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FULL PAPER

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Chemical Composition and Antioxidant Activity of *Tuber indicum* from Different Geographical Regions of China

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Tuber indicum, an endemic truffle species in eastern Asian, is an edible mushroom that is both an important export and widely distributed across China. Many existing studies on truffles focus on analyzing their taxonomy, population genetics, volatile organic compounds and artificial cultivation of the truffles, while little information is available about their nutrient composition and pharmacological activity, especially the relationship between chemical composition in ascocarps and their geographic distributions. This study presents a comprehensive investigation of the chemical composition of *T. indicum,* including free sugars, fatty acids, organic acids, phenolic acids, flavonoids, and polysaccharides, and tracks the antioxidant activity of *T. indicum* ascocarps collected from five geographical regions of four provinces in P. R. China: Hebei, Tibet, Yunnan, and Liaoning province. Our results showed that *T. indicum* collected from Qujing, Yunnan province, possessed the highest amount of free sugars (23.67 mg/g dw), total flavonoids (2.31 mg/g dw), total phenolics (4.46 mg/g dw) and the highest DPPH and ABTS radical-scavenging activities. The amount of water-soluble polysaccharides was the highest (115.24 mg/g dw) in ascocarps from Tibet, the total organic acids was the highest (22.073 mg/g dw) in ascocarps from Gongshan, and polyunsaturated fatty acids were most abundant in those from Hebei province. This study reveals that the quantity of chemical compounds in *T. indicum* varies by geographical origin. Detecting differences in chemical composition may provide important data for understanding the relationship between environmental factors and truffle formation, as well as quality evaluation of the commercial species *T. indicum* throughout China.

Keywords: *Tuber indicum*, Chinese black truffles, polysaccharides, radical-scavenging activity, quality evaluation, antioxidants.

Introduction

The genus *Tuber* (Ascomycota, Pezizales), known as the 'true truffle', is a rare and highly valued hypogeous fungi that usually establishes an ectomycorrhizal symbiotic relationship with tree roots of gymnosperms and angiosperms.[1] The ascocarps of truffles can emit a unique and seductive aroma, making truffles highly priced in the international food market.[2,3] In the past several decades, most studies have focused on understanding truffle biology by using 'omics' tools,[4–7] the identification of volatile organic aromas,[8–14] and the

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cultivation of several commercial truffle species such as *T. melanosporum*, *T. magnatum*, *T. aestivum*, and *T. borchii*.[15–17] In China, researchers have focused mostly on the taxonomy, resources and ecology of truffles.[18–20] Research on the nutritional value and active ingredients of truffles is relatively less but has received increasing attention recently.

Truffles are rich in protein, dietary fiber, ash, essential amino acids and other nutrients.[2,3, 21] Nutritional analysis of *T. indicum* shows that protein accounts for 27.9% of dry matter.[22] In addition, studies show that truffles have pharmaceutical relevance including antiviral, antimicrobial, anti-mutagenic, anti-inflammatory, and antioxidant activities including hydroxyl radical-scavenging and ferrous ion-chelating activities.[2,23–25] For example, a new protein-binding polysaccharide from the Chinese truffle (*T. sinense*) has for the first time been extracted and shown to have tumor-inhabiting activity.[26] These biological activities are related to compounds in ascocarps such as polysaccharides, phenolic acids and flavonoids.[2,25,27,28] A higher percentage of oxidation inhibition than that of common food antioxidants (alpha-tocopherol, BHA, BHT, and propyl gallate) has also been detected in two desert truffles: *Terfezia claveryi* and *Picoa juniperi*.[29] These results indicate that more research is necessary in order to discover the broad and untapped potential of truffles as functional foods.

In China, there are more than sixty species of *Tuber*, but only *T. indicum* can be exported as a major commercial truffle because of its high production and established distribution channels, not to mention its close similarity to the renowned European truffle species *T. melanosporum*, which shares its morphological and phylogenetic relationships and volatile organic compound (VOC) composition.[9,30–32]

T. indicum is mainly distributed in southwestern China (i.e. Yunnan and Sichuan province), and has been found in northeastern and northern China as investigations show.[33] Large quantities of *T. indicum* from the wild are exported from China, and unethical, unscientific collection practices are rapidly leading to the endangerment of the endemic species in China. Having no systematic studies on the nutrient composition of the species also limits its effective utilization. Therefore, comprehensive research of the nutrient composition of *T. indicum* and reasonable guidelines for utilization are essential to the conservation of the truffle as a resource.

Historically, the geographical origin of ascocarps, together with species, size, maturity, quality and the quantity harvested during the season have determined the market value of truffles.[34,35] Chemical constituents such as phenolics, flavonoids, and carotenoids in the case of desert truffles, vary according to geographical and climatic differences.[25] Previous research shows that geographical origin could affect the chemical composition of *T. aestivum*[36] and the proportion of volatile organic compounds in *T. magnatum*.[10,37] To the best of our knowledge, few studies are available that address whether geographical origin affects the chemical composition and quality of the Chinese truffle *T. indicum*. This study analyses the chemical composition – including water-soluble polysaccharides, free sugars, total phenolics, flavonoids, organic acids, fatty acids, and antioxidant activities – of *T. indicum* samples originating from five different regions in four provinces of China. The study also provides fundamental data for understanding the role of environment play in the quality of truffles.

Results and Discussion

In this study, we characterized the chemical composition of *T. indicum* from different regions in China, including Gongshan, Qujing, Liaoning, Hebei, and Tibet. The effects of genetic and geographical factors on chemical composition and antioxidant activity were described below.

Phylogenetic Analysis of T. indicum *Collected from Different Regions*

A phylogenetic tree was constructed based on nrDNA-ITS sequences of *T. indicum* samples from different regions in our study using the Maximum Likelihood (ML) method in software MEGA 7.0. *T. borchii* was selected as the outgroup (*Figure 1*). All analyzed *T. indicum* samples were divided into two groups (Clade 1 and 2), and samples collected by ourselves in this study were clustered into the same clade with a high bootstrap value (BP = 99%).

Free Sugars

Research on free sugars, an important part of chemical composition, has been carried out on fruits and mushrooms in the past several decades.[38–41] As shown in *Table 1*, the contents of total free sugars in *T. indicum* ascocarps were not significantly different between those of Qujing, Hebei, and Liaoning truffles, ranging from 22.10 to 23.67 mg/g dw. They were,

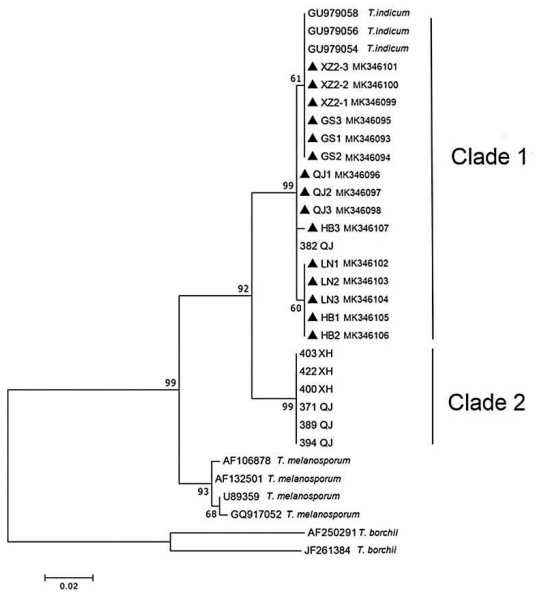

Figure 1. The phylogenetic tree based on ITS sequences of *Tuber indicum* by Maximum Likelihood method. Numbers near branches indicate bootstrap support above 50%. The sequences analyzed at this study are sample numbers with black triangle. Samples from Liaoning province: LN1 (HKSA104215), LN2 (HKSA104216), LN3 (HKSA104366); Samples from Qujing city, Yunnan province: QJ1 (HKAS104373), QJ2 (HKAS104374), QJ3 (HKAS104375); Samples from Gongshan Lisu Autonomous Prefecture, Yunnan province: GS1 (HKAS104370), GS2 (HKAS104371), GS3 (HKAS104372); Samples from Hebei province: HB1 (HKAS96779), HB2 (HKAS96780), HB3 (HKAS96781); Samples from Tibet: XZ1 (HKAS104367), XZ2 (HKAS104368), XZ3 (HKAS104369).

however, about two times lower in truffles from Gongshan and Tibet. Three kinds of free sugars – sucrose, fructose, and glucose – were detected in all specimens, while xylose and rhamnose were detected only in Qujing truffles. The highest levels of sucrose (8.18 mg/g dw), fructose (12.12 mg/g dw), and glucose (13.70 mg/g dw) were found in Hebei, Liaoning, and Qujing truffles, respectively. According to Saltarelli et al.,[42] the monosaccharide contents in *Tuber* range between 25 and 53 mg/g dw, and our results correlate well with those of *T. melanosporum* samples from central Italy.[42]

It has been reported that the sugar content of fruits and plants is affected by geographical factors.[43–45] Given the similarities between all examined samples in respect to maturity and genotype, geographical origin is a reasonable explanation for the variation in sugar types and contents of *T. indicum* from different regions, especially those from within a single province (Qujing and Gongshan).

Fatty Acids

Fatty acid is an essential nutrient component in foods. Unsaturated fatty acids can maintain the structure and function of biofilms, regulate lipid metabolism in the human body, treat and prevent cardiovascular and cerebrovascular diseases; they contain anti-cancer and anti-aging properties and can have important physiological effects, including immune-system regulation.[46–49] Therefore, studying fatty acids in *T. indicum* moves us toward a better understanding of its nutritional and functional properties.

In this study, the contents of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were detected in *T. indicum* from different regions (*Table 2*). The major fatty acids in the tested truffles are PUFA (64.20–85.53%), followed by MUFA (0.77–20.32%) and SFA (10.63–15.93%). Content variability of fatty acids was observed among samples from different regions. The highest PUFA content (85.53%) was found in *T. indicum* from Hebei as a result of the highest percentage of linoleic acid (18:2n6). Lower PUFA contents were detected in truffles from Liaoning (70.94%) then Gongshan (67.77%), followed by Tibet (64.92%) and Qujing (64.20%). Unlike PUFA, the MUFA content was lowest in Hebei truffles (0.77%) due to

Table 1. Content of free sugars detected in ascocarps of *Tuber indicum* from different regions in China.

Region	Sucrose (mg/g dw)	Fructose (mg/g dw)	Glucose (mg/g dw)	Xylose (mg/g dw)	Rhamnose (mg/g dw)	Total sugars (mg/g dw)
Gongshan, Yunnan	1.68 ± 0.16b[a]	3.33 ± 0.16a	6.33 ± 0.48b	nd[b]	nd	11.34 ± 0.62a
Tibet	0.51 ± 0.21a	5.21 ± 1.56a	4.13 ± 0.41a	nd	nd	9.85 ± 2.04a
Hebei	8.18 ± 0.66c	6.96 ± 3.14a	8.04 ± 0.57c	nd	nd	23.18 ± 3.33b
Liaoning	1.06 ± 0.27ab	12.12 ± 4.18b	8.93 ± 0.28d	nd	nd	22.10 ± 4.70b
Qujing, Yunnan	1.13 ± 0.65ab	4.18 ± 1.29a	13.70 ± 0.17e	1.74 ± 0.30	2.93 ± 0.21	23.67 ± 1.82b

[a] Each value is the mean value of three biological replicates (mean ± SD), mean values with different letters (a, b, c, d, e) within a column are significantly different ($P < 0.05$). [b] nd: not detected.

Table 2. Fatty acids detected in *Tuber indicum* from different regions.

Regions	C16:0	C18:0	C18:1	C18:2n6[c]	SFA (% of total FA)	MUFA (% of total FA)	PUFA (% of total FA)
Gongshan, Yunnan	9.89 ± 0.56c[a]	2.61 ± 0.18b	17.94 ± 1.09a	76.52 ± 1.02c	13.85 ± 0.76b	18.38 ± 0.48b	67.77 ± 1.46ab
Tibet	8.40 ± 0.31b	3.84 ± 0.07c	19.70 ± 0.62a	64.61 ± 1.05a	14.76 ± 0.61b	20.32 ± 0.54b	64.92 ± 0.94a
Hebei	8.34 ± 0.24b	1.98 ± 0.17ab	nd[b]	85.25 ± 0.24d	13.71 ± 0.38a	0.77 ± 0.07a	85.53 ± 0.31c
Liaoning	6.13 ± 0.04a	1.61 ± 0.09a	17.87 ± 1.73a	70.72 ± 1.63ab	10.63 ± 0.11b	18.42 ± 1.72b	70.94 ± 1.61b
Qujing, Yunnan	6.49 ± 1.03a	2.52 ± 0.55b	15.52 ± 3.80a	56.05 ± 13.28a	15.93 ± 3.25c	19.87 ± 0.74b	64.20 ± 3.98a

[a] Each value is the mean value of three biological replicates (mean ± SD), mean values with different letters (a, b, c) within a column are significantly different ($P < 0.05$). [b] nd: not detected. [c] palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6).

Table 3. Organic acids composition in *Tuber indicum* from different regions.

Regions	Oxalic acid (mg/g dw)	Succinic acid (mg/g dw)	Lactic acid (mg/g dw)	Formic acid (mg/g dw)	Acetic acid (mg/g dw)	Total organic acids (mg/g dw)
Gongshan, Yunnan	0.008 ± 0.007a[a]	0.192 ± 0.032a	0.237 ± 0.006a	0.289 ± 0.107a	21.347 ± 1.280c	22.073 ± 1.322c
Tibet	nd[b]	nd	nd	0.413 ± 0.093a	7.784 ± 0.830a	8.197 ± 0.901a
Hebei	0.022 ± 0.009ab	0.277 ± 0.043b	0.365 ± 0.046a	0.282 ± 0.154a	15.483 ± 1.084b	16.428 ± 1.059b
Liaoning	0.036 ± 0.012b	nd	nd	0.306 ± 0.030a	16.805 ± 0.388b	17.148 ± 0.386b
Qujing, Yunnan	0.014 ± 0.009a	nd	nd	1.011 ± 0.146b	16.913 ± 1.522b	17.938 ± 1.439b

[a] Each value is the mean value of three biological replicates (mean ± SD), mean values with different letters (a, b, c) within a column are significantly different ($P < 0.05$). [b] nd: not detected.

the lack of oleic acid, but much higher in samples from other regions, ranging from 13.38 to 20.32%. SFA content in Qujing truffles was the highest, while the main SFA, stearic acid (C18:0) and palmitic acid (C16:0), were more abundant in samples from Gongshan and Tibet, respectively.

Previous studies have shown that MUFA content in *T. aestivum*, *T. indium*, *T. himalaynese,* and *T. borchii* range between 65.54 and 75.84% of the total fatty acid content.[50] Similar work has also proved that MUFA is the main component in *Tuber*.[3,51–54]. In this study, *T. indicum* from different regions are rich in MUFA, which provides certain benefits to human health. Differences exist in the content of fatty acids in ascocarps from different regions, which may be one of the criteria for the quality assessment of truffles.

Organic Acids

Organic acids play an important role in maintaining vegetable and fruit quality and sensory characteristics,[55] their particular qualities and concentration may also impact mushroom flavor.[56,57] Organic acids in edible fungi are also closely related to the metabolic processes of synthetic phenols, amino acids, esters, and aromatics.[58] Moreover, organic acids have a lower susceptibility to change during processing and storage than other compounds such as pigments and flavor compounds.[55] Therefore, as an important part of the chemical composition of *T. indicum*, organic acids are also characterized in this study (*Table 3*). The content of total organic acids was highest in Gongshan truffles (22.073 mg/g dw) and lowest in Tibetan truffles (8.197 mg/g dw). Five types of organic acids (oxalic, succinic, lactic, formic, and acetic acid) were detected in Gongshan and Hebei truffles. Oxalic, formic, and acetic acid were detected in Qujing and Liaoning truffles, and two acids (formic acid and acetic acid) were detected in Tibetan truffles. The distribution of different organic acids was uneven among samples from different regions: acetic acid, formic acid, and oxalic acid were significantly higher in ascocarps from Gongshan, Qujing, and Liaoning ($P < 0.05$), respectively.

Previous studies show that certain organic acids such as succinic, malic, tartaric, and citric acids have antioxidant abilities by way of chelating metals and delocalization of the electronic charge coming from free radicals.[59] In our study, succinic acid, although low in content, was detected in specimens from Gongshan and Hebei, which implies that organic acids in *T. indicum* may contribute to its antioxidant activity. To the best of our knowledge, little to no research has

Table 4. Contents of bioactive compounds in ascocarps of *Tuber indicum* from different regions.

Regions	Gallic acid (μg/g dw)	*p*-Coumaric Acid (μg/g dw)	Erucic acid (μg/g dw)	Total phenolic acids (μg/g dw)	Total phenolics (mg/g dw)	Total flavonoids (mg/g dw)	Polysaccharides (mg/g dw)
Gongshan, Yunnan	31.35 ± 8.47a[a]	0.42 ± 0.06a	nd	31.77 ± 8.45a	1.65 ± 0.12a	0.95 ± 0.08b	85.41 ± 5.87ab
Tibet	15.69 ± 2.67a	nd[b]	2.19 ± 0.77b	16.55 ± 2.74a	1.63 ± 0.13a	0.64 ± 0.17a	115.24 ± 12.25c
Hebei	13.77 ± 5.26a	nd	nd	13.76 ± 5.26a	2.24 ± 0.13b	1.34 ± 0.05c	104.73 ± 29.66b
Liaoning	22.94 ± 8.84a	0.59 ± 0.10a	0.87 ± 0.23a	25.72 ± 8.33a	2.00 ± 0.20b	1.84 ± 0.07d	75.79 ± 9.69a
Qujing, Yunnan	228.79 ± 19.99b	1.00 ± 0.21b	nd	229.79 ± 19.90b	4.46 ± 0.10c	2.31 ± 0.21e	70.23 ± 7.31a

[a] Each value is the mean value of three biological replicates (mean ± SD), mean values with different letters (a, b, c, d, e) within a column are significantly different ($P < 0.05$). [b] nd: not detected.

been done on organic acids in the *Tuber* genus. Therefore, more information is necessary to discover the function of organic acids play in *T. indicum*. In addition, the content of low-molecular-weight organic acids in plants and mushrooms differs noticeably according to factors including species, climate, soil, pollution, etc.[56,60] The variations in organic acid content detected in this study leads us to believe that there is a direct correlation between these content levels and the factor of geography.

Bioactive Compounds and Antioxidant Activity of T. indicum

Free radicals are atoms, molecules, or ions with unpaired electrons that are active and highly unstable, and are susceptible to chemical reactions with other molecules. The production and elimination of free radicals are balanced in the case of a normal human metabolism. However, this balance can be offset when certain external factors, such as smoking, environmental pollution, radiation, etc., promote the production of free radicals in the human body.[61,62] This leads to premature effects of aging as well as cancer, atherosclerosis, rheumatoid arthritis, emphysema and other diseases.[63] In truffle ascocarps, flavonoids, polysaccharides, and total free phenolics have been proven to be in direct correlation with antiradical activities by radical-scavenging.[2,24,25,27] This indicates that there is great potential for *Tuber* species to be used as natural antioxidants. In this study, we examined the content of phenolic acids, total phenolics, flavonoids and water-soluble polysaccharides of *T. indicum* from different regions (*Table 4*).

Phenolic Acids

The concentration of total phenolic acids in *T. indicum* from Gongshan, Tibet, Hebei and Liaoning did not vary greatly (ranging from 13.76 and 31.77 μg/g dw), with levels significantly lower than that of Qujing truffles (229.79 μg/g dw). The difference in phenolic acid content between Gongshan and Qujing was particularly remarkable as the two regions belong to the same province. The distribution of three kinds of phenolic acids detected in each regional sample also varied. Gallic acid was present in all samples, *p*-coumaric acid was found in Gongshan, Liaoning, and Qujing truffles, and erucic acid was found only in Tibetan and Liaoning truffles.

In previous *Tuber* studies, gallic acid content has been measured at 0.2 μg/g dw in *T. magnatum* from Serbia[2] and 9.11 μg/g dw and 10.16 μg/g dw in *T. subglobosum* and *T. pseudohimalayense* from Yunnan province, respectively.[3] These were all lower than the gallic acid content found in *T. indicum* in this study.

Phenolics and Flavonoids

The content of total phenolics in *T. indicum* from different regions ranged from 1.43 to 4.46 mg/g dw, and the content of total flavonoids ranged from 0.64 to 2.31 mg/g dw (*Table 4*). The highest content of phenolics and flavonoids was detected in Qujing truffles, which was significantly different from that of other regions ($P < 0.05$). The lowest content was found in samples from Tibet. In the findings similar to ours, the total phenolics in desert truffles (*Tirmania nivea*) from various middle eastern origins ranged between 2.57 and 3.26 mg/g, and the total phenolic and flavonoid contents in the ethanolic crude extract of *T. indicum* were 2.62 and 1.97 mg/g dw, respectively.[24] The content of flavonoids in mushrooms is a con-

Table 5. Antioxidant properties (EC_{50} value) of *Tuber indicum* from different regions and different truffles from references.

	EC_{50} value [mg/mL] Gongshan, Yunnan	Tibet	Hebei	Liaoning	Qujing, Yunnan	Vc
DPPH Scavenging activity	3.50 ± 0.69d[a]	3.87 ± 0.37d	2.64 ± 0.41c	3.09 ± 0.22cd	0.93 ± 0.11b	0.01 ± 0.01a
ABTS Scavenging activity	2.84 ± 0.42d	2.60 ± 0.63d	1.94 ± 0.09c	2.44 ± 0.44d	0.68 ± 0.13b	0.02 ± 0.01a

[a] Each value is the mean value of three biological replicates (mean ± SD), mean values with different letters (a, b, c, d, e) within a row are significantly different ($P < 0.05$).

troversial topic. Some studies have detected various flavonoids in the fruiting bodies of mushrooms,[3,64] while others claim that mushrooms do not contain flavonoid compounds.[65] The literature about flavonoids in edible mushrooms mainly focuses on content determination by aluminum chloride colorimetric analysis and in a few cases on the isolation and structural identification of flavonoids. In our study, aluminum chloride colorimetric analysis method that used normally in foods[66,67] was used to detect the content of flavonoids of *T. indicum* primarily. But this method also has certain drawbacks, such as the influence of various phenols to some extent. In addition, extraction conditions including extraction method, solvent type, solvent concentration, etc. also tend to affect content results.[68] For this reason, further work on the isolation of exact flavonoid compounds and content determination using the chromatography method is necessary. Even so, the results give us guidelines moving forward into a deeper exploration of antioxidant activity and bioactive compounds.

Previous studies show that climate conditions, including average annual precipitation and temperatures, have a significant impact on the chemical composition of *T. aestivum* fruiting bodies.[36] In this study, the influence of climate conditions on the chemical constituents in *T. indicum* fruiting bodies was unclear, because even in cases of similar climate conditions, for instance those of Qujing (with an average annual temperature of 14.7 °C and average annual precipitation of 900–1000 mm) and Gongshan (with an average annual temperature of 13.5–15 °C and average annual precipitation of 1300 mm), there were significant differences in the samples taken. Thus, the factors that affect the chemical composition of *T. indicum* can be determined as multifactorial.

Polysaccharides

Polysaccharides represent the most important bioactive composition in medicinal mushrooms. These present in mushrooms belong to the 1,3-β-glucan family and have anti-tumor activity that operates by enhancing and blocking cellular immune pathways.[69,70] The content of water-soluble polysaccharides in *T. indicum* from different regions ranges from 70.23 to 115.24 mg/g dw (*Table 4*). Tibetan truffles possess the highest polysaccharide content, followed by truffles from Hebei, Gongshan, Liaoning and Qujing.

Antioxidant Activity

T. indicum is described as being an excellent natural source of antioxidants due to its ability to scavenge free radicals.[24,71] The ABTS and DPPH assay methods that use spectrophotometry to determine the antioxidant activity of foods[72,73] were used in this study to examine the antioxidant activities of *T. indicum* methanolic extracts. The results are shown by EC_{50} value, which is the concentration of a compound that will induce half of the maximum action (*Table 5*). Qujing truffles had the highest DPPH and ABTS radical-scavenging activities because of the lowest EC_{50} value of 0.93 mg/mL and 0.68 mg/mL, respectively, both of which were significantly different from the levels in samples from other regions. Hebei and Liaoning truffles had the median level of radical-scavenging activity, while the lowest appeared in Gongshan and Tibetan truffles. Note that the EC_{50} value of ABTS radical-scavenging activity was lower than that of DPPH in this study showed *T. indicum* has greater ABTS radical-scavenging activity. In this study, ascorbic acid (Vc) was selected as a positive control. The results show that Vc possessed stronger DPPH and ABTS free-radical-scavenging abilities than *T. indicum*. The radical-scavenging activity of truffles has different outcomes when different solvents are selected.[2,71,74] In a previous study, the DPPH scavenging activity of *T. indicum* collected from Yunnan (whose EC_{50} value ranges between 1.19 and 2.87 mg/mL) was comparable with the results of this study.[75] Both showed activity that is stronger than that of other *Tuber* species such as *T. pseudohimalayense*, *T. liyuanum*, etc.,[3] but weaker than that of *T. aestivum*, *T. magna-*

tum, and desert truffles.[74,76] Thus, different *Tuber* species may possess different antioxidant activities.

Taking into account the Pearson coefficient and significant analysis, the antioxidant activity of *T. indicum* could be correlated with its total content of phenolic acids ($r = 0.905$, $P < 0.05$) and total phenolics ($r = 0.978$, $P < 0.01$). This result was consistent with previous studies that showed phenolic acids and total phenolics possess strong antioxidant potential.[71,75] In contrast, the relationship between flavonoids and water-soluble polysaccharide content and antioxidant activity was not significant. In the past several decades, polysaccharides have been studied for their strong antioxidant activity in truffles such as *T. indicum*, *T. sinense,* and *T. huidongense* from China.[23,28,77] In our study, however, *T. indicum* from Tibet had the highest content of polysaccharides but the lowest radical-scavenging activity. More samples are needed to verify the polysaccharide content of *T. indicum* samples from different regions, and an analysis of monosaccharide composition will also be necessary in order to better explain the correlation between polysaccharide content and antioxidant activity.[23] The antioxidant activity of *T. indicum* may be the product of the co-operation of various antioxidant substances such as tocopherols and β-carotene, both of which are associated with antioxidant action. Therefore, polysaccharides could just play one part of the role of antioxidants in *T. indicum*.[78] The complex interactions between all of the antioxidants in *T. indicum* call for more in-depth research.

The antioxidant compounds in plants and mushrooms have been proved to be associated with seasonal variations, climatic conditions, and geographical origins.[79,80] As our results show, *T. indicum* distributed across different natural habitats contain different levels of bioactive substances, and show variation in their antioxidant activities. Seeing as geographic factors include differences in soil, climate, host plants, etc., a single factor test must be conducted in the future to determine whether the bioactive substance of *T. indicum* is related to a specific environmental factor.

Conclusions

The chemical composition and antioxidant activities of *T. indicum* from various geographical regions have been the focus of this investigation. It appears that no *T. indicum* sample from a specific region has yielded the highest values in all assays characterized, but truffles from Qujing have seen the highest scores in most of the test parameters (total free sugars, glucose, phenolic acids, total phenolics and flavonoids, radical-scavenging activity). Furthermore, there is a significant correlation between phenolic content and radical-scavenging activity of *T. indicum*.

The study initially verified the effects of geographic distribution on chemical constituents of *T. indicum*, and following this, a larger sample size that includes more geographic regions is the next step. However, a clear pattern has emerged, showing that the evaluation of different chemical compounds may provide a theoretical basis for assessing *T. indicum* truffle quality (i.e. gustatory and nutritional values) across China. This comprehensive study of the geographical distribution, chemical composition, and quality assessment of Chinese black truffles may prove to be a useful tool to develop and expand further studies. Further research may be able to effectively promote the protection of the genetic diversity of truffles (via germplasm resources) and upgrade its status in the international market.

Experimental Section

Fungal Samples

The equivalent amounts of fresh, mature ascocarps (the ratio between the number of asci that contained melanized spores and the total number of asci was 70–80%) of *T. indicum* tested in this study were selected from natural habitats in five regions of China from October to November of 2017. Samples (excluding those with signs of pests) were carefully cleaned with sterile water. All the specimens were first macroscopically checked and microscopically identified by the method of Chen et al.,[33] and were sealed in storage bags, stored at −20 °C and analyzed as soon as possible. The dried specimens were deposited with the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Science(HKAS): Linzhi, Tibet (HKAS104367-HKAS104369), Chaoyang, Liaoning province (HKAS104215, HKAS104216, HKAS104366), Zunhua, Hebei province (HKAS96779-HKAS96781), Qujing, Yunnan province HKAS104373-HKAS104375) and Gongshan, Yunnan province (HKAS104370-HKAS104372). Sampling location information is listed in *Figure 2a*. The data regarding average annual temperature and precipitation as follows: *i*) Zayü County (Tibet): average annual precipitation 650 mm; average annual temperature 8.7 °C; *ii*) Pengdang Township (Gongshan): average annual precipitation

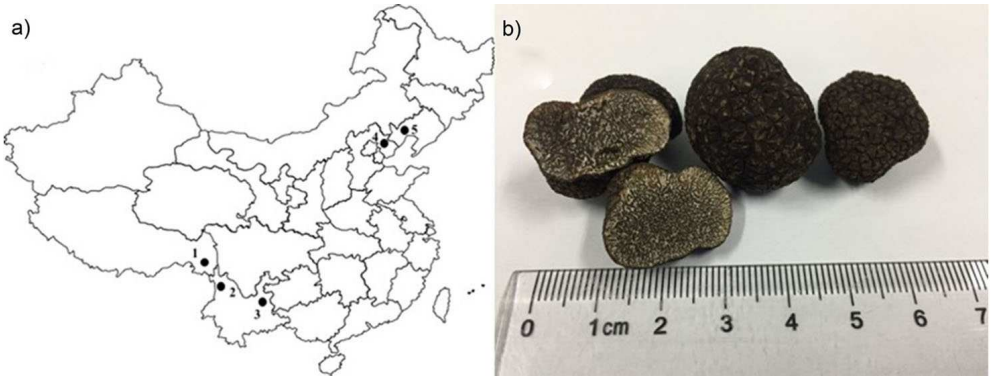

Figure 2. a) Origins of *Tuber indicum* samples in China: 1. Zayü County, Linzhi City, Tibet; 2, Pengdang Township, Gongshan Lisu Autonomous Prefecture, Yunnan Province; 3, Luliang County, Qujing City, Yunnan Province; 4, Zunhua City, Hebei Province; 5, Chaoyang City, Liaoning Province. b) Ascocarps of *T. indicum* in China.

1300 mm; average annual temperature 13.5–15 °C; *iii*) Luliang County (Qujing): average annual precipitation 900–1000 mm; average annual temperature 14.7 °C; *iv*) Zunhua (Heibei): average annual precipitation 724.7 mm; average annual temperature 10.9 °C; *v*) Chaoyang (Liaoning): average annual precipitation 450–580 mm; average annual temperature 5.4–8.7 °C.

Standards and Reagents

Methanol of HPLC grade was purchased from Fisher Scientific (Waltham, MA, USA). The fatty acids methyl ester (FAME) reference standard mixture (standard MIDI No. 1300), ascorbic acid, individual fatty acid isomers, sugars (sucrose, (−)-D-fructose, (+)-D-glucose, (+)-D-xylose, (+)-L-rhamnose), phenolic compounds (gallic acid, *p*-coumaric acid, and erucic acid), organic acids (oxalic acid, succinic acid, lactic acid, acetic acid, and formic acid) and Folin & Ciocalteu's phenol reagent were purchased from Sigma (St. Louis, Mo, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Sinosec Micro Test Instrument Sci & Tech Co., Ltd., Beijing, P. R. China).

DNA Extraction, PCR Amplification and Phylogenetic Analyses

Genomic DNA was extracted from fresh ascocarps by using the E.Z.N.A.® Fungal DNA kit (Omega Bio-Tek, Doraville, GA, USA) following the manufacturer's protocol. The DNA extracted was confirmed by PCR amplification using primer pairs ITS1 and ITS4 for the ITS sequences.[81] The sequences obtained in this study were blasted against the GenBank data, then download the sequences with high similarity for further phylogenetic analyses. The reference sequences used in the phylogenetic analyses are mainly those sequences reported by Roux et al.,[82] Paolocci et al.,[83] Bonito et al.,[84] and Zeppa et al.[85] All acquired sequences have been submitted to GeneBank with the accession numbers from MK346096 to MK346107.

Chemical Parameters Assay

Total Polysaccharides. Total polysaccharides were determined by phenol-sulfuric acid colorimetry.[86] The lyophilized *T. indicum* powder (0.5 g) was extracted with water (10 mL) by boiling for 30 min, and filtered through Whatman No. 4 paper. The residue was then extracted with an additional aqueous solution. The combined extracts were then diluted with water to 25 mL. The extract solution (1 mL) was added to 1 mL of 5% phenol and concentrated sulfuric acid (10 mL). The mixture was shaken and allowed to stand in a boiling water bath for 2 min before cooling. The absorbance was measured at 490 nm. The results were expressed as mg of glucose equivalents (GE) per g of dry weight (dw).

Total Phenolics. The lyophilized *T. indicum* samples (1.0 g) were extracted with 30 mL methanol/water (80:20, v/v) at 25 °C for 30 min, and filtered through Whatman No. 4 paper. Then the residue was extracted with an additional methanol aqueous solution. The combined methanolic extracts were evaporated under

reduced pressure (rotary evaporator Heidolph Laborota 4002-digital; Germany), re-dissolved in initial flow phase in 10 mL, then stored at 4 °C for further analyses (total phenolics, total flavonoids, free sugars, organic acids, phenolic acids and antioxidant assays). Total phenolics were estimated by Folin-Ciocalteu method.[87]

Total Flavonoids. Taking 1 mL methanolic extract and make up to 5 mL with 30% methanol, adding 0.3 mL of 5% sodium nitrite, then 0.3 mL of 10% aluminum nitrate. The mixture was left at room temperature for 60 min, and then 2 mL of 1.0 mol/L sodium hydroxide was added, after which the absorbance of the reaction mixture was measured at 485 nm. The results were expressed as mg of quercetin equivalents (QE) per g of dry weight (dw).

Free Sugars. Free sugars were assessed by an UltiMate 3000 liquid chromatograph (Thermo Fisher Scientific) coupled to a differential refractive index detector (HPLC-RID, Knauer Smartline system 1000; Berlin, Germany). Separation was achieved on a Hi-Plex Pb column (Agilent, 300×7.7 mm) at 65 °C with water in the mobile phase, using a flow rate of 0.6 mL/min. Detection was carried out by a refractive index detector (RID). The results were expressed as mg per g of dry weight (dw).

Organic Acids. Organic acids were determined by HPLC passed through a diode array detector (DAD, VWD-3000) using 210 nm as the preferred wavelength. Separation was achieved using a Rezex ROA-Organic Acid H^+ column (8%, 7.8×300 mm, 8 μm). The mobile phase was 5 mmol/L H_2SO_4 aqueous solution with 0.4 mL/min flow rate at 35 °C. The results were expressed as mg per g of dry weight (dw).

Phenolic Acids. Phenolic acids were detected using HPLC passed through a DAD using 280 nm and 320 nm as the preferred wavelengths. Separation was achieved using a Thermo Acclaim 120 reverse phase C18 column (4.6×250 mm, 5 μm) thermostated at 40 °C. The solvents used were: (*A*) methanol and (*B*) 1.5% acetic acid in water. The elution gradient established was 96% *B* to 89% *B* over 5 min, 89% *B* isocratic over 17 min, 89–96% *B* over 27 min, isocratic 96% *B* for 2 min, 96–74% *B* over 25 min, and re-equilibration of the column, using a flow rate of 1.0 mL/min. The results were expressed as mg per g of dry weight (dw).

Fatty Acids. The lyophilized truffle powder (0.04 g) was extracted with 1 mL of methanol/sodium hydroxide/water 3:10:10 (v/v/v) for 30 min in a bath at 100 °C. After cooling, 2 mL of hydrochloric acid/methanol/water 38:55:27 (v/v/v) was placed into the bath for 10 min at 80 °C; in order to obtain phase separation, 1.25 mL of n-hexane/methyl *tert*-butyl ether 1:1 (v/v/v) was added after ice-cooling, then shaken for 10 min before the bottom phase was discarded. This was again shaken for 5 min after adding 3 mL of 1.2% sodium hydroxide and a couple of drops of saturated sodium chloride solution, and the upper phase was moved into an amber vial and filtered through a 0.2 μm Whatman nylon filter. Fatty acids were determined by gas chromatography with flame ionization detection (GC-FID) with a GC HP6890 instrument equipped with a split/splitless injector, a flame ionization detector (FID) and an Ultra-2 column (25 m×0.2 mm ID×0.33 μm film thickness). The oven temperature guidelines used started with an initial column temperature of 170 °C, followed by a 5 °C/min ramp to 260 °C, then ramp to 310 °C followed by a 40 °C/min and held for 1.5 min. The carrier gas (hydrogen) flow-rate was 0.5 mL/min. Split injection (100:1) was carried out at 250 °C. For each analysis 2 μL of the sample was injected in GC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using HP Chem-Station Ver. A 5.01 and expressed as a relative percentage of each fatty acid.

Antioxidant Activity. Antioxidant activity was evaluated by DPPH$^\bullet$ radical-scavenging activity and ABTS$^\bullet$ radical-scavenging activity. Assays were performed according to the method of Liang.[88] The extract concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated using antioxidant activity graphs. Trolox was used as a standard.

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Author Contribution Statement

J.-M. Li and H.-Q. Liang contributed equally to this work. J. Chen, S.-X. Guo designed the study and revised the manuscript, J.-M. Li and H.-Q. Liang performed the experiments, collected the test data and drafted the manuscript, P. Qiao, K.-M. Su, and P.-G. Liu provided the fungal materials.

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