroughly with the base
499 fertilizer (diamine phosphate 10.6 g per pot, organic fertilizer 23.6 g per pot). Put the
500 mixed soil up to 4~6 cm lower the container mouth, layered compacted. 8 seedlings of
501 each pot, with a depth of 3 cm and an interval of about 4 cm. Cover the seeds with
502 topsoil and apply a small amount of water (about 50 mL) to moisten the seeding layer.
503 During whole trial period, no fungicides were sprayed, manual weeding and preventing
504 pests and diseases in time. When drought occurred 2 cm below the topsoil, irrigating
505 each pot with the same amount of water. During 2018, *A. sinensis* plants were allowed
506 to grow for 180 days. Carefully uprooted the fresh plants from the soil and slightly
507 shaken to remove loosely attached soil [30]. The rhizosphere soil tightly attached to
508 tuberous roots was collected. The fresh plants and soil samples were then transferred to
509 the laboratory in an ice cooler and stored at -80 °C until analysis.

510 **DNA extraction, PCR amplification and sequencing**

511 A total of 36 soil samples collected from the 8 groups of the pot experiment (GN,
512 GS, YN, YS, GN-ck, GS-ck, YN-ck, YS-ck) were sent to 100015 (China) Beijing
513 Novogene Biotech Co., Ltd. to perform 16S rRNA gene amplicon sequencing through
514 Ion S5TM XL platform. Use CTAB or SDS method to extract genomic DNA, use agarose
515 gel electrophoresis to detect the purity and concentration of DNA, and then dilute it
516 with sterile water to 1 ng·μL⁻¹. The variable region V4 of the bacterial 16S rRNA gene
517 was amplified with degenerate PCR primers 515F and 806R (Walters et al. 2016). The

samples were separated on a 2% (w/v) agarose gel, and mixed together in equivalent amounts according to the concentration of PCR products. The mixed PCR products were purified using the GeneJET™ Gel Extraction Kit (Thermo Scientific). Briefly, 16S rRNA gene amplicons were extracted by Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific) according to the manufacturer's instructions. The built DNA amplicon library was sequenced on the Ion S5™ XL platform after Qubit quantification and examination of genomic libraries.

Data processing and analysis

The raw sequencing data from Ion AmpliSeq were processed by using Cutadapt (V 1.9.1) (Martin M., 2011). Remove the barcode and primer sequence for preliminary quality control to obtain raw reads. The chimera sequences were detected and deleted from the dataset by using UCHIME algorithm to finally get clean reads. The clean reads were clustered into operational taxonomic units (OTUs) at a sequence similarity level of 97% by using Uparse software (v7.0.1001). Taxonomic annotation through the SSUrRNA database was performed by Mothur (version 1.25.1) and SILVA132 (<http://www.arb-silva.de/>). Use MUSCLE (V 3.8.31) software to perform multiple sequence alignment to obtain the phylogenetic relationship of all OTUs sequences. Use QIIME (V 1.9.1) and R software (V 2.15.3) for sample diversity analysis. α -diversity was calculated considering OTU tables. Spearman rank correlation analysis was performed by R using the vegan packages.

Multi-index comprehensive evaluation of the chemical composition of *A. sinensis*

Method 1: preparation of Sample Solutions for secondary metabolite analysis.

The accurately weighed 0.2 g dried powder (40 mesh) was sonicated (100 Hz, 25°C) with 4 mL 70% methanol for 45 mins in a 50 mL glass-stoppered conical flask. The samples were centrifuged (12000 r/min, 10 mins), and the supernatants were injected directly onto a A Thermo Synchronis C₁₈ (2.1 mm×100 mm, 1.7 μm) column. The mobile phase consisted of A (0.1% formic acid) and B (acetonitrile). The optimized elution conditions were as follows: 0~2 min, 5%~10% B; 2~10 min, 10%~40% B; 10~14 min, 40%~58% B; 14~20 min, 58%~90% B; 20~22 min, 90%~90% B; 22~22.5 min, 90%~5% B; 22.5~24 min, 5%~5% B. The flow rate is 0.4 mL·min⁻¹, column temperature remained at 35°C, and the injection volume was 2 μL. Mass spectrometric detection was carried out on a Waters ACQUITY™ Synapt Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) source. ESI-MS spectra were obtained in both positive (ESI⁺) and negative (ESI⁻) ion modes by scanning from 100 to 1000 Da. The optimized conditions referenced to published articles in Chemistry Central Journal [59].

Method 2: preparation of Nucleosides, Nucleobases and Amino Acids analysis samples and standard solutions for qualitative and quantitative analysis.

Referenced to published articles in Molecules [17]. A mixed standard stock solution of 26 reference compounds was prepared in methanol/water (9:1, v/v), the concentration of which was thymine 4.1 μg·mL⁻¹, thymidine 3.8 μg·mL⁻¹, 2'-deoxyguanosine 3.7 μg·mL⁻¹, cytidine 4.4 μg·mL⁻¹, 2'-deoxyuridine 5.3 μg·mL⁻¹,

560 cytidine-5'-monophosphate $4.1 \mu\text{g}\cdot\text{mL}^{-1}$, guanine $3.8 \mu\text{g}\cdot\text{mL}^{-1}$, ornithine $3.6 \mu\text{g}\cdot\text{mL}^{-1}$,
561 leucine $15.0 \mu\text{g}\cdot\text{mL}^{-1}$, iso-leucine $15.0 \mu\text{g}\cdot\text{mL}^{-1}$, phenylalamine $15.3 \mu\text{g}\cdot\text{mL}^{-1}$,
562 tryptophan $15.8 \mu\text{g}\cdot\text{mL}^{-1}$, aminobutyric acid $19.0 \mu\text{g}\cdot\text{mL}^{-1}$, methionine $17.0 \mu\text{g}\cdot\text{mL}^{-1}$,
563 proline $18.5 \mu\text{g}\cdot\text{mL}^{-1}$, valine $17.2 \mu\text{g}\cdot\text{mL}^{-1}$, trans-4-hydroxy-L-proline $21.0 \mu\text{g}\cdot\text{mL}^{-1}$,
564 glutamincacid $15.7 \mu\text{g}\cdot\text{mL}^{-1}$, glutamine $13.8 \mu\text{g}\cdot\text{mL}^{-1}$ and asparagine $23.8 \mu\text{g}\cdot\text{mL}^{-1}$,
565 tyrosine $15.3 \mu\text{g}\cdot\text{mL}^{-1}$, threonine $17.3 \mu\text{g}\cdot\text{mL}^{-1}$, lysine $18.5 \mu\text{g}\cdot\text{mL}^{-1}$, serine $15.5 \mu\text{g}\cdot\text{mL}^{-1}$,
566 ¹, citrulline $17.3 \mu\text{g}\cdot\text{mL}^{-1}$, arginine $32.3 \mu\text{g}\cdot\text{mL}^{-1}$. The working standard solution for the
567 calibration curves were prepared by diluting the mixed standard solution with 10%
568 methanol at different concentrations.

569 **Method 3: Preparation of organic acids and phthalides analysis samples and**
570 **mixed control solutions.**

571 0.2 g dried powder (40 mesh) was accurately weighed and extracted in the
572 ultrasound assisted condition at 25°C with 20 mL methanol for 40 mins in a 50 mL
573 glass-stoppered conical flask. After centrifugation (13,000 r/min, 10 mins) and filtration
574 ($0.22 \mu\text{m}$ membrane filter), the supernatants were injected into the UPLC system for
575 analysis. A mixed standard stock solution of 9 reference compounds was prepared in
576 methanol/water (9:1, v/v), the concentration of which was chlorogenic acid 24.1313
577 $\text{mg}\cdot\text{L}^{-1}$, ferulic acid $15.8838 \text{ mg}\cdot\text{L}^{-1}$, senkyunolide I $7.5600 \text{ mg}\cdot\text{L}^{-1}$, senkyunolide H
578 $3.0208 \text{ mg}\cdot\text{L}^{-1}$, coniferyl ferulate $18.0340 \text{ mg}\cdot\text{L}^{-1}$, senkyunolide A $5.3025 \text{ mg}\cdot\text{L}^{-1}$, n-
579 butylphthalide $199.9788 \text{ mg}\cdot\text{L}^{-1}$, Z-ligustilide $17.9196 \text{ mg}\cdot\text{L}^{-1}$, butylidenephthalide
580 $15.0 \text{ mg}\cdot\text{L}^{-1}$. The working standard solution for calibration curves were prepared by

diluting the mixed standard solution with methanol at different concentrations. The separation was performed on a Thermo Synchronis C₁₈ (2.1 mm × 100 mm, 1.7 μm) column; mobile phase consisted of A (0.1% formic acid) and B (acetonitrile) with a gradient elution: 0~2 min, 5%~10% B; 2~10 min, 10%~40% B; 10~13 min, 40%~40% B; 13~19 min, 40%~50.8% B; 19~20 min, 50.8%~90% B; 20~21 min, 90%~90% B; 21~23 min, 90%~5% B. Column temperature maintained at 35 °C; the flow rate was 0.4 mL·min⁻¹, with detection wave length of 260 nm, 280 nm, 320 nm and injection volume of 2 μL. Linear regression models between sample concentration and peak area were established to obtain linear regression equation and linear ranges of chlorogenic acid, ferulic acid, senkyunolide I, senkyunolide H, coniferyl ferulate, senkyunolide A, n-butylphthalide, Z-ligustilide, and butylidenephthalide respectively.

Statistical analysis

Statistical analysis was performed in R software (V 2.15.3) (R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS 20.0 (IBM Corporation, USA). Analysis of variance (ANOVA) and multiple comparison analysis were used to calculate the mean and standard deviation and perform statistical tests. The differences of parameter variance were estimated by ANOVA and Kruskal Wallis rank sum test based on the distribution of parameter statistics. Tukey's honest significant differences tests and Wilcoxon matched-pairs signed-rank test were used for post-hoc comparison. R (V 3.2.2) performed heat map, PCoA, and hierarchical clustering. The R language performed comparison of microorganisms through the 'adonis' function from R's

‘vegan’ package. The multiple null hypothesis testing of the prior group was performed using the ‘anosim’ or ‘adonis’ function from R’s ‘vegan’ package. According to the method of Franzosa et al [52], we performed clustering of differentially abundant features using a custom approach, then the correlation analysis between the differentially abundant chemical components and the differentially abundant microorganisms was carried out (For all clustering analyses, we applied Spearman’s rank correlation as a similarity measure with a threshold of $r = 0.7$). Unless otherwise stated, the significance level was set at $p < 0.05$.

Supplementary Information

Additional file 1. Table S1.

Additional file 2. Supplementary Figure S1, Table S2. Tentative markers for discriminating *A. sinensis* samples between every two groups. Note: A, GN and YN group; B, GS and YS group; C, GN and GS group; D, YN and YS group.

Additional file 3. Table S3.

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Authors’ contributions

Zhu L and Yan H participated in the design of the study. Zhu L carried out the experiments, data acquisition, and drafted the manuscript. Yan H, Zhou GS, Jiang CH also contributed to figures generation, literature search, and manuscript editing. Liu P, Yu G performed the statistical data analysis. Guo S, Wu QN, Duan JA contributed to the discussion of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The online version contains datasets available at <http://www.ncbi.nlm.nih.gov/bioproject/690235>.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Photographs of *A. sinensis* grown in different soil microenvironments. GN (below, A2) and YN (above, A1); GS (below, B2) and YS (above, B1); GN (below, C2) and GS (above, C1); YN (below, D2) and YS (above, D1). (Notice: GN represent Gansu non-sterilized group; YN represent Yunnan non-sterilized group; GS represent Gansu sterilized group; YS represent Yunnan sterilized group).

Figure 2. The structure of metabolites and microbial communities of *A. sinensis* samples. PCA scores for the comparison of metabolomic profiles between GN, GS, YN, YS group (A); principal coordinates analysis (PCoA) based on unweighted UniFrac (UUF) distance metric (B); and unweighted pair-group method with arithmetic mean (UPGMA) clustering analyses at phylum level of samples from GN, GS, YN, YS, GNck, GSck, YNck, YSck group (n=6) (C).

Figure 3. Potentially mechanistic associations between rhizosphere microbes and metabolites. Hierarchical clustering heatmap shows a consistent clustering pattern within individual groups and a diverse clustering pattern between different groups (A); covariation between microbes and small molecules in *A. sinensis*, specifically those of differentially abundant microbes and metabolites matched against standards between GS and YS group (Spearman's rank correlation with two-tailed nominal P values) (n=6) (B).

Figure 4. The composition of bacteria rhizosphere microbial communities. Shannon index (A); simpson index (B); Phylum distribution (C); heatmap distribution and hierarchical clustering at the genus level (D) of samples from GN, GS, YN, YS, GNck, GSck, YNck, YSck group (n=6).

Figure. 1

Figure. 2

Figure. 3

Figure. 4

Table 1. Root system growth of *A. sinensis* from different soil microenvironments.

Group	Average surviving rate /%	Average dry weight /g	Average root diameter /mm	Average rootlet number	Average root length /cm
GN	0.77±0.09 a	2.94±0.85 a	7.86±1.66 a	6.94±1.64 a	11.14±2.34 a
YN	0.63±0.18 a	0.88±0.25 b	5.12±0.88 b	1.72±0.80 b	7.65±0.70 bc
GS	0.48±0.15 b	1.30±0.62 b	6.14±1.13 b	5.98±2.01 ab	10.36±1.13 c
YS	0.46±0.19 b	0.36±0.05 b	3.01±0.74 c	1.00±0.00 b	9.01±0.79 b

Values are mean ± standard error (n = 6). Values with the same letter are not statistically significantly different, ANOVA, $p < 0.05$.

Table 2. Comparison of the analytes in different groups of *A. sinensis* samples (mg/g).

Group	chlorogeni c acid	ferulic acid	senkyunol ide I	senkyunol ide H	coniferyl ferulate	senkyunol ide A	butylphtha lide	Z- ligustilide	butylidene phthalide
GN	1.89±0.15 c	0.48±0.15 c	0.22±0.09 a	0.08±0.04 a	0.51±0.42 a	0.02±0.03 b	-	6.25±1.34 b	0.35±0.08 a
YN	3.62±0.35 a	0.72±0.11 b	0.18±0.05 a	0.07±0.02 a	0.08±0.04 b	0.96±0.33 a	0.35±0.26 a	9.68±1.49 a	0.23±0.06 b
GS	2.05±0.23 c	0.87±0.07 ab	0.23±0.09 a	0.10±0.04 a	0.04±0.03 b	0.07±0.09 b	-	6.96±1.52 b	0.26±0.09 b
YS	2.88±0.43 b	0.97±0.24 a	0.04±0.00 b	0.01±0.00 b	0.10±0.07 b	0.32±0.25 b	0.03±0.04 b	11.01±2.7 8a	0.07±0.02 c

Values are mean ± standard error (n = 6). Values with the same letter are not statistically significantly different, ANOVA, $p < 0.05$.

- Not detected.

Figures

Figure. 1

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