

Article

Profiling and Pharmacokinetic Studies of Alkaloids in Rats After Oral Administration of *Zanthoxylum nitidum* Decoction by UPLC-Q-TOF-MS/MS and HPLC-MS/MS

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Abstract: *Zanthoxylum nitidum* (Roxb.) DC (Rutaceae), called as “liangmianzhen” in China, is well known for its anti-inflammation and analgesic effect. Alkaloids are its main active constituents. However, little has been known about the absorption of main alkaloids in vivo. In this study, an ultra-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry was employed for identification of absorbed alkaloids in rats after oral administration of *Z. nitidum* decoction. By analyzing the fragmentation patterns, a total of nineteen alkaloids were exactly or tentatively identified in rat plasma after treatment, of which magnoflorine, α -alocryptopine, and skimmianine are dominant. Moreover, a high performance liquid chromatography coupled mass spectrometry method was developed for simultaneous quantification of magnoflorine, α -alocryptopine, and skimmianine, and successfully applied to pharmacokinetic study in rats after oral administration of *Z. nitidum* decoction. The research would contribute to comprehensive understanding of the material basis and function mechanism of *Z. nitidum* decoction.

Keywords: *Zanthoxylum nitidum*; UPLC-Q-TOF-MS/MS; pharmacokinetic study; HPLC-MS/MS; magnoflorine

1. Introduction

Zanthoxylum nitidum (Roxb.) DC (Rutaceae), locally called as “liangmianzhen” belongs to the genus *Zanthoxylum* of family Rutaceae. Its roots are traditionally used for treating various ailments such as toothache, stomachache, fever, rheumatism, paresis, and boils, and can be used as an insecticide [1]. Our previous studies indicated that *Z. nitidum* decoction has anti-contusion injury, analgesic, anti-inflammation, anti-gastritis, gastric mucosal protection, and gastrointestinal movement promotion effects [2,3]. Alkaloids are proved to be the major bioactive components of *Z. nitidum* [4–20]. Recently, alkaloid profiling of *Z. nitidum* by HPLC-Q-TOF-MS had been reported [21,22]. Until now, up to 50 alkaloids were isolated and identified, which mainly belong to aporphine, benzyloisoquinoline, protoberberine, protopine, benzophenanthridine, and quinoline alkaloids (Table S1). Our study gave similar results. On the other hand, it is generally accepted that only the components absorbed in blood

might contribute to the therapeutic effects. Despite of critical pharmacological function of *Z. nitidum* decoction, its absorbed alkaloid profile as well as pharmacokinetic behavior *in vivo* remain unknown. Moreover, in order to understand the material basis and function mechanism of *Z. nitidum* decoction, it is necessary to depict the absorption and pharmacokinetics of the major bioactive alkaloids *in vivo* after oral administration.

Ultra-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) has been widely applied for characterization of the components in Chinese medicines and prescriptions [23,24]. Owing to its high resolution and sensitivity, UPLC-Q-TOF-MS/MS can provide a simple and efficient approach for speculating unknown components besides identifying the known ones [25]. In this paper, the absorbed alkaloids *in vivo* were analyzed by UPLC-Q-TOF-MS/MS. Based on the fragmentation patterns of five authentic alkaloids and those reported in literatures, nineteen alkaloids were exactly identified or tentatively identified in rat plasma after oral administration of *Z. nitidum* decoction. Five of them were reported for the first time in *Z. nitidum*. Meanwhile, pharmacokinetic behavior of *Z. nitidum* decoction was further investigated by HPLC-MS/MS for the first time. The study would provide key information for understanding of the function mechanism of *Z. nitidum* decoction as well as quality control.

2. Results and Discussion

2.1. Identification of Absorbed Alkaloids of *Z. nitidum* Decocotion in Rat Plasma

To identify the absorbed alkaloids *in vivo*, the rat plasma after oral administration of *Z. nitidum* decoction was analyzed using the target (Table S1) and untarget strategy reported by Zhang et al. [26]. As a result, a total of 19 prototype alkaloids were identified, including 2 aporphinoid, 3 protopine, 7 benzophenanthridine, and 7 quinoline alkaloids (Table 1). Among 19 compounds, magnoflorine, α -allocryptopine, nitidine, chelerythrine, and skimmianine were unambiguously characterized by comparison with authentic standards. Other compounds were tentatively deduced based on accurate mass of quasimolecular, MS² spectra and fragmentation pathway, and some isomers were further differentiated by considering relative retention time and molecular polarity. The total ion chromatograms (TICs) of these components are shown in Figure 1 and their chemical structures are shown in Figure 2. The extract ion chromatograms (EICs) and MS² spectra are given in Figure S1.

Table 1. MS data and identification results of the alkaloids in rat plasma after oral administration of *Z. nitidum* decoction.

NO.	RT (min)	Mass Found	Error (ppm)	Selected ion	Formula	MS ² Ions	Identification	Types
1	3.2 *	518.2016	0.3	[M + H] ⁺	C ₂₆ H ₃₂ NO ₁₀	356.1491, 338.1389, 188.0704	glucothalictisine/ glucohunnemannine	protopine
2	4.2	342.1702	−1	[M] ⁺	C ₂₀ H ₂₄ NO ₄	297.1111, 282.0876, 265.0848, 237.0900	magnoflorine	aporphine
3	5.3 *	356.1494	−1.2	[M + H] ⁺	C ₂₀ H ₂₂ NO ₅	338.1389, 275.0698, 206.0809, 188.0809	thalictisine/hunnemannine	protopine
4	8.1	370.1664	0.8	[M + H] ⁺	C ₂₁ H ₂₃ NO ₅	352.1555, 206.0813, 189.0773, 188.0704	α-allocryptopine	protopine
5	8.4 *	260.1284	1.1	[M + H] ⁺	C ₁₅ H ₁₇ NO ₃	242.1180, 188.0709, 176.0706, 134.0609	2,2,6-trimethy-3,4,5,6-tetrahydro- 2H-pyrano[3,2-c]quinoline	quinoline
6	9.8 *	246.0761	0.2	[M + H] ⁺	C ₁₃ H ₁₂ NO ₄	231.0526, 213.0419, 185.0471	4-hydroxy-7,8-dimethoxy- furanquinoline	quinoline
7	9.9	334.1072	−2	[M] ⁺	C ₂₀ H ₁₆ NO ₄	319.0831, 291.0886, 276.0663	isofagaridine	benzophenanthrine
8	11.3	348.1239	0.7	[M] ⁺	C ₂₁ H ₁₈ NO ₄	332.0934, 304.0985, 290.0806	nitidine	benzophenanthrine
9	11.4 *	260.1283	0.9	[M + H] ⁺	C ₁₅ H ₁₇ NO ₃	242.1174, 188.0709, 176.0706, 134.0609	ribalinine	quinoline
10	11.6	348.1231	0.5	[M] ⁺	C ₂₁ H ₁₈ NO ₄	332.0923, 304.0975, 290.0817	chelerythrine	benzophenanthrine
11	12.2	276.0656	0.5	[M + H] ⁺	C ₁₇ H ₉ NO ₃	248.0698, 218.0594, 190.0636	liriodenine	aporphine
12	13.1	246.0759	−0.5	[M + H] ⁺	C ₁₃ H ₁₂ NO ₄	231.0526, 216.0286, 188.0346	haplopine	quinoline
13	15.5	260.0916	0.9	[M + H] ⁺	C ₁₄ H ₁₃ NO ₄	245.0682, 227.0575, 199.0627	skimmianine	quinoline
14	15.8	230.0809	−0.9	[M + H] ⁺	C ₁₃ H ₁₁ NO ₃	230.0809, 215.0809, 186.0539, 172.0573	γ-fagarine	quinoline
15	16.6	200.0703	−1.5	[M + H] ⁺	C ₁₂ H ₉ NO ₂	185.0471, 129.0579, 364.1177, 354.1321,	dictamine	quinoline
16	16.8	382.1287	0.4	[M + H] ⁺	C ₂₁ H ₁₉ NO ₆	349.0947, 323.0913, 292.0724, 348.1232, 333.0983,	isoarnottianamide	benzophenanthrine
17	17.4	366.1340	1	[M + H] ⁺	C ₂₁ H ₁₉ NO ₅	320.0920, 305.0700, 292.0739, 275.0700, 364.1179, 354.1334,	O-demethyl-becconoline	benzophenanthrine
18	17.5	382.1288	0.7	[M + H] ⁺	C ₂₁ H ₁₉ NO ₆	339.1098, 336.1225, 292.0718	arnottianamide	benzophenanthrine
19	19.1	334.1076	0.8	[M + H] ⁺	C ₂₀ H ₁₆ NO ₄	319.0848, 318.0760, 290.0840	Norchelerythrine	benzophenanthrine

RT mean retention time, and * mean that the compound was reported for the first time in *Z. nitidum*.

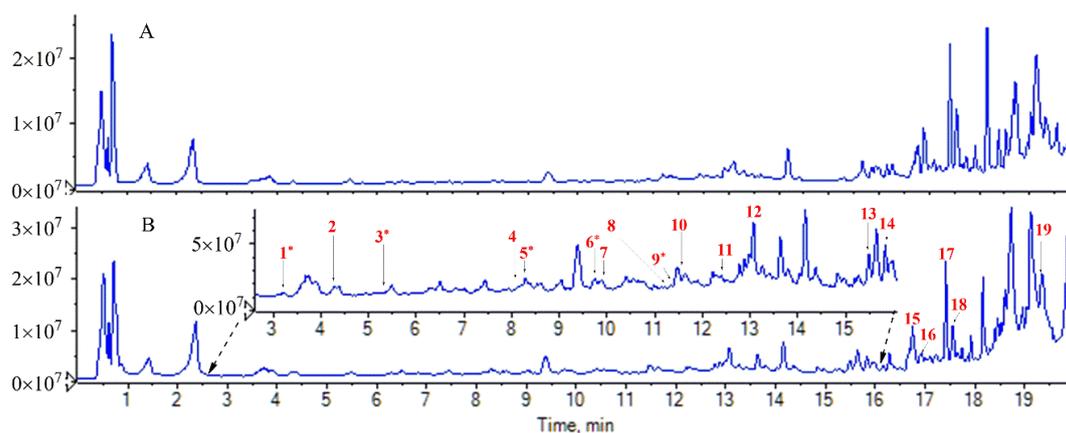
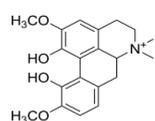
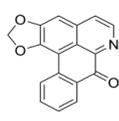


Figure 1. TIC chromatograms. (A) Blank plasma; (B) plasma after oral administration of *Z. nitidum* decoction.

Aporphine

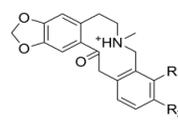


2 magnoflorine



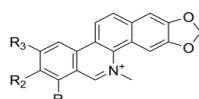
11 liriodenine

Protopine

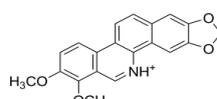


1 glucotalictrisine/glucohunnemannine $R_1=OCH_3, R_2=OGlu, R_1=OGlu, R_2=OCH_3$
 3 thalictisine/hunnemannine $R_1=OCH_3, R_2=OH/R_1=OH, R_2=OCH_3$
 4 α -allocryptopine $R_1=R_2=OCH_3$

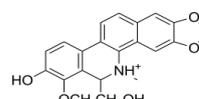
Benzophenanthrine



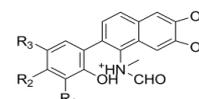
7 isofagaridine $R_1=H, R_2=OH, R_3=OCH_3$
 8 nitidine $R_1=H, R_2=R_3=OCH_3$
 10 chelerythrine $R_1=R_2=OCH_3, R_3=H$



19 norchelerythrine

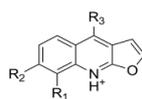


17 O-demethyl-becononine

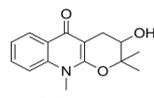


16 isoarnottianamide $R_1=H, R_2=R_3=OCH_3$
 18 arnottianamide $R_1=R_2=OCH_3, R_3=H$

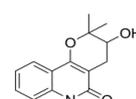
Quinoline



6 4-hydroxy-7,8-dimethoxy-furanquinoline $R_1=R_2=OCH_3, R_3=OH$
 12 haplopine $R_1=OH, R_2=R_3=OCH_3$
 13 skimmianine $R_1=R_2=R_3=OCH_3$
 14 γ -fagarine $R_1=R_3=OCH_3, R_2=H$
 15 dictamine $R_1=R_2=H, R_3=OCH_3$



9 ribalinine



5 2,2,6-trimethyl-3,4,5,6-tetrahydro-2H-pyrano[3,2-c]quinoline

Figure 2. Chemical structure of the alkaloids in rat plasma after oral administration of *Z. nitidum* decoction.

To facilitate alkaloid identification, 5 authentic standards representing 5 known alkaloids in *Z. nitidum*, including one aporphine (magnoflorine), one protopine (α -allocryptopine), two benzophenanthridine (nitidine and chelerythrine), and one quinoine (skimmianine) were selected and analyzed thoroughly to illustrate the proposed fragmentation pathways for references (Figures 3–5). Among them, the fragment pathway of skimmianine is given in our previous published reference [27].

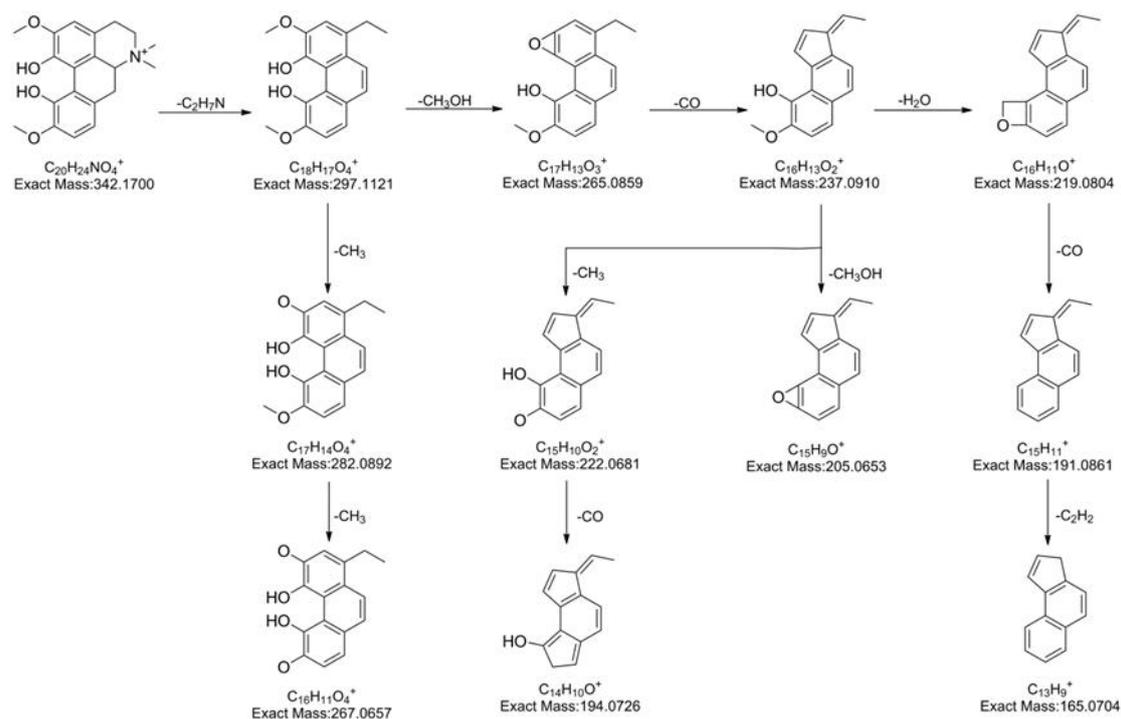


Figure 3. The proposed fragmentation pathway of magnoflorine.

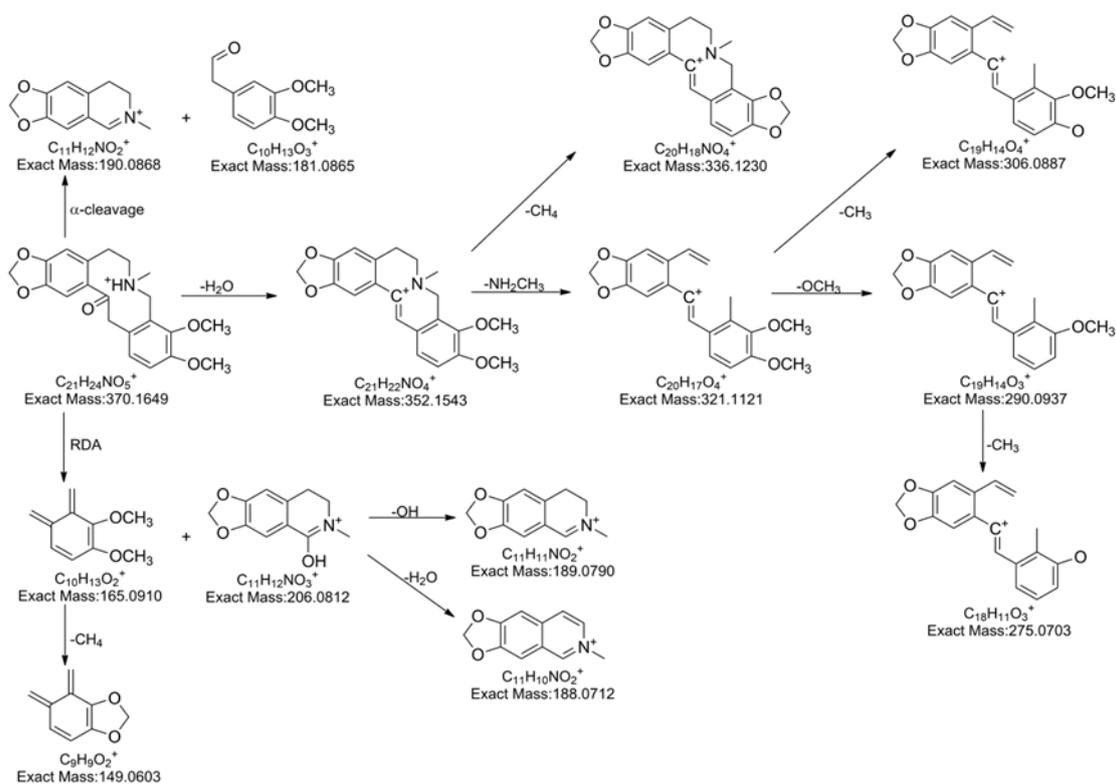


Figure 4. The proposed fragmentation pathway of α -allocryptopine.

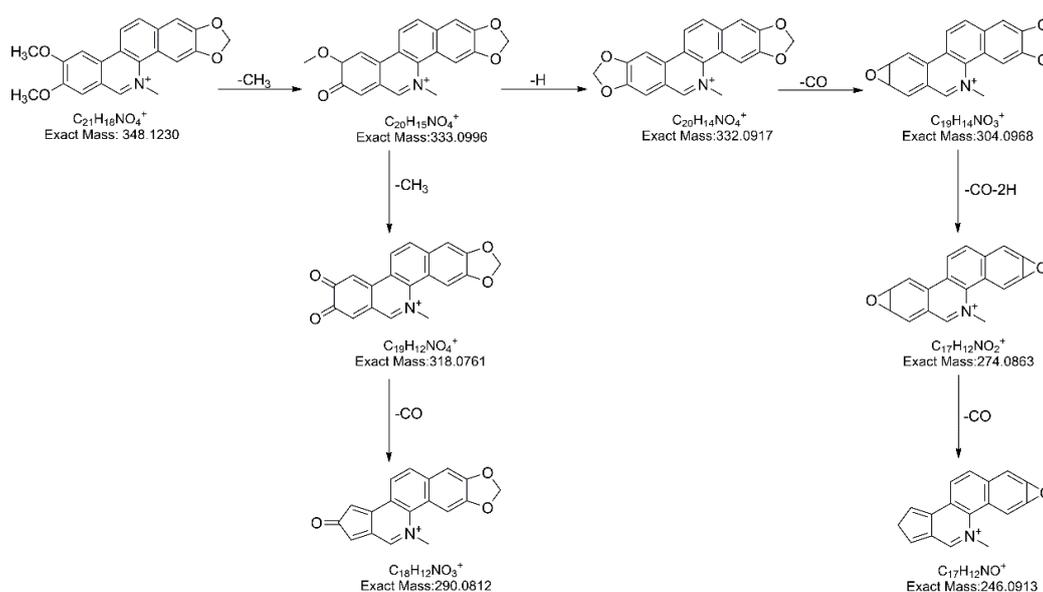


Figure 5. The proposed fragmentation pathway of nitidine.

Magnoflorine was eluted at 4.2 min with the parent ion at m/z 342.1706 ($C_{20}H_{24}NO_4^+$). The fragment ion at m/z 297.1121 ($C_{18}H_{17}O_4^+$) was attributed to the elimination of $(CH_3)_2NH$, which might be an important characteristic of aporphine alkaloid fragmentation pathway [28]. Subsequently, the fragment ion at m/z 265.0859 ($C_{17}H_{13}O_3^+$) was observed as the base peak due to the loss of CH_3OH . Because of the electron-withdraw inductive effect and the minimal energy of ion, the expulsion of CH_3OH could occur from vicinal hydroxyl and methoxy groups on C1 and C2 [29]. The fragment ion at m/z 237.0910 ($C_{16}H_{13}O_2^+$) was produced by the neutral loss of CO from the fragment ion at m/z 265.0859 ($C_{17}H_{13}O_3^+$). The removal of CH_3OH followed by CO in vicinal hydroxyl and methoxy groups is an important fragmentation pathway of aporphine alkaloids. The fragment ion at m/z 237.0910 ($C_{16}H_{13}O_2^+$) further fragmented in three ways. The first way yielded fragment ions at m/z 219.0804 ($C_{16}H_{11}O^+$), 191.0861 ($C_{15}H_{11}^+$), 165.0704 ($C_{13}H_9^+$) after the consecutive loss of H_2O , CO, and C_2H_2 . The second produced fragment ions at m/z 222.0681 ($C_{15}H_{10}O_2^+$), 194.0726 ($C_{14}H_{10}O^+$) by the successive elimination of CH_3 and CO. While the ion at m/z 205.0853 ($C_{15}H_9O^+$) was the result of the loss of CH_3OH through the third way. The fragment ion at m/z 297.1121 ($C_{18}H_{17}O_4^+$) might break up into the ions at m/z 282.0892 ($C_{17}H_{14}O_4^+$), 267.0657 ($C_{16}H_{11}O_4^+$) via the consecutive loss of CH_3 and CH_3 .

α -allocryptopine gave parent ion at m/z 370.1651 ($C_{21}H_{24}NO_5^+$) at 8.1 min. Subsequently, the parent ion fragmented in three main ways. The first way generated fragment ions at m/z 206.0811 ($C_{11}H_{12}NO_3^+$), 165.0916 ($C_{10}H_{13}O_2^+$) by retro-Diels-Alder (RDA) reaction. The ion at m/z 206.0811 ($C_{11}H_{12}NO_3^+$) further fragmented in two ways, generating fragment ions at m/z 189.0780 ($C_{11}H_{11}NO_2^+$) and 188.0704 ($C_{11}H_{10}NO_2^+$). The loss of OH led to the formation of ion at m/z 189.0780 ($C_{11}H_{11}NO_2^+$), while the ion at m/z 188.0704 ($C_{11}H_{10}NO_2^+$) was produced via the loss of H_2O . The ions at m/z 206.0811 ($C_{11}H_{12}NO_3^+$), 189.0780 ($C_{11}H_{11}NO_2^+$) and 188.0704 ($C_{11}H_{10}NO_2^+$) were the dominant peaks in the MS^2 spectrum of α -allocryptopine, indicating that the loss of H_2O or OH following the RDA reaction must be the major fragmentation pathway. Concerning the second fragmentation way, the parent ion might undergo α -cleavage, producing the ions at m/z 181.0865 ($C_{10}H_{13}O_3^+$). Otherwise, the parent ion might break up into the ions at m/z 352.1543 ($C_{21}H_{22}NO_4^+$), 321.1124 ($C_{20}H_{17}O_4^+$), 290.0937 ($C_{19}H_{14}O_3^+$) and 275.0703 ($C_{18}H_{11}O_3^+$) after the consecutive loss of H_2O , NH_2CH_3 , OCH_3 and CH_3 . The minor peaks at m/z 336.1230 ($C_{20}H_{18}NO_4^+$) and 306.094 ($C_{19}H_{14}O_4^+$) might be produced by the loss of CH_4 from the ion at m/z 352.1543 ($C_{21}H_{22}NO_4^+$) and by the loss of CH_3 from the ion at m/z 321.1124 ($C_{20}H_{17}O_4^+$), respectively. The loss of H_2O or OH following the RDA reaction, as well as

the loss of H₂O, were notable fragmentation pattern of α -allocryptopine. This notable fragmentation pattern is consistent with the published fragmentation of protopine-type alkaloids [30].

Two isomers, nitidine and chelerythrine at retention time 11.3 and 11.6 min, respectively, both showed parent ion at m/z 348.1236 (C₂₁H₁₈NO₄⁺) and similar fragment pathway. Due to its vicinal methoxy groups, the fragment ion at m/z 333.0996 (C₂₀H₁₅NO₄⁺), 332.0917 (C₂₀H₁₄NO₄⁺), 304.0968 (C₁₉H₁₄NO₃⁺), 274.0863 (C₁₈H₁₂NO₂⁺) and 246.0913 (C₁₇H₁₂NO⁺) were observed as a result of the elimination of CH₃, H, CO, CH₂O and CO. Another route produced 333.0996 (C₂₀H₁₅NO₄⁺), 318.0761 (C₁₉H₁₂NO₄⁺), and 290.0812 (C₁₈H₁₂NO₃⁺) via the successive loss CH₃, CH₃ and CO.

Thus, compounds (**2**, **4**, **8**, **10**, **13**) were unambiguously identified as magnoflorine, α -allocryptopine, nitidine, chelerythrine, and skimmianine, respectively.

Compound **11** displayed the protonated molecule ion at m/z 276.0655 (C₁₇H₁₀NO₃⁺) at retention time of 12.2 min. It produced the fragment ions at m/z 248.0706 (C₁₆H₁₀NO₂⁺), 218.0600 (C₁₅H₈NO⁺) and 190.0651 (C₁₄H₈N⁺) via the successive loss of CO, CH₂O and CO. By comparing with the data in literature, compounds **11** was tentatively identified as liriodenine [5].

Besides α -allocryptopine, two other protopine alkaloids (**1**, **3**) were observed. Compounds **1** and **3** were reported for the first time in *Z. nitidum*. These two compounds were following notable fragmentation pathway: the loss of H₂O or OH following the RDA reaction and the loss of H₂O from parent ion.

Compound **3** exhibited the protonated molecule ion at m/z 356.1498 (C₂₀H₂₂NO₅⁺) at retention time of 5.3 min, 14 u less than that of α -allocryptopine. The fragment ions at m/z 206.0811 (C₁₁H₁₂NO₃⁺), 189.0780 (C₁₁H₁₁NO₂⁺) and 188.0704 (C₁₁H₁₀NO₂⁺) resulted from the characteristic losses of H₂O or OH following the RDA reaction. The fragment ion at m/z 338.1389 (C₂₀H₂₀NO₄⁺) was the result of the loss of H₂O. Compound **3** was identified as hunnemannine or thalictisine [31]. Compound **1** eluted at 3.2 min, had molecular formula (C₂₆H₃₂NO₁₀⁺), 162 u more than that of compound **3**. The main fragment ions were identical to those of compound **3**. Compound **1** was speculated to be glucothalictisine or glucohunnemannine.

In addition to nitidine and chelerythrine, five benzophenanthridine alkaloids (**7**, **16**, **17**, **18**, **19**) were observed. Benzophenanthridine alkaloids strictly keep their nitrogen inside the highly aromatic ring during fragmentation. The fragment ions were mainly produced by the peripheral loss from methoxyl, methylenedioxy and N-CH₃ groups and so on.

Compound **7** exhibited molecule ion at m/z 334.1079 (C₂₀H₁₆NO₄⁺) at retention times of 9.9 min. It produced the fragment ions at m/z 319.0839 (C₁₉H₁₃NO₄⁺), 291.0890 (C₁₈H₁₃NO₃⁺) and 276.0655 (C₁₇H₁₀NO₃⁺) via the successive loss of CH₃, CO and CH₃. Compound **7** was identified as isofagaridine after compared with data in the literature [14].

Eluted at 19.1 min, compound **19** showed the protonated molecular ion at m/z 334.1074 (C₂₀H₁₆NO₄⁺). It produced the fragment ions at 319.0839 (C₁₉H₁₃NO₄⁺), 318.0761 (C₁₉H₁₂NO₄⁺) and 290.0812 (C₁₈H₁₂NO₃⁺) due to the successive loss of CH₃, H and CO, suggesting that compound **19** contained vicinal methoxy groups. Compound **19** was identified as norchelerythrine after compared with the literature [6].

Compound **17** was eluted at 17.4 min, and displayed the protonated molecule ion at m/z 366.1336 (C₂₁H₂₀NO₅⁺). It produced the fragment ions at m/z 348.1220 (C₂₁H₁₈NO₄⁺), 320.0917 (C₁₉H₁₄NO₄⁺) via the successive loss of H₂O and C₂H₄ on C8 and N, respectively. Compound **17** was identified as 10-O-demethyboconoline [17].

Compounds **16** and **18** were deduced to be isoarnottianamide and arnottianamide [5]. These two compounds were derivatives of benzophenanthridine containing N-methylformamide group, whose mass spectrum a minor peak corresponding to the loss of HCONHCH₃ was observed. Compounds **16** and **18** displayed the same protonated molecule ion at m/z 382.1291 (C₂₁H₂₀NO₆⁺) at retention times of 16.8 and 17.5 min, respectively. Both of them produced the fragment ions at 364.1185 (C₂₀H₂₀NO₅⁺) and 323.0913 (C₁₈H₁₂NO₃⁺) resulted from the loss of H₂O and HCONHCH₃, respectively.

Besides skimmianine, six quinoline alkaloids (**5**, **6**, **9**, **12**, **14**, **15**) were detected. Compounds **5**, **6**, and **9** were reported for the first time in *Z. nitidum*.

Compounds (**6**, **12**, **14**, **15**) were furanquinoline alkanoids. For furanquinoline compounds containing vicinal methoxy groups at C7 and C8, the successive loss of H₂O following CH₃ was a typical characteristic [27]. The main fragment pattern of the other furanquinoline alkanoids was the loss of CH₃, CO, and CO, the final loss of CO might come from phenolic hydroxyl or furan ring [32]. Compounds **6** and **12** displayed the same protonated molecule ion at m/z 246.0761 (C₁₃H₁₂NO₄⁺) at retention times of 9.8 and 13.1 min, respectively. Compound **6** yielded the fragment ions at 231.0531 (C₁₂H₉NO₄⁺), 213.0419 (C₁₂H₇NO₃⁺) and 185.0470 (C₁₁H₇NO₂⁺) via the consecutive loss of CH₃, H₂O and CO. The fragmentation pathway was like that of skimmianine, suggesting the existence of adjacent methoxy groups at C7 and C8. Compound **6** was identified as 4-hydroxy-7, 8-dimethoxyl-furanquinoline [27]. Compound **12** generated the fragment ions at 231.0531(C₁₂H₉NO₄⁺), 216.0289 (C₁₁H₆NO₄⁺), and 188.0327 (C₁₀H₆NO₃⁺) due to the consecutive losses of CH₃, CH₃, and CO. The fragmentation pathway showed that compound **12** do not possess adjacent methoxy groups at C7 and C8. So, it might contain C7-OH or C8-OH. Because C7-OH is less polar than C8-OH, isomer with C7-OH must be eluted later. Comparing the retention time of two isomers with our previous published reference [27], compound **12** was speculated as the isomer with C7-OH. Thus, compound **12** was identified as haplopine [7].

Compound **14** displayed the protonated molecule ion at m/z 230.0817 (C₁₃H₁₂NO₃⁺) at retention times of 15.8 min. The subsequent fragment ions at m/z 215.0575 (C₁₂H₉NO₃⁺), 200.0341 (C₁₁H₆NO₃⁺), 172.0391 (C₁₀H₆NO₂⁺), 144.0403 (C₉H₆NO⁺), and 116.0499 (C₈H₆N⁺) were observed as a result of the losses of CH₃, CH₃, CO, CO and CO. Compound **14** was identified as γ -fagarine [16].

Compound **15** yielded the protonated molecule ion at m/z 200.0706 (C₁₂H₁₀NO₂⁺) at retention times of 16.6 min. The fragment ions at m/z 185.0471 (C₁₁H₇NO₂⁺), 157.0522 (C₁₀H₇NO⁺) and 129.0573 (C₉H₇N⁺) resulted from the consecutive losses of CH₃, CH₃ and CO. Compound **15** was tentatively identified as dictamine [9].

Compounds **5** and **9** eluted at 8.4 and 11.4 min, respectively, must contain dihydropyran ring with hydroxyl. Both shared the same protonated molecule ion at m/z 260.1287 (C₁₅H₁₈NO₃⁺). These two compounds all produced the fragment ions corresponding to the loss of H₂O and C₄H₈O. The loss of C₄H₈O were produced by RDA reaction of dihydropyran ring. Therefore, compounds **5** and **9** were identified as 3-hydroxy, 2, 2, 6-trimethyl-3, 4, 5, 6-tetrahydro-2H-pyrano [3, 2-c] quinoline 5-one and ribalinine, which had been reported to be isolated from *Skimmia laureola* Hook, a plant of family Rutaceae [33].

2.2. Quantitative Method Validation

After oral administration of *Z. nitidum* decoction, a total of 19 prototype alkaloids were identified. Considering the quality control component of *Z. nitidum* suggested by Chinese Pharmacopoeia 2015, content in *Z. nitidum*, plasma exposure level and the availability of reference standard, magnoflorine, α -allocryptopine, nitidine, chelerythrine, and skimmianine were selected to perform pharmacokinetic experiments by HPLC-MS/MS. However, the results of the preliminary experiment showed that nitidine and chelerythrine had poor absorption, as reported in the literatures [34,35]. Moreover, obvious interference was observed from endogenous material at the retention times of nitidine and chelerythrine during the chromatographic separation, despite various sample preparation methods were applied. Finally, magnoflorine, α -allocryptopine, and skimmianine were selected for the further pharmacokinetic study. The corresponding quantification method using HPLC-MS/MS were developed.

The typical chromatograms of blank plasma, blank plasma spiked with three analytes and internal standard (IS), and plasma after oral administration of *Z. nitidum* decoction were shown in Figure 6. No obvious interference was observed from endogenous material at the retention times of analytes and IS. As shown in Table 2, magnoflorine, α -allocryptopine, and skimmianine showed good

linearity ($r > 0.999$) over the linear range. The lower limit of quantification (LLOQ) of magnoflorine, α -allocryptopine and skimmianine were 2, 2, and 0.5 ng/mL, respectively. The intra- and inter-day precision and accuracy were summarized in Table 3. All analytes displayed relative standard deviation (RSD%) below 11.23% and relative error (RE%) ranged from 8.05% to 11.23%, which were within the acceptable criteria. The extraction recovery of magnoflorine, α -allocryptopine and skimmianine were in the range of 89.87–98.32% and IS was 93.90%. The matrix effects of three analytes were in the range of 92.73–108.46% and IS was 93.88%. The stability of analytes under four storage conditions were assessed and the results were listed in Table 4. All analytes exhibited RSD% below 11.32% and RE% ranged from 10.00% to 12.53%, indicating that the analytes were stable. In conclusion, the developed method was validated and satisfactory for pharmacokinetic study.

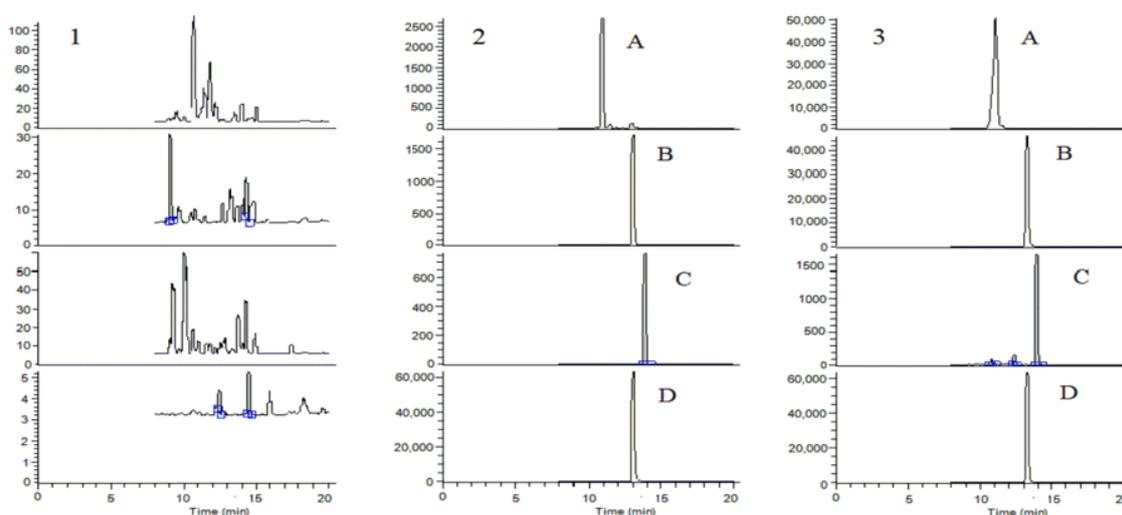


Figure 6. The typical SRM chromatograms. 1. Blank plasma; 2. Blank plasma spiked with three analytes at LLOQ level and IS; 3. Plasma collected at 1 h after oral administration of *Z. nitidum* decoction. A. Magnoflorine, B. α -allocryptopine, C. Skimmianine and D. internal standard (IS).

Table 2. Regression equation, correlation coefficients, linear range and lower limit of quantification (LLOQ) of analytes.

Analyte	Linear Regression Equation	r	Linear Range (ng/mL)	LLOQ		
				Concentration (ng/mL)	RSD (%)	RE (%)
magnoflorine	$y = 0.011x + 0.018$	0.9990	2–200	2	8.72	−6.88
α -allocryptopine	$y = 0.012x + 0.010$	0.9995	2–200	2	13.04	−7.25
skimmianine	$y = 0.014x - 0.010$	0.9996	0.5–50	0.5	14.81	5.63

Table 3. Precision, accuracy, extraction recovery and matrix effect of analytes in rat plasma ($n = 6$).

Analyte	Concentration (ng/mL)	Intraday		Interday		Extraction Recovery (Mean \pm SD, %)	Matrix Effect (Mean \pm SD, %)	Related Matrix Effect (RSD, %)
		RSD (%)	RE (%)	RSD (%)	RE (%)			
magnoflorine	5	4.22	−8.05	10.0	11.03	89.87 \pm 7.75	108.46 \pm 9.4	6.51
	50	2.89	5.62	8.12	−3.45	90.52 \pm 3.16	98.91 \pm 5.59	4.76
	160	6.78	3.16	5.26	6.64	95.57 \pm 3.82	94.47 \pm 7.18	5.43
α -allocryptopine	5	1.83	9.33	3.96	7.56	92.68 \pm 5.38	102.38 \pm 8.43	8.37
	50	3.75	−3.87	5.77	−1.57	96.08 \pm 6.77	99.32 \pm 4.35	7.12
	160	2.23	1.82	7.02	−8.02	94.30 \pm 4.16	93.82 \pm 5.63	5.56
skimmianine	1	2.62	4.41	4.01	6.33	93.41 \pm 4.41	101.10 \pm 6.42	6.39
	10	2.84	6.51	11.23	−7.75	98.32 \pm 6.47	99.39 \pm 5.84	8.41
	32	3.56	4.97	2.65	4.39	94.06 \pm 3.56	92.73 \pm 5.52	5.17

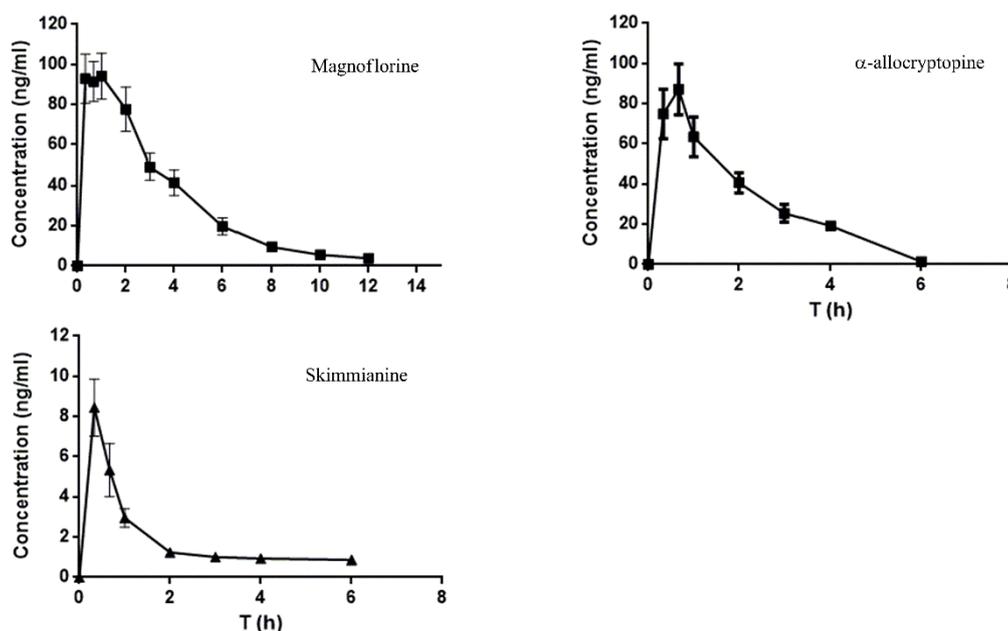
SD: standard deviation.

Table 4. Stability of analytes in rat plasma ($n = 6$).

Analyte	Concentration (ng/mL)	Three Freeze-Thaw Cycle		8 h at Room Temperature		24 h at 4 °C		40 Days at = −80 °C	
		RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
magnoflorine	5	7.53	−10.00	8.25	−5.14	4.46	−5.59	6.43	10.17
	50	4.58	11.03	3.26	6.87	3.81	9.24	3.68	−8.92
	160	5.54	9.16	1.53	5.74	9.95	4.61	4.15	5.74
α -alloclptopine	5	8.61	8.97	6.55	12.53	2.07	2.99	5.88	8.59
	50	6.62	−5.58	3.68	−7.13	6.14	−6.21	3.71	−4.82
	160	1.19	4.24	1.59	3.27	1.26	7.75	2.73	−6.91
skimmianine	1	9.22	10.43	11.06	−9.41	8.18	9.47	8.57	9.69
	10	8.51	−5.95	10.44	6.62	11.32	−4.39	9.92	−6.48
	32	7.13	−9.01	8.43	9.51	4.31	−1.09	8.01	−4.07

2.3. Pharmacokinetic Study

The validated HPLC-MS/MS method was successfully applied for the pharmacokinetic study of magnoflorine, α -alloclptopine, and skimmianine in rat plasma after oral administration of *Z. nitidum* decoction. The plasma concentration-time curve was shown in Figure 7. The main pharmacokinetic parameters were processed by Drug and Statistics (DAS) 2.0 software and listed in Table 5.

**Figure 7.** Plasma concentration-time curves of analytes.**Table 5.** Pharmacokinetic parameters of analytes in rats after oral administration.

Parameters	Analytes (Mean \pm SD, $n = 6$)		
	Magnoflorine	α -ALloclptopine	Skimmianine
C _{max} (ng/mL)	112.69 \pm 18.79	100.28 \pm 49.91	8.91 \pm 1.89
T _{max} (h)	1.05 \pm 0.71	0.47 \pm 0.13	0.38 \pm 0.12
T _{1/2} (h)	3.24 \pm 1.31	0.78 \pm 0.17	5.99 \pm 1.62
AUC _{0-t} (h*ng/mL)	408.13 \pm 91.34	180.361 \pm 96.32	11.08 \pm 2.02
AUC _{0-∞} (h*ng/mL)	437.99 \pm 106.29	186.41 \pm 98.68	17.05 \pm 6.32

AUC: area under curve.

As shown in Figure 7, the three components were absorbed rapidly after oral administration, with T_{max} ranged from 0.38 to 1.05 h. The C_{max} of magnoflorine and α -alloclptopine were about

ten times higher than that of skimmianine. Combined with their contents in *Z. nitidum* decoction (6.7, 1.1 and 0.4 mg/mL), the absorption rate of α -allocryptopine might be the highest of these three alkaloids. The $T_{1/2}$ of magnoflorine, α -allocryptopine and skimmianine were 3.24 ± 1.31 , 0.78 ± 0.17 and 5.99 ± 1.62 h, respectively. The total exposure area under curve (AUC)_{0-∞} of magnoflorine was the largest of the three components. The relatively higher plasma concentration and AUC_{0-∞} indicated that magnoflorine and α -allocryptopine might have favorable drug-like properties.

Magnoflorine has been reported for its diverse pharmacological properties, such as anti-inflammatory, anti-bacteria and immunomodulatory effects [36–39]. Some of these properties might contributed to anti-inflammation and analgesic effect of *Z. nitidum* decoction in clinic use. Limited information is known about pharmacological properties of α -allocryptopine. Available studies indicate that α -allocryptopine possesses antiarrhythmic effects [40–42]. Skimmianine has been reported to possess anti-inflammatory and non-narcotic analgesic effects etc. [43–45], but its plasma concentration and AUC_{0-∞} was relatively lower in this study. Whether there exist synergistic action of them needs further investigation.

As for nitidine, due to poor absorption, its functional mechanism in vivo needs further study.

3. Experimental

3.1. Chemical, Reagents and Materials

The roots of *Z. nitidum* were collected from Guangdong, China, and authenticated by RT Zhan. Magnoflorine (No. 130611, purity 98.0%) and α -allocryptopine (No.150510, purity 98.0%) were purchased from Sichuan Victory Bio-Technology Ltd. (Chengdu, China) Co., nitidine chloride (No.110848, purity 98.0%) and chelerythrine (No.110718, purity 98.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Product (Beijing, China), skimmianine (No.160106, purity 98.0%) was purchased from Shanghai yuanmu Bio-Technology Ltd. (Shanghai, China) Co. Acetonitrile and formic acid were of HPLC grade agents and obtained from Merck (Darmstadt, Germany). All other agents were of analytical grade and purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Triple deionized water was prepared using a Milli-Q system (Millipore, Billerica, MA, USA). The HyperSep C18 solid-phase extraction (SPE) column (1000 mg, 6 mL) was purchased from Thermo Electron Corporation (Waltham, MA, USA).

3.2. Instrumentation and Analytical Conditions

3.2.1. Qualitative Analysis

The UPLC analysis was performed on a Shimadzu Nexera UHPLC LC-30A system (Shimadzu Corporation, Tokyo, Japan). The separation was executed by an ODS column (Shimadzu, 2.0 mm i.d. \times 100 mm, 1.9 μ m) maintained at 35 °C. The mobile phase consisted of Solvent A (0.1% formic acid solution) and Solvent B (0.1% formic acid acetonitrile). The flow rate was 0.4 mL/min. The gradient program was as follows: 5–20% B at 0–10 min; 20–35% B at 10–15 min; 35–100% B at 15–20 min.

MS spectra were achieved on an AB SCIEX Triple TOF 5600 (AB Sciex Pte. Ltd., Singapore, Singapore) with electrospray ionization (ESI) source in positive mode. The following parameters of mass spectra were used: source temperature at 500 °C; nebulizer and heater gas pressure at 50 psi; curtain gas pressure at 40 psi; ion spray voltage at 5500 V; declustering potential at 100 V, collision energy 10 eV, and mass range 100–800 amu. The collision energy in “product ion” scan was set at 35 v with a collision energy spread of 10 eV. Data acquisition was controlled with AB SCIEX Analyst TF (Version 1.7) software (AB Sciex Pte. Ltd., Singapore, Singapore). Data processing was performed with Peakview (Version 2.0) software (AB Sciex Pte. Ltd., Singapore, Singapore).

3.2.2. Quantitative Analysis

The HPLC analysis performed on a Surveyor plus HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The separation was executed by a Thermo Accucore aq C18 column (100 × 2.1 mm, 2.6 μm) (Thermo Fisher Scientific, Waltham, MA, USA) maintained at 30 °C. The mobile phase consisted of Solvent A (0.1% formic acid solution) and Solvent B (0.1% formic acid acetonitrile). The flow rate was 0.3 mL/min. The gradient program was as follows: 5–10% B at 0–5 min; 10–100% B at 5–15 min; 100–5% B at 15–15.1 min; 5% B at 15–20 min.

MS data were achieved on a triple-quadrupole mass spectrometer: TSQ Quantum Access (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionization (ESI) source in positive mode. The instrument parameters were as follows: spray voltage at 3000 V, sheath gas and auxiliary gas with a flow of 30 and 5 arbitrary units, capillary temperature at 350 °C, collision gas pressure at 1.0 mTorr, skimmer offset at 2 V. The selected reaction monitoring (SRM) transitions used for MS analysis were as follows: m/z 342→297 for magnoflorine, 370→188 for α -allocryptopine, 260→227 for skimmianine, 356→192 for IS; the collision energies at 15 eV for magnoflorine, 30 eV for α -allocryptopine, skimmianine and IS. The data acquisition and processing was performed with Xcalibur 2.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

3.3. Preparation of Samples

3.3.1. Preparation of *Z. nitidum* Decoction

100 g of the roots of *Z. nitidum* were weighed and decocted with 1.2 L of water for 3 h. The filtrate was collected and residue was decocted in 1.2 L of water for 2 h again. Subsequently, the filtrates from each decoction were combined and concentrated to 50 mL. The contents of magnoflorine, α -allocryptopine, nitidine, chelerythrine, and skimmianine in *Z. nitidum* decoction were 6.7, 1.1, 1.8, 3.5, and 0.4 mg/mL, respectively.

3.3.2. Preparation of Plasma Samples

Qualitative Analysis

The 2 mL plasma sample was loaded on a pretreated SPE column which was eluted with 20 mL methanol followed by 20 mL water. After being washed off by 6 mL of water, the cartridge was eluted using 6 mL methanol. The methanol eluting was evaporated to dryness at 35 °C in vacuum using SpeedVac Concentration (Savant SPD 1010, Thermo scientific). The residue was reconstituted in 300 μL acetonitrile and water (50:50, v/v) and centrifuged at 13,000 rpm (15,493 × g) for 15 min.

Quantitative Analysis

100 μL plasma sample, 10 μL IS solution (200 ng/mL) and 300 μL acetonitrile were added to a 1.5 mL eppendorf tube and vortex-mixed for 5 min, then centrifuged at 13,000 (15,493 × g) rpm for 10 min. The supernatant was transferred into another eppendorf tube and evaporated to dryness at 35 °C in vacuum using SpeedVac Concentration ((RVC 2-18, Christ). The residue was reconstituted in 100 μL acetonitrile and water (50:50, v/v) and centrifuged at 13,000 rpm (15,493 × g) for 15 min.

3.3.3. Calibration Samples and Quality Control Samples

The stock solutions of magnoflorine, α -allocryptopine and skimmianine were prepared in acetonitrile at 1.06, 1.02, and 0.98 mg/mL, respectively. A series of working solutions were obtained by diluting with acetonitrile. The IS solution was prepared at a concentration of 200 ng/mL in acetonitrile.

The calibration working solution were prepared by spiking the working solution into blank plasma to obtain concentrations ranges from 2–200 ng/mL for magnoflorine and α -allocryptopine, from 0.5–50 ng/mL for skimmianine. Quality control (QC) samples were obtained at 5, 50, 160 ng/mL for magnoflorine and α -allocryptopine, at 1, 10, 32 ng/mL for skimmianine.

3.4. Method Validation

Validation of the analytical method was assessed on selectivity, linearity, sensitivity, accuracy, precision, recovery, matrix effect, and stability according to Pharmacopoeia of the People's Republic of China 2015 guidelines.

The selectivity was assessed by analyzing the chromatograms of blank plasma of six different rats, a blank plasma with magnoflorine, α -allocryptopine, skimmianine and IS, and a plasma after dose. The linearity was determined by plotting the peak areas ratios (y) of each analyte to IS against the concentrations, and evaluated by least-squares linear regression. The lower limit of quantification (LLOQ) was defined as the lowest concentration point of the calibration curve ($S/N > 10$) with the accuracy within $\pm 20\%$ and precision lower than 20%. The accuracy and precision were evaluated by analyzing the six replicate QC samples on the same day (intra-day) and three consecutive days. The accuracy and precision were expressed as relative error (RE%) and relative standard deviation (RSD%), respectively. The extraction recovery was determined by comparing peak areas of the extracted QC samples with those of post-extracted spiked samples. The matrix effects was measured by calculated the analytes peak area ratios of post-extracted spiked samples to those of pure work solution. The stability was investigated by analyzing samples stability under diverse storage conditions: three freeze-thaw cycles, 8 h at 25 °C, -80 °C for 40 days and in autosampler for 24 h.

3.5. Animal Experiments

Male Sprague–Dawley rats (250 ± 20 g) used in this study were provided by the Experimental Animal Center of Guangzhou University of Chinese Medicine. The laboratory animal license number is SCXK 2013-0020. These animals were maintained in an air-conditioned animal facility at 23 ± 2 °C, with a humidity of $55\% \pm 5\%$ and a 12 h light/dark cycle for 5 days before use. The rats had free access to water and a standard diet. Animal welfare and experimental procedures were strictly in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in China and the related ethical regulations of Guangzhou University of Chinese Medicine.

For profile study, twelve rats were randomly divided into 2 group, blank control and experimental groups. Before administration, the rats were fasted for 12 h but allowed water ad libitum. *Z. nitidum* decoction was orally administered to experimental group at a dose of $15 \text{ mL} \cdot \text{kg}^{-1}$ (15 mL decoction equal to 30 g crude drug) body weight, while distilled water was orally administered to control group. The rats of experimental group were anesthetized at 0.5 h, 1 h, 2 h, 3 h, 4 h, and 6 h after dose, respectively. The blood samples were collected from aorta abdominalis in heparinized tube. All blood samples were then centrifuged at 3500 rpm ($1274 \times g$) for 15 min at 4 °C. Blank plasma samples were prepared following the same procedures. All samples were stored at -80 °C.

For pharmacokinetic study, six rats were orally administered *Z. nitidum* decoction at a dose of $5.4 \text{ mL} \cdot \text{kg}^{-1}$ (5.4 mL decoction equal to 10.8 g crude drug) body weight. The blood samples were collected from orbital vein before dose and 0.17, 0.33, 0.67, 1, 2, 3, 4, 6, 8, 10, 12, and 24 after dose. The blood samples were then centrifuged at 3500 rpm ($1274 \times g$) for 15 min at 4 °C. The samples were stored at -80 °C. Data analysis was performed by Drug and Statistics (DAS) 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

4. Conclusions

In this study, the UPLC-Q-TOF-MS/MS method was used to identify the absorbed alkaloids *in vivo* after oral administration *Z. nitidum* decoction for the first time. The fragmentation pathway of magnoflorine, α -allocryptopine and nitidine was proposed, and a total of 19 alkaloids were exactly or tentatively identified in rat plasma after dose, including 2 aporphinoid, 3 protopine, 7 benzophenanthridine, 7 quinoline alkaloids. Among them, five constituents were reported for the first time in *Z. nitidum*. In addition, a HPLC-MS/MS method was developed to simultaneous determination of three main absorbed components, including magnoflorine, α -allocryptopine, and

skimmianine for the first time. This HPLC-MS/MS method was applied to pharmacokinetic study after oral administration *Z. nitidum* decoction. These results would be helpful for a better understanding about material basis and function mechanism of *Z. nitidum* decoction, and also provided important information for the quality control and further pharmacological study.

Supplementary Materials: The following are available online.

Author Contributions: R.Z., H.X. and W.C. designed the research; M.W., J.Q. and L.O. collected the data; A.H., Y.C., Z.Z., J.L. analyzed and interpreted the data; A.H. and H.X. wrote the paper.

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