JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Functional Structure/Activity Relationships

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b02173 • Publication Date (Web): 31 Jul 2019

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Synthesis and biological evaluation of (+)-usnic acid derivatives as potential anti-*Toxoplasma gondii* agents

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Abstract

Six series of (+)-usnic acid derivatives were synthesized. The IC_{50} values of these compounds were determined in *T. gondii*-infected HeLa cells (μM) and in HeLa cells (μM), and their selectivity indexes (SI) were calculated. *In vitro*, most of the derivatives tested in this study exhibited more anti-*T. gondii* activity than that of the parent compound (+)-usnic acid and the positive control drugs. Among these derivatives, methyl(E)-(1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene) ethyl)phenylalaninate (**D3**) showed the most effective anti-*T. gondii* activity (Selectivity: > 2.77). And compared with the clinically used positive control drugs sulfadiazine (Selectivity: 1.15), pyrimethamine (Selectivity: 0.89), spiramycin (Selectivity: 0.72) and the lead compound (+)-usnic acid (Selectivity: 0.96), **D3** showed better results *in vitro*. Furthermore, **D3** and (E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-(quinolin-6-ylamino)ethylidene)dibenzo[b,d]furan-1,3 (2H,9bH)-dione (**F3**) had more inhibitory effects on *T. gondii* (inhibition rate: 76.0% and 64.6%) *in vivo* than spiramycin (inhibition rate: 55.2%) and in the peritoneal cavity of mice, the number of tachyzoites was significantly reduced ($p < 0.001$) *in vivo*. Additionally, some biochemical parameters were measured, spleen indexes were comprehensively evaluated, and the results indicated that mice treated with both compound **D3** and compound **F3** showed reduced hepatotoxicity and significantly enhanced antioxidative effects compared to the normal group. And granuloma and cyst-formation were effected by the inhibition of compound **D3** and compound **F3** in liver sections. Overall, these results indicated that **D3** and **F3** for use as anti-*T. gondii* agents are promising lead compounds.

Key words: Usnic acid, Derivatives, *Toxoplasma gondii*, Tachyzoite

1. Introduction

Toxoplasma gondii (*T. gondii*) can cause toxoplasmosis to affect the healthy of between humans and animals [1,2]. The life cycle of *T. gondii* is complex, it can be differentiated from a rapid replication form (tachyzoite) to a less metabolically active form (bradyzoite) encased in a tissue cyst. Tachyzoites can spread through the placenta of pregnant women, by eating contaminated water or food by parasitic oocysts transmitted by infected felines, by ingesting uncooked meat with parasitic tissue cysts, thereby establish acute infections in humans and other animals [3-5]. Toxoplasmosis is a zoonosis affecting a third of humankind [6-7]. However, nowadays there is not any reliable drugs to prevent and treat toxoplasmosis due to different biological characteristics, its multifarious pathogenesis, and the complication of the life cycle of *T. gondii*. The current anti-*T. gondii* drugs have many defects, like failure in immuno-compromised individuals, frequent recurrence, high toxicity and killing protozoa and oocysts is not thorough [8,9]. Over the past few years, in the market, the proportion of drugs based on natural products has increased. In this background, natural products have proven useful as core sources of novel compositions. Additionally, The found and development of anti-*T. gondii* drug make us known the function of strong anti-*T. gondii from* natural products and their derivatives. [10,11]. Therefore, the discovery of more effective and less toxic derivatives through structural modification of natural products in the study of anti-*T. gondii* drugs is promising.

Usnic acid (**Figure 1**) is produced by *Alectoria* (Alectoriaceae), *Parmelia* (Parmeliaceae), *Evernia*, *Ramalina* (Ramalinaceae), *Usnea* (Usneaceae), *Cladonia* (Cladoniaceae), and other lichen genera. It is the most well-known lichen secondary metabolite [12]. Many physiological and biological activities can be found in usnic acid, like analgesic and anti-pyretic [13], anti-antimicrobial [14], anti-inflammatory [15], anti-proliferative [16], anti-mitotic [17], anti-parasitic [18]. Usnic acid is present as (+) and (-) enantiomers. Compared with (-) enantiomers, the (+) enantiomeric activity is better, and it produces selective activity against *Staphylococcus mutants* [19]. According to recent research reports, usnic acid has a lower concentration of

inhibitory effects on *Trichomonas vaginalis*, *Leishmania spp*, and pathogenic protozoan in vitro [12]. The excellent inhibitory effect of usnea sodium on *T. gondii* in vitro has been proved by Chinese researchers [20]. But, the toxicity of usnic acid, especially liver failure[21]and cultured mouse hepatocyte necrosis [22], has also been reported. Nevertheless, poor solubility in water [23] and hepatotoxicity [24] limit the application of usnic acid to some extent. In addition, as we all known, there is no research of usnic acid derivatives for anti-*T. gondii.* Therefore, in the present study, we introduced different crucial fragments in (+)-usnic acid in order to obtain compounds with low toxicity and high efficiency against *T. gondii*.

Due to the effective biological and synthetic importance of triazole and its fused phenyl derivatives, its chemical properties have obtained considerable attention. [25-27]. A series of 1,2,3-triazole-conjugated phenyl derivatives promote the latent antiparasitic drugs developing and in the *T. gondii* model, five of these derivatives showed outstanding selectivity *in vitro*, according to Sharling et al. [27]. The above studies show that triazole compounds have latent anti-*T. gondii* activity. Amino acids as the fundamental functional and biological units to play a quite major role in human metabolism and make up proteins. Some of biological functions were found in amino acid derivatives, such as immunomodulatory [28], anti-parasitic [29-31], bactericidal [32], anti-tuberculosis [33], anti-cancer [34,35] and so on. The combine of molecular structure of a drug and an amino acid brings two advantages. For the one hand, the method could increase biological activity and reduce toxic effects on cells [36-40]. For the another hand, the approach should enhance bioavailability of drug due to increased solubility and the active transport [41,42]. In usually, the number of polar groups for a compound would affect solubility, such as amide groups, amino, carboxyl, and hydroxyl. The biological activity of a compound may change as its solubility changes. In addition, an amide group can be used as a hydrogen donor, decreasing their sensibility to enzymatic hydrolysis, and it can also improve the stability of compounds. Similarly, a drug with a phosphonate introduced can display enhanced solubility and drug-like properties by regulating the

distribution coefficient [43,44]. In addition, pyrazole and quinolines have been reported to possess anti-*T. gondii* activity [45,46].

The idea of designing and synthesizing six series of (+)-usnic acid derivatives was came out of the combination principles of drugs and the findings above. The IC_{50} values of the target compounds were determined in *T. gondii*-infected HeLa cells (μM) and HeLa cells (μM), and their selectivity index (SI) was calculated. It is generally reflected the efficacy of a compound against *T. gondii* and toxicity for host cells. [47]. Our synthesis and screening goal was to obtain compounds with greater selectivity than the leading compound. Because testing *in vivo* is an important aspect in assessing activity, the two most active anti-*T. gondii* derivatives were selected for animal studies.

2. **Experimental Section**

2.1. General procedures

The chemical reactions involved in this experiment were examined using thin-layer chromatography (TLC). The determination of the melting point using an open capillary tube and temperature was uncorrected. Deuterated chloroform ($CDCl_3$) was used as a solvent for the determination of ^{13}C-NMR and ^{1}H-NMR spectra on BRUKER AV-300 (Bruker, Switzerland) with ppm as a chemical shift unit. Thermo Scientific LTQ Orbitrap XL in ESI mode was used as the measurement of high-resolution mass spectra. All analytical grade solvent and chemicals were used directly without further processing and were obtained commerically.

2.2. Representative Intermediates ^{1}H-NMR Spectra

*2.2.1. (1-phenyl-1H-1,2,3-triazol-4-yl)methanamine (**1a**)*

^{1}H-NMR ($CDCl_3$, 300MHz, ppm): δ 7.91 (s, 1H, -N-CH=), 7.79-7.72 (m, 2H, Ar-OH), 7.59-7.42 (m, 3H, Ar-OH), 4.10 (s, 2H, =C-CH_2-), 1.71 (s, 2H, -CH_2-$\underline{NH_2}$).

*2.2.2. Diethyl (amino(phenyl)methyl)phosphonate (2**a**)*

^{1}H-NMR ($CDCl_3$, 300MHz, ppm): δ 7.51-7.30 (m, 5H, Ar-H), 4.27 (d, *J* = 17.1 Hz, 1H, Ar-CH-), 4.15-3.81 (m, 4H, -O-CH_2-), 1.88 (s, 2H, -CH-NH_2), 1.24 (dt, 6H, *J* = 29.1 Hz, *J* = 7.2 Hz, -CH_2-CH_3).

*2.3. Procedure for the preparation of compound **A1-A8***

The (+)-usnic acid (172 mg, 0.5 mmol), and different (1-phenyl-1*H*-1,2,3-triazol-4-yl)methanamines (0.6 mmol) were stirred in 10 mL absolute ethanol at 78℃ for 2-3 h. The crude product was obtained under reduced pressure by evaporation of the solvent after confirming completion of the reaction by TLC. The silica gel column chromatography was used for products purification (silica gel, methanol/dichloromethane, 1:200-1:100 v/v). The characteristics of the spectroscopic and physical data were as shown below.

2.3.1.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-(((1-phenyl-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)dibenzo[b,d]furan-1,3(2H,9bH)-dione (**A1**)*

Yellow crystal. M.p. 129-130℃. Yield: 62%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.98 (brs, 1H, Ar-OH), 13.58 (s, 1H, -C-NH-CH_2-), 11.86 (brs, 1H, Ar-OH), 8.02 (s, 1H, triazole-H), 7.74 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.58-7.46 (m, 3H, Ar-H), 5.80 (s, 1H, O=C-CH=), 4.92-4.90 (m, 2H, -NH-CH_2-), 2.80 (s, 3H, CH_3-C=O), 2.67 (s, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-CH_3), 1.71 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.63, 198.47, 175.40 , 174.15, 163.44, 158.12, 155.76, 142.85, 136.62, 129.85 (2C), 129.19 (2C), 120.57 (2C), 120.26, 107.99, 104.90, 102.61, 102.37, 101.30, 57.18, 39.42, 31.94, 31.26, 18.63, 7.47. ESI-HRMS (m/z): calcd for $C_{27}H_{25}N_4O_6^+$ $[M+H]^+$: 501.17686; found: 501.17672.

2.3.2.

*(E)-6-acetyl-2-(1-(((1-(4-chlorophenyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione (**A2**)*

Yellow crystal. M.p. 135-136℃. Yield: 58%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.92 (brs, 1H, Ar-OH), 13.35 (s, 1H, -C-N<u>H</u>-CH_2-), 11.81 (brs, 1H, Ar-OH), 8.07 (s, 1H, triazole-H), 7.69 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.50 (d, *J* = 8.4 Hz, 2H, Ar-H), 5.76 (s, 1H, O=C-CH=), 4.90 (d, *J* = 5.1 Hz, 2H, -NH-C<u>H</u>$_2$-), 2.78 (s, 3H, CH_3-C=O), 2.65 (s, 3H, Ar-CH_3), 2.07 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.68 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.67, 198.49, 190.23, 175.40, 174.16, 163.44, 158.08, 155.75, 143.14, 135.08, 135.00, 130.04 (2C), 121.71 (2C), 120.23, 107.98, 104.88, 102.62, 102.35, 101.30, 57.20, 39.39, 31.97, 31.29, 18.66, 7.51. ESI-HRMS (m/z): calcd for $C_{27}H_{24}ClN_4O_6^+$ $[M+H]^+$: 535.13789; found: 535.13745.

2.3.3.

*(E)-6-acetyl-2-(1-(((1-(2-chlorophenyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione (**A3**)*

Yellow crystal. M.p. 137-138℃. Yield: 55%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.99 (brs, 1H, Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.89 (brs, 1H, Ar-OH), 8.04 (s, 1H, triazole-H), 7.67-7.59 (m, 2H, Ar-H), 7.53-7.48 (m, 2H, Ar-H), 5.80 (s, 1H, O=C-CH=), 4.93 (d, *J* = 5.7 Hz, 2H, -NH-C<u>H</u>$_2$-), 2.80 (s, 3H, CH_3-C=O), 2.67 (s, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.72 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.60, 198.45, 190.17, 175.40, 174.12, 163.40, 158.08, 155.73, 141.90, 134.41, 131.08, 130.78, 128.44, 128.02, 127.69, 124.10, 107.95, 104.88, 102.59, 102.34, 101.37, 57.14, 39.37, 31.90, 31.22, 18.63, 7.43. ESI-HRMS (m/z): calcd for $C_{27}H_{24}ClN_4O_6^+$ $[M+H]^+$: 535.13789; found: 535.13748.

2.3.4.

*(E)-6-acetyl-2-(1-(((1-(4-fluorophenyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione (**A4**)*

Yellow crystal. M.p. 128-129℃. Yield: 73%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.97 (brs, 1H, Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.84 (brs, 1H, Ar-OH), 7.98 (s, 1H, triazole-H), 7.74-7.69 (m, 2H, Ar-H), 7.28-7.22 (m, 2H, Ar-H), 5.79 (s, 1H, O=C-CH=), 4.91-4.89 (m, 2H, -NH-C<u>H</u>$_2$-), 2.79 (s, 3H, CH_3-C=O), 2.67 (s, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.71 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 220.63, 198.48, 175.37, 174.14, 164.24, 163.43, 160.93, 158.09, 155.74, 142.99, 132.91, 132.87, 122.66, 122.54, 120.44, 117.00, 116.70, 107.98, 104.88, 102.33, 101.29, 57.24, 39.38, 31.93, 31.24, 18.61, 7.47. ESI-HRMS (m/z): calcd for $C_{27}H_{24}FN_4O_6^+$ $[M+H]^+$: 519.16744; found: 519.16785.

2.3.5.

*(E)-6-acetyl-2-(1-(((1-(4-bromophenyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione (**A5**)*

Yellow crystal. M.p. 134-135℃. Yield: 47%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.98 (brs, 1H, Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.83 (brs, 1H, Ar-OH), 8.01 (s, 1H, triazole-H), 7.66 (q, *J* = 9.0 Hz, 4H, Ar-H), 5.79 (s, 1H, O=C-CH=), 4.91-4.89 (m, 2H, -NH-C<u>H</u>$_2$-), 2.79 (s, 3H, CH_3-C=O), 2.67 (s, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.71 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.54, 198.58, 198.49, 175.37, 174.15, 163.49, 158.11, 155.76, 143.16, 135.62 (2C), 132.99, 122.88, 121.91 (2C), 120.07, 108.03, 104.88, 102.33, 101.33, 90.35, 57.19, 39.35, 31.90, 31.14, 18.50, 7.43. ESI-HRMS (m/z): calcd for $C_{27}H_{24}BrN_4O_6^+$ $[M+H]^+$: 579.08737; found: 579.08765.

2.3.6.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-(((1-(4-(trifluoromethyl)phenyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)dibenzo[b,d]furan-1,3(2H,9bH)-dione (**A6**)*

Yellow crystal. M.p. 159-160℃. Yield: 47%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.96 (brs, 1H, Ar-OH), 13.35 (s, 1H, -C-N<u>H</u>-CH_2-), 11.80 (brs, 1H, Ar-OH), 8.03 (s, 1H, triazole-H), 7.73 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.50 (s, 2H, Ar-H), 5.78 (s, 1H, O=C-CH=), 4.89 (d, *J* = 5.1 Hz, 2H, -NH-C<u>H</u>$_2$-), 2.78 (s, 3H, CH_3-C=O), 2.67 (s, 3H, Ar-CH_3), 2.09 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.70 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.67, 198.49, 197.11, 175.30, 163.39, 157.99, 155.69, 153.14, 143.14, 130.34, 126.20, 126.16, 125.94, 121.12, 120.97, 120.85, 120.30 (2C), 119.96, 108.00, 104.79, 101.27, 39.29, 31.89, 31.22, 18.58, 7.55, 7.47. ESI-HRMS (m/z): calcd for $C_{28}H_{24}F_3N_4O_6^+$ $[M+H]^+$: 569.16425; found: 569.16456.

2.3.7.

*(E)-6-acetyl-7,9-dihydroxy-2-(1-(((1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione (**A7**)*

Yellow crystal. M.p. 132-134℃. Yield: 53%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.97 (brs, 1H, Ar-OH), 13.38 (s, 1H, -C-N<u>H</u>-CH_2-), 11.89 (brs, 1H, Ar-OH), 7.95 (s, 1H, triazole-H), 7.64 (d, *J* = 9.0 Hz, 2H, Ar-H), 7.05 (d, *J* = 9.0 Hz, 2H, Ar-H), 5.81 (s, 1H, O=C-CH=), 4.92-4.90 (m, 2H, -NH-C<u>H</u>$_2$-), 3.90 (s, 3H, -OCH_3), 2.81 (s, 3H, CH_3-C=O), 2.69 (s, 3H, Ar-CH_3), 2.11 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.73 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.55, 198.50, 175.37, 163.51, 160.16, 158.18, 155.80, 142.61, 130.11, 122.24 (2C), 120.33, 114.88 (2C), 108.05, 104.94, 102.47, 102.39, 102.34, 102.24, 101.35, 55.62, 39.47 (2C), 31.90, 31.14, 18.52, 7.42. ESI-HRMS (m/z): calcd for $C_{28}H_{27}N_4O_7^+$ $[M+H]^+$: 531.18743; found: 531.18777.

2.3.8.

*(E)-6-acetyl-7,9-dihydroxy-2-(1-(((1-(2-methoxyphenyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethylidene)-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione (**A8**)*

Yellow crystal. M.p. 129-130℃. Yield: 58%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.92 (brs, 1H, Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.92 (brs, 1H, Ar-OH), 8.17 (s, 1H, triazole-H), 7.80 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.49-7.43 (m, 1H, Ar-H), 7.16-7.10 (m, 2H, Ar-H), 5.79 (s, 1H, O=C-CH=), 4.91-4.89 (m, 2H, -NH-C<u>H</u>$_2$-), 3.92 (s, 3H, -OCH_3), 2.80 (s, 3H, CH_3-C=O), 2.67 (s, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.72 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.65, 198.43, 190.13, 175.38, 174.07, 163.41, 158.14, 155.79, 150.88, 130.45, 125.81, 125.31, 124.32, 121.28, 112.32, 112.22, 107.93, 104.96, 102.58, 102.43, 101.29, 57.11, 56.00, 39.53, 31.95, 31.27, 18.71, 7.47. ESI-HRMS (m/z): calcd for $C_{28}H_{27}N_4O_7^+$ $[M+H]^+$: 531.18743; found: 531.18776.

*2.4. Procedure for the preparation of compound **B1-B5***

The (+)-usnic acid (172 mg, 0.5 mmol), and different 2-aminoethyl secondary amines (0.6 mmol) were stirred in 10 mL absolute ethanol at 78℃ for 2-3 h. The crude product was obtained under reduced pressure by evaporation of the solvent after confirming completion of the reaction by TLC. The silica gel column chromatography was used for products purification (silica gel, methanol/dichloromethane, 1:200-1:100 v/v). The characteristics of the spectroscopic and physical data were as shown below.

2.4.1.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-((2-(pyrrolidin-1-yl)ethyl)amino)ethylidene)dibenzo[b,d]furan-1,3(2H,9bH)-dione (**B1**)*

Yellow crystal. M.p. 96-97℃. Yield: 82%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.36 (brs, 1H, Ar-OH), 12.04 (brs, 1H, Ar-OH), 5.78 (s, 1H, O=C-CH=), 3.62-3.56 (m, 2H, -NH-C$\underline{H}_2$-), 2.84-2.80 (m, 2H, -NH-CH_2-C$\underline{H}_2$-), 2.67 (s, 3H, CH_3-C=O), 2.64 (s, 3H, Ar-CH_3), 2.62-2.56 (m, 4H, pyrrole-H), 2.09 (s, 3H, -NH-C-C$\underline{H}_3$), 1.92-1.80 (m, 4H, pyrrole-H), 1.70 (s, 3H, O=C-C-CH_3), 1.25 (s, 1H, -C-N$\underline{H}$-CH_2-). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.57, 198.11, 174.51, 173.86, 163.47, 158.36 (2C), 155.91, 107.91, 105.16, 102.54 (2C), 101.35, 56.94, 54.03 (2C), 53.96, 43.29, 31.94, 31.14, 23.71 (2C), 18.55, 7.41. ESI-HRMS (m/z): calcd for $C_{24}H_{29}N_2O_6^+$ $[M+H]^+$: 441.20201; found: 441.20211.

2.4.2.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-((2-(piperidin-1-yl)ethyl)amino)ethylidene)dibenzo[b,d]furan-1,3(2H,9bH)-dione (**B2**)*

Yellow crystal. M.p. 98-99℃. Yield: 87%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.37 (brs, 1H, Ar-OH), 12.06 (brs, 1H, Ar-OH), 5.79 (s, 1H, O=C-CH=), 3.58-3.52 (m, 2H, -NH-C$\underline{H}_2$-), 2.68 (s, 3H, CH_3-C=O), 2.66-2.62 (m, 5H, Ar-CH_3, -NH-CH_2-C$\underline{H}_2$-), 2.49-2.46 (m, 4H, piperidine-H), 2.10 (s, 3H, -NH-C-C$\underline{H}_3$), 1.71 (s, 3H, O=C-C-CH_3), 1.68-1.61 (m, 4H, piperidine-H), 1.51-1.46 (m, 2H, piperidine-H), 1.26 (s, 1H, -C-N$\underline{H}$-CH_2-). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 220.53 (2C), 197.99, 174.29, 173.70, 163.41, 158.35, 155.89, 107.83, 105.16, 102.59 (2C), 101.29, 56.46, 54.44 (2C), 41.59, 31.90, 31.09, 25.90 (3C), 24.20, 18.63, 7.37. ESI-HRMS (m/z): calcd for $C_{25}H_{31}N_2O_6^+$ $[M+H]^+$: 455.21766; found: 455.21793.

2.4.3.

*(E)-6-acetyl-2-(1-((2-(dimethylamino)ethyl)amino)ethylidene)-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione (**B3**)*

Yellow crystal. M.p. 83-84℃. Yield: 84%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.37 (brs, 1H, Ar-OH), 12.05 (brs, 1H, Ar-OH), 5.79 (s, 1H, O=C-CH=), 3.58-3.49 (m, 2H, -NH-C$\underline{H}_2$-), 2.68 (s, 3H, CH_3-C=O), 2.65 (s, 3H, Ar-CH_3), 2.64-2.62 (m, 2H, -NH-CH_2-C$\underline{H}_2$-), 2.35 (s, 6H, -N-CH_3), 2.10 (s, 3H, -NH-C-C$\underline{H}_3$), 1.71 (s, 3H, O=C-C-CH_3), 1.26 (s, 1H, -C-N$\underline{H}$-CH_2-). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 220.69 (2C), 198.15, 174.39, 173.90, 163.40, 158.31, 155.89, 114.27, 107.83, 105.16, 102.50, 101.31, 57.11 (2C), 45.32 (2C), 41.99, 31.99, 31.30, 18.79, 7.48. ESI-HRMS (m/z): calcd for $C_{22}H_{27}N_2O_6^+$ $[M+H]^+$: 415.18636; found: 415.18656.

2.4.4.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-((2-(4-methylpiperazin-1-yl)ethyl)amino)ethylidene) dibenzo[b,d]furan-1,3(2H,9bH)-dione (**B4**)*

Yellow crystal. M.p. 80-82℃. Yield: 72%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.35 (brs, 1H, Ar-OH), 12.03 (brs, 1H, Ar-OH), 5.77 (s, 1H, O=C-CH=), 3.58-3.53 (m, 2H, -NH-C$\underline{H}_2$-), 2.72-2.59 (m, 16H, -NH-CH_2-C$\underline{H}_2$-, CH_3-C=O, -NH-C-C$\underline{H}_3$, piperazine-H), 2.35 (s, 3H, -N-CH_3), 2.08 (s, 3H, Ar-CH_3), 1.69 (s, 3H, O=C-C-CH_3), 1.24 (s, 1H, -C-N$\underline{H}$-CH_2-). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.64 (2C), 197.99, 174.32, 173.75, 163.37, 158.28, 155.87, 107.81, 105.11, 102.61, 102.56, 101.28, 55.48, 54.85 (2C), 52.63 (2C), 45.75, 41.19, 31.95, 31.89, 31.23, 18.81, 7.43. ESI-HRMS (m/z): calcd for $C_{25}H_{32}N_3O_6^+$ $[M+H]^+$: 470.22856; found: 470.22876.

2.4.5.

*(E)-6-acetyl-2-(1-((2-(diethylamino)ethyl)amino)ethylidene)-7,9-dihydroxy-8,9b-dimethyldibenzo[b, d]furan-1,3(2H,9bH)-dione (**B5**)*

Yellow crystal. M.p. 106-107℃. Yield: 96%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.36 (brs, 1H, Ar-OH), 12.08 (brs, 1H, Ar-OH), 5.78 (s, 1H, O=C-CH=), 3.55-3.49 (m, 2H, -NH-C$\underline{H}_2$-), 2.77-2.73 (m, 2H, -NH-CH_2-C$\underline{H}_2$-), 2.67 (s, 3H, CH_3-C=O), 2.64 (s, 3H, Ar-CH_3), 2.62-2.58 (m, 4H,

-N-C<u>H</u>$_2$-CH$_3$), 2.08 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.69 (s, 3H, O=C-C-CH$_3$), 1.25 (s, 1H, -C-N<u>H</u>-CH$_2$-), 1.07 (t, 6H, J = 7.2, -N-CH$_2$-C<u>H</u>$_3$). ^{13}C-NMR (CDCl$_3$, 75 MHz, ppm): δ 200.58 (2C), 198.05, 174.35, 173.74, 163.46, 158.39, 155.94, 107.87, 105.20, 102.62, 102.41, 101.34, 56.86, 51.15, 47.01 (2C), 42.62, 31.95, 31.14, 18.69, 11.82 (2C), 7.41. ESI-HRMS (m/z): calcd for $C_{24}H_{31}N_2O_6^+$ $[M+H]^+$: 443.21766; found: 443.21754.

*2.5. Procedure for the preparation of compound **C1-C5***

The (+)-usnic acid (172 mg, 0.5 mmol), and different diethyl (amino(phenyl)methyl)phosphonates (0.6 mmol) was stirred in 10 mL absolute ethanol at 78℃ for 2-3 h. The crude product was obtained under reduced pressure by evaporation of the solvent after confirming completion of the reaction by TLC. The silica gel column chromatography was used for products purification (silica gel, methanol/dichloromethane, 1:200-1:100 v/v). The characteristics of the spectroscopic and physical data were as shown below.

2.5.1.

*Diethyl(E)-(((1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene)ethyl)amino)(phenyl)methyl)phosphonate (**C1**)*

Yellow crystal. M.p. 98-99℃. Yield: 50%. ^{1}H-NMR (CDCl$_3$, 300 MHz, ppm): δ 14.64 (brs, 1H, Ar-OH), 13.35 (s, 1H, -C-N<u>H</u>-CH$_2$-), 11.78 (d, J = 3.3 Hz, 1H, Ar-OH), 7.47-7.41 (m, 5H, Ar-H), 5.86 (d, J = 5.7 Hz, 1H, O=C-CH=), 5.23-5.12 (m, 1H, -NH-C<u>H</u>-), 4.12-3.98 (m, 4H, -O-CH$_2$-), 3.49 (s, 3H, CH$_3$-C=O), 2.69 (s, 3H, Ar-CH$_3$), 2.12-2.09 (m, 3H, -NH-C-C<u>H</u>$_3$), 1.77-1.69 (m, 3H, O=C-C-CH$_3$), 1.31-1.22 (m, 6H, -CH$_2$-C<u>H</u>$_3$). ^{13}C-NMR (CDCl$_3$, 75 MHz, ppm): δ 200.60, 198.57, 190.90, 174.52, 174.46, 163.47 (2C), 158.13, 132.77, 129.15, 128.91, 127.71, 127.65, 127.58, 108.08, 104.89, 102.26, 102.16, 101.32, 64.04, 57.16, 55.12, 31.84, 31.28 (2C), 18.38, 16.38, 16.31, 7.45. ESI-HRMS (m/z): calcd for $C_{29}H_{33}NO_9P^+$ $[M+H]^+$: 570.18874; found: 570.18855.

296

297 *2.5.2.*

298 *Diethyl(E)-(((1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(*

299 *1H)-ylidene)ethyl)amino)(4-chlorophenyl)methyl)phosphonate (**C2**)*

300 Yellow crystal. M.p. 120-121℃. Yield: 64%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 14.65 (brs, 1H,
301 Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.71 (d, *J* = 3.6 Hz, 1H, Ar-OH), 7.42-7.38 (m, 4H, Ar-H),
302 5.86 (d, *J* = 6.0 Hz, 1H, O=C-CH=), 5.20-5.08 (m, 1H, -NH-C<u>H</u>-), 4.15-4.03 (m, 4H, -O-CH_2-),
303 2.69 (s, 3H, CH_3-C=O), 2.59 (s, 3H, Ar-CH_3), 2.10-2.09 (m, 3H, -NH-C-C<u>H</u>$_3$), 1.71 (d, *J* = 10.0 Hz,
304 3H, O=C-C-CH_3), 1.33-1.25 (m, 6H, -CH_2-C<u>H</u>$_3$). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.48,
305 198.71, 198.64, 190.91, 174.57, 163.54, 158.10, 155.68, 134.98, 131.55, 129.32, 128.99, 128.93,
306 128.86, 108.18, 104.80, 103.05, 102.20, 101.35, 64.07, 57.55, 56.55, 54.54, 31.77, 31.14, 18.23,
307 16.34, 16.27, 7.38. ESI-HRMS (m/z): calcd for $C_{29}H_{32}ClNO_9P^+$ $[M+H]^+$: 604.14977; found:
308 604.14996.

309

310 *2.5.3.*

311 *Diethyl(E)-(((1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(*

312 *1H)-ylidene)ethyl)amino)(3,4-dichlorophenyl)methyl)phosphonate (**C3**)*

313 Yellow crystal. M.p. 101-102℃. Yield: 66%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 14.70-14.65 (m,
314 1H, Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.66 (d, *J* = 2.1 Hz, 1H, Ar-OH), 7.56-7.49 (m, 2H,
315 Ar-H), 7.37-7.31 (m, 1H, Ar-H), 5.88 (d, *J* = 4.6 Hz, 1H, O=C-CH=), 5.19-5.06 (m, 1H, -NH-C<u>H</u>-),
316 4.22-4.09 (m, 4H, -O-CH_2-), 2.69 (s, 3H, CH_3-C=O), 2.60 (s, 3H, Ar-CH_3), 2.10 (s, 3H,
317 -NH-C-C<u>H</u>$_3$), 1.77-1.71 (m, 3H, O=C-C-CH_3), 1.37-1.30 (m, 6H, -CH_2-C<u>H</u>$_3$). ^{13}C-NMR ($CDCl_3$, 75
318 MHz, ppm): δ 200.63, 198.85, 198.78, 191.14, 191.09, 174.81, 163.61, 158.11, 155.75, 133.44,
319 131.16, 129.63, 126.84, 108.30, 104.83, 103.26, 103.21, 102.27, 102.19, 101.41, 64.32, 57.68,

56.16, 54.12, 31.86, 31.31, 18.38, 16.37, 7.49. ESI-HRMS (m/z): calcd for $C_{29}H_{31}Cl_2NO_9P^+$ $[M+H]^+$: 638.11080; found: 638.11063.

2.5.4.

*Diethyl(E)-(((1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene)ethyl)amino)(p-tolyl)methyl)phosphonate (**C4**)*

Yellow crystal. M.p. 106-107℃. Yield: 61%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 14.61 (brs, 1H, Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.81 (d, *J* = 5.1 Hz, 1H, Ar-OH), 7.38-7.30 (m, 2H, Ar-H), 7.23-7.20 (m, 1H, Ar-H), 5.85 (d, *J* = 6.0 Hz, 1H, O=C-CH=), 5.19-5.07 (m, 1H, -NH-C<u>H</u>-), 4.13-3.99 (m, 4H, -O-CH_2-), 2.69 (s, 3H, CH_3-C=O), 2.60 (s, 3H, Ar-CH_3), 2.36 (d, *J* = 5.4 Hz, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.73-1.69 (m, 3H, O=C-C-CH_3), 1.30-1.23 (m, 6H, -CH_2-C<u>H</u>$_3$). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.61, 198.52, 190.86, 174.47, 163.46, 158.16, 138.90, 129.83 (2C), 127.61, 127.54 (2C), 127.47, 108.07, 104.92, 102.26, 102.16, 101.31, 90.33, 63.98, 63.80, 56.93, 54.89, 31.84, 31.67, 31.28, 21.14, 18.38, 16.33, 7.45. ESI-HRMS (m/z): calcd for $C_{30}H_{35}NO_9P^+$ $[M+H]^+$: 584.20439; found: 584.20471.

2.5.5.

*Diethyl(E)-(((1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene)ethyl)amino)(4-methoxyphenyl)methyl)phosphonate (**C5**)*

Yellow crystal. M.p. 128-129℃. Yield: 51%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 14.58 (brs, 1H, Ar-OH), 13.36 (s, 1H, -C-N<u>H</u>-CH_2-), 11.81 (d, *J* = 3.9 Hz, 1H, Ar-OH), 7.42-7.34 (m, 2H, Ar-H), 6.98-6.92 (m, 2H, Ar-H), 5.85 (d, *J* = 6.3 Hz, 1H, O=C-CH=), 5.17-5.06 (m, 1H, -NH-C<u>H</u>-), 4.09-4.04 (m, 4H, -O-CH_2-), 3.82 (d, *J* = 4.5 Hz, 3H, -O-CH_3), 2.69 (s, 3H, -NH-C-C<u>H</u>$_3$), 2.61 (s, 3H, CH_3-C=O), 2.10 (s, 3H, Ar-CH_3), 1.73-1.69 (m, 3H, O=C-C-CH_3), 1.31-1.22 (m, 6H, -CH_2-C<u>H</u>$_3$). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.56, 198.58, 190.85, 163.65 (2C), 160.05,

158.22, 155.82, 128.96 (2C), 128.89, 124.69, 114.63 (2C), 108.15, 104.95, 103.00, 102.27, 101.38, 64.02, 63.90, 56.67, 55.32, 54.60, 31.82, 31.18, 18.28, 16.40, 16.33, 7.42. ESI-HRMS (m/z): calcd for $C_{30}H_{35}NO_{10}P^+$ $[M+H]^+$: 600.19931; found: 600.19914.

*2.6. Procedure for the preparation of compound **D1-D4***

The (+)-usnic acid (172 mg, 0.5 mmol), Et_3N (1.0 mmol), and different L-amino acid methyl ester hydrochlorides (0.6 mmol) was stirred in 10 mL ethanol and 2 mL H_2O at 78℃ for 3-5 h. The mixture was obtained under reduced pressure by evaporation of the solvent after confirming completion of the reaction by TLC. Next, the mixture was poured into water (20 mL), extracted by dichloromethane (15×3 mL), and the organic phase was washed with saturated NaCl solution (50 mL) and dried by sodium sulfate. The sodium sulfate was removed by filtration. The crude product was obtained under reduced pressure by evaporation of the solvent. The silica gel column chromatography was used for Products purification (silica gel, methanol/dichloromethane, 1:400-1:200 v/v). The characteristics of the spectroscopic and physical data were as shown below.

2.6.1.

*Methyl(E)-(1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene)ethyl)glycinate (**D1**)*

Yellow crystal. M.p. 141-142℃. Yield: 57%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.89 (brs, 1H, Ar-OH), 13.37 (s, 1H, -C-N<u>H</u>-CH_2-), 11.83 (brs, 1H, Ar-OH), 5.83 (s, 1H, O=C-CH=), 4.29-4.27 (m, 2H, -NH-C<u>H</u>$_2$-), 3.87 (s, 3H, -O-CH_3), 2.68 (s, 3H, CH_3-C=O), 2.60 (s, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-C<u>H</u>$_3$), 1.72 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.62, 200.55, 198.62, 175.04, 174.28, 167.45, 163.51, 158.13, 155.78, 108.04, 104.88, 102.25, 102.21, 101.35,

62.40, 52.98, 45.02, 31.11, 18.60, 14.03, 7.36. ESI-HRMS (m/z): calcd for $C_{21}H_{22}NO_8^+$ $[M+H]^+$: 416.13399; found: 416.13364.

2.6.2.

*Methyl(E)-(1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene)ethyl)tryptophanate (**D2**)*

Yellow crystal. M.p. 253-254℃. Yield: 48%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 14.04 (brs, 1H, Ar-OH), 13.38 (s, 1H, -C-N<u>H</u>-CH_2-), 11.86 (brs, 1H, Ar-OH), 8.17 (s, 1H, indole-NH), 7.58 (d, *J* = 7.2 Hz, 1H, indole-H), 7.39 (d, *J* = 7.8 Hz, 1H, indole-H), 7.26-7.14 (m, 3H, indole-H), 5.82 (s, 1H, O=C-CH=), 4.85-4.74 (m, 1H, -NH-C<u>H</u>-), 3.84 (s, 3H, -O-CH_3), 3.59-3.50 (m, 2H, -NH-CH-C<u>H_2</u>-), 2.70 (s, 3H, CH_3-C=O), 2.23 (s, 3H, Ar-CH_3), 2.10 (s, 3H, -NH-C-C<u>H_3</u>), 1.69 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.65, 198.45, 198.35, 174.59, 170.06, 163.48 (2C), 158.16, 155.77, 136.19, 126.70, 123.72, 122.55, 119.96, 118.02, 117.87, 111.53, 108.65, 108.03, 104.96, 102.23, 101.33, 62.35, 57.39, 53.04, 31.75, 31.13, 29.42, 18.37, 7.38. ESI-HRMS (m/z): calcd for $C_{30}H_{29}N_2O_8^+$ $[M+H]^+$: 545.19184; found: 545.19165.

2.6.3.

*Methyl(E)-(1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene)ethyl)phenylalaninate (**D3**)*

Yellow crystal. M.p. 94-95℃. Yield:50%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 14.08 (d, *J* = 7.8 Hz, 1H, Ar-OH), 13.35 (s, 1H, -C-N<u>H</u>-CH_2-), 11.80 (brs, 1H, Ar-OH), 7.31-7.29 (m, 3H, Ar-H), 7.19-7.16 (m, 2H, Ar-H), 5.30 (s, 1H, O=C-CH=), 4.68-4.66 (m, 1H, -NH-C<u>H</u>-), 3.84 (s, 3H, -O-CH_3), 3.38-3.32 (m, 1H, -NH-CH-C<u>H_2</u>-), 3.14-3.06 (m, 1H, -NH-CH-C<u>H_2</u>-), 2.68 (s, 3H, CH_3-C=O), 2.24 (s, 3H, Ar-CH_3), 2.09 (s, 3H, -NH-C-C<u>H_3</u>), 1.68 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.59, 198.52, 198.43, 174.62, 174.56, 169.63, 163.41, 158.08, 155.74,

134.58, 129.18 (2C), 128.93 (2C), 127.75, 107.97, 104.90, 102.37, 102.32, 101.28, 62.41, 58.32, 53.09, 39.46, 31.77, 31.22, 18.19, 7.41. ESI-HRMS (m/z): calcd for $C_{28}H_{28}NO_8^+$ $[M+H]^+$: 506.18094; found: 506.18137.

*2.6.4. Methyl(E)-(1-(6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1H)-ylidene)ethyl)tyrosinate (**D4**)*

Yellow crystal. M.p. 155-156℃. Yield: 52%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.83 (brs, 1H, Ar-OH), 13.34 (s, 1H, -C-N<u>H</u>-CH_2-), 11.76 (brs, 1H, Ar-OH), 7.07-7.00 (m, 2H, Ar-H), 6.79-6.75 (m, 2H, Ar-H), 5.83 (s, 1H, O=C-CH=), 4.73-4.62 (m, 1H, -NH-C<u>H</u>-), 3.83-3.81 (m, 3H, -O-CH_3), 3.29-3.22 (m, 1H, -NH-CH-C<u>H_2</u>-), 3.13-3.05 (m, 1H, -NH-CH-C<u>H_2</u>-), 2.66-2.65 (m, 3H, CH_3-C=O), 2.37 (s, 3H, Ar-CH_3), 2.08 (s, 3H, -NH-C-C<u>H_3</u>), 1.68 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.72, 200.65, 198.65, 174.80, 174.64, 169.72, 169.27, 163.52, 158.13, 155.76, 130.56, 130.49, 130.44, 126.08, 115.95, 115.91, 108.17, 104.92, 102.12, 101.38, 62.45, 58.50, 53.07, 38.51, 31.15, 18.46, 14.07, 7.42. ESI-HRMS (m/z): calcd for $C_{28}H_{28}NO_9^+$ $[M+H]^+$: 522.17586; found: 522.17633.

*2.7. Procedure for the preparation of compound **E1-E4***

The (+)-usnic acid (172 mg, 0.5 mmol), Et_3N (1.0 mmol), and different phenylhydrazine hydrochlorides (0.6 mmol) was stirred in 10 mL absolute ethanol at 78℃ for 3-5 h. The mixture was obtained under reduced pressure by evaporation of the solvent after confirming completion of the reaction by TLC. Next, the mixture was poured into water (20 mL), extracted by dichloromethane (15×3 mL), and the organic phase was washed with saturated NaCl solution (50 mL) and dried by dry Na_2SO_4. Filtering to remove Na_2SO_4. The crude product was obtained under

reduced pressure by evaporation of the solvent. The silica gel column chromatography was used for products purification (silica gel, ethyl acetate/petroleum ether, 1:25-1:20 v/v). The characteristics of the spectroscopic and physical data were as shown below.

2.7.1.

8-acetyl-5,7-dihydroxy-3,4a,6-trimethyl-1-phenyl-1,4a-dihydro-4H-benzofuro[3,2-f]indazol-4-one ***(E1)***

Yellow crystal. M.p. 179-180℃. Yield: 70%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.32 (s, 1H, Ar-OH), 11.12 (s, 1H, Ar-OH), 7.58-7.45 (m, 5H, Ar-H), 6.25 (s, 1H, O=C-CH=), 2.64 (s, 3H, CH_3-C=O), 2.59 (s, 3H, -N=C-CH_3), 2.12 (s, 3H, Ar-CH_3), 1.82 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.35, 196.16, 172.93, 163.53, 157.61, 156.24, 151.42, 148.18, 137.85, 130.87, 129.65, 128.78, 128.73, 123.89, 110.67, 108.24, 103.96, 101.49, 89.27, 60.31, 31.19, 30.43, 13.26, 7.45. ESI-HRMS (m/z): calcd for $C_{24}H_{21}N_2O_5^+$ $[M+H]^+$: 417.14450; found: 417.14472.

2.7.2.

*8-acetyl-1-(3-chlorophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1,4a-dihydro-4H-benzofuro[3,2-f]indazol-4-one (****E2****)*

Yellow crystal. M.p. 128-129℃. Yield: 47%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.33 (s, 1H, Ar-OH), 11.02 (s, 1H, Ar-OH), 7.62 (s, 1H, Ar-H), 7.54-7.43 (m, 3H, Ar-H), 6.25 (s, 1H, O=C-CH=), 2.66 (s, 3H, CH_3-C=O), 2.58 (s, 3H, -N=C-CH_3), 2.12 (s, 3H, Ar-CH_3), 1.82 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.36, 196.15, 173.43, 163.59, 157.52, 156.17, 151.75, 148.34, 138.88, 135.46, 130.65, 128.79, 124.05, 121.81, 110.85, 108.39, 103.85, 101.52, 88.98, 60.47, 31.24, 30.43, 13.23, 7.46. ESI-HRMS (m/z): calcd for $C_{24}H_{20}ClN_2O_5^+$ $[M+H]^+$: 451.10553; found: 451.10593.

441

442 *2.7.3.*

443 *8-acetyl-1-(3-fluorophenyl)-5,7-dihydroxy-3,4a,6-trimethyl-1,4a-dihydro-4H-benzofuro[3,2-f]indaz*

444 *ol-4-one (**E3**)*

445 Yellow crystal. M.p. 166-167℃. Yield: 44%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.32 (s, 1H,
446 Ar-OH), 11.03 (s, 1H, Ar-OH), 7.54-7.50 (m, 1H, Ar-H), 7.40-7.34 (m, 2H, Ar-H), 7.22-7.16 (m,
447 1H, Ar-H), 6.28 (s, 1H, O=C-CH=), 2.66 (s, 3H, CH_3-C=O), 2.58 (s, 3H, -N=C-CH_3), 2.12 (s, 3H,
448 Ar-CH_3), 1.82 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.34, 196.15, 173.39,
449 164.59, 163.57, 157.52, 156.17, 151.70, 148.32, 131.04, 119.22, 115.81, 115.53, 111.66, 111.33,
450 110.86, 108.37, 101.51, 89.05, 60.46, 31.21, 30.42, 13.22, 7.45. ESI-HRMS (m/z): calcd for
451 $C_{24}H_{20}FN_2O_5^+$ $[M+H]^+$: 435.13508; found: 435.13555.

452

453 *2.7.4.*

454 *8-acetyl-5,7-dihydroxy-3,4a,6-trimethyl-1-(p-tolyl)-1,4a-dihydro-4H-benzofuro[3,2-f]indazol-4-one*

455 *(**E4**)*

456 Yellow crystal. M.p. 183-184℃. Yield: 46%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 13.32 (s, 1H,
457 Ar-OH), 11.14 (s, 1H, Ar-OH), 7.44 (d, J = 8.4 Hz, 2H, Ar-H), 7.34 (d, J = 8.1 Hz, 2H, Ar-H), 6.21
458 (s, 1H, O=C-CH=), 2.63 (s, 3H, CH_3-C=O), 2.58 (s, 3H, -N=C-CH_3), 2.45 (s, 3H, Ar-CH_3), 2.11 (s,
459 3H, Ar-CH_3), 1.81 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.37, 196.12,
460 172.72, 163.49, 157.63, 156.27, 151.25, 148.10, 138.93, 135.37, 130.16 (2C), 123.76 (2C), 110.52,
461 108.17, 104.00, 101.46, 89.35, 60.24, 31.19, 30.43, 21.16, 13.27, 7.45. ESI-HRMS (m/z): calcd for
462 $C_{25}H_{23}N_2O_5^+$ $[M+H]^+$: 431.16015; found: 431.16064.

463

464 *2.8. Procedure for the preparation of compound **F1-F4***

The (+)-usnic acid (172 mg, 0.5 mmol), CH_3COOH (0.125 mL), and different aminoquinolines (0.6 mmol) was stirred in 10 mL absolute ethanol at 78℃ for 12 h. The crude product was obtained under reduced pressure by evaporation of the solvent after confirming completion of the reaction by TLC. The silica gel column chromatography was used for products purification (silica gel, methanol/dichloromethane, 1:250-1:100 v/v). The characteristics of the spectroscopic and physical data were as shown below.

2.8.1.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-(quinolin-3-ylamino)ethylidene)dibenzo[b,d]furan-1,3(2H,9bH)-dione (**F1**)*

Yellow crystal. M.p. 257-258℃. Yield: 62%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 15.42 (brs, 1H, Ar-OH), 13.37 (s, 1H, -C-N<u>H</u>-CH_2-), 11.63 (brs, 1H, Ar-OH), 8.80 (s, 1H, quinoline-H), 8.18 (d, *J* = 9.0 Hz, 1H, quinoline-H), 8.02-8.02 (m, 1H, quinoline-H), 7.89-7.80 (m, 2H, quinoline-H), 7.70-7.64 (m, 1H, quinoline-H), 5.94 (s, 1H, O=C-CH=), 2.71 (s, 3H, CH_3-C=O), 2.67 (s, 3H, -NH-C-C<u>H</u>$_3$), 2.12 (s, 3H, Ar-CH_3), 1.79 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.45, 198.96, 191.09, 175.05, 174.15, 163.62, 158.01, 155.64, 147.44, 147.08, 131.47, 130.45, 129.77, 129.55, 128.05, 127.68, 108.36, 104.69, 103.27, 102.12, 101.39, 90.35, 57.70, 31.78, 31.13, 20.52, 7.41. ESI-HRMS (m/z): calcd for $C_{27}H_{23}N_2O_6^+$ $[M+H]^+$: 471.15506; found: 471.15523.

2.8.2.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-(quinolin-5-ylamino)ethylidene)dibenzo[b,d]furan-1,3(2H,9bH)-dione (**F2**)*

Yellow crystal. M.p. 120-121℃. Yield: 21%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 15.41 (brs, 1H, Ar-OH), 13.37 (s, 1H, -C-N<u>H</u>-CH_2-), 11.71 (brs, 1H, Ar-OH), 9.04 (m, 1H, quinoline-H), 8.22 (m, 2H, quinoline-H), 7.83-7.78 (m, 1H, quinoline-H), 7.56-7.52 (m, 1H, quinoline-H), 7.46-7.43 (m,

1H, quinoline-H), 5.96 (s, 1H, O=C-CH=), 2.72 (s, 3H, CH_3-C=O), 2.53 (s, 3H, -NH-C-C$\underline{H}_3$), 2.12 (s, 3H, Ar-CH_3), 1.82 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.28, 198.83, 191.33, 175.29, 163.57, 158.06, 151.44, 148.64, 130.64, 130.39, 128.78 (2C), 124.50, 124.38, 122.41, 108.29, 105.17, 104.73, 103.01, 102.18, 101.36, 98.27, 57.65, 31.92, 31.29, 20.73, 7.48. ESI-HRMS (m/z): calcd for $C_{27}H_{23}N_2O_6^+$ $[M+H]^+$: 471.15506; found: 471.15536.

2.8.3.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-(quinolin-6-ylamino)ethylidene)dibenzo[b,d]furan-1,3 (2H,9bH)-dione (**F3**)*

Yellow crystal. M.p. 124-125℃. Yield: 54%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 15.35 (brs, 1H, Ar-OH), 13.37 (s, 1H, -C-N$\underline{H}$-CH_2-), 11.75 (brs, 1H, Ar-OH), 9.02-9.00 (m, 1H, quinoline-H), 8.21 (t, J = 9.0 Hz, 2H, quinoline-H), 7.68-7.67 (m, 1H, quinoline-H), 7.57-7.49 (m, 2H, quinoline-H), 5.92 (s, 1H, O=C-CH=), 2.71 (s, 3H, CH_3-C=O), 2.66 (s, 3H, -NH-C-C$\underline{H}_3$), 2.12 (s, 3H, Ar-CH_3), 1.79 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.50, 200.48, 198.87, 191.05, 174.88, 173.81, 163.58, 158.07, 155.69, 151.40, 147.13, 135.80, 131.40, 128.24, 127.15, 123.89, 122.26, 108.25, 104.77, 102.94, 102.15, 101.38, 57.64, 31.81, 31.14, 20.66, 7.41. ESI-HRMS (m/z): calcd for $C_{27}H_{23}N_2O_6^+$ $[M+H]^+$: 471.15506; found: 471.15576.

2.8.4.

*(E)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-2-(1-(quinolin-8-ylamino)ethylidene)dibenzo[b,d]furan-1,3 (2H,9bH)-dione (**F4**)*

Yellow crystal. M.p. 118-119℃. Yield: 59%. ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 15.69 (brs, 1H, Ar-OH), 13.38 (s, 1H, -C-N$\underline{H}$-CH_2-), 11.95 (brs, 1H, Ar-OH), 9.04-9.02 (m, 1H, quinoline-H), 8.27-8.24 (m, 1H, quinoline-H), 7.88-7.85 (m, 1H, quinoline-H), 7.63-7.61 (m, 2H, quinoline-H), 7.56-7.52 (m, 1H, quinoline-H), 5.95 (s, 1H, O=C-CH=), 2.71 (s, 3H, CH_3-C=O), 2.68 (s, 3H,

-NH-C-C$\underline{H}_3$), 2.12 (s, 3H, Ar-CH_3), 1.80 (s, 3H, O=C-C-CH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz, ppm): δ 200.53, 198.75, 190.91, 173.86, 163.51, 158.23, 151.02, 147.25, 142.69, 136.19, 133.68, 129.01, 127.78, 125.83, 125.24, 122.29, 121.20, 115.90, 109.95, 108.06, 105.06, 102.47, 101.36, 31.72, 31.16, 21.41, 7.42. ESI-HRMS (m/z): calcd for $C_{27}H_{23}N_2O_6^+$ $[M+H]^+$: 471.15506; found: 471.15561.

2.9. In vitro anti-T. gondii activity

The published methyl thiazolyl tetrazole (MTT) method was used to study in vitro the cytotoxicity of compounds to host cells (HeLa) and the median inhibitory concentration of compounds to *T. gondii*-infected HeLa cells. Then the selectivity index (SI) was calculated. Cells were inoculated with appropriate densities in 96-well plates to ensure that the cells grew exponentially during the whole experiment period (3,000 cells/well). And then adhered for 24 h at 37℃. Infection of HeLa cells with *T. gondii* (15,000 tachyzoites/well), and then incubated *T. gondii*-infected HeLa cells for 24 h. Dissolved 10 mM compound in DMSO and stored it, and tested its serial dilution (1-1000 μM). Positive controls used spiramycin (spi), pyrimethamine and sulfadiazine. After plates were incubated for 24 hours, each well added 10 μL of MTT solution. Then a further 4 h of incubation. The optical density (OD) was read at 540 nm wavelength from microplate reader.

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2.10. In vivo anti-T. gondii activity

Divide 36 female KM mice into 6 groups (normal group, infected untreated group, infected with spi-treated group, infected with (+)-usnic acid-treated group, infected with **D3-**treated group, infected with **F3-**treated group), each group had six mice, and invasion of 2,000 tachyzoites of the *T.gondii* RH strain into mice by intraperitoneal injection *in vivo*. An animal model of acute infection of *T.gondii* was prepared by intraperitoneal injection of tachyzoites for 4 hours in mice.

Then, mice were gavaged once a day for 4 consecutive days with 100 mg/kg of compound, and the same dose of distilled water was given to the untreated group. After the fifth day of infection in mice, the mice were sacrificed by cervical dislocation and the abdominal parasites were collected by rinsing with sterile physiological saline. The inhibition rate of compounds against *T.gondii* was calculated by counting the number of tachyzoites in mouse peritoneal fluid using optical microscopy. Anatomize the liver and spleen. The liver and spleen index, aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and malonaldehyde (MDA) and liver homogenate glutathione (GSH) were measured. And, pathological examination was performed through liver sections treated with hematoxylin and eosin (H&E).

2.11. Statistical analysis

All data were expressed as mean ± standard deviation (SD) triplicate. GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA) and SPSS 16.0 software (SPSS Inc., Chicago, USA) were used for statistical analysis and charts in the study. When $p < 0.05$, it was considered statistically signifificant.

3. Results and discussion

3.1. Chemistry

(+)-usnic acid has an insecticidal effect *in vitro*, and trace amounts of (+)-usnic acid exhibit a strong anti-*T. gondii* tachyzoite activity [48]. According to the antibacterial and insecticidal experiments and clinical application results of (+)-usnic acid, it is speculated that (+)-usnic acid may also have a good anti-insect effect on *T. gondii* in humans and animals. Conveniently, (+)-usnic acid was also commercially available, so the (+) isomer was selected in our study.

The reaction of various primary amines with (+)-usnic acid highly reactive methyl ketone [49] to form enamine compounds is our strategic basis. **Scheme 1**. shown the synthetic method for obtaining target products. Compounds **A1-A8** are obtained by the reaction between various aminotriazoles and (+)-usnic acid in anhydrous ethanol at 78℃, and the yields of **A1-A8** ranged between 47%-66%. Compounds **B1-B5** are obtained by the reaction between various 2-aminoethyl secondary amines and (+)-usnic acid in anhydrous ethanol at 78℃, and the yields of **B1-B5** ranged between 72%-96%. Compounds **C1-C5** are obtained by the reaction between various phosphoramidates and (+)-usnic acid in anhydrous ethanol at 78℃, and the yields of **C1-C5** ranged between 50%-66%. Compounds **D1-D4** were obtained by an amide condensation reaction with different amino acid esters, catalyzed by Et_3N in ethanol and water at 78℃, and the yields of **D1-D4** ranged between 48%-57%. Compounds **E1-E4** are obtained by the reaction between various benzoquinones and (+)-usnic acid, catalyzed by Et_3N in anhydrous ethanol at 78℃, and the yields of **E1-E4** ranged between 44%-70%. Compounds **F1-F4** are obtained by the reaction between different aminoquinolines and (+)-usnic acid, catalyzed by CH_3COOH in anhydrous ethanol at 78℃, and the yields of **F1-F4** ranged between 21%-62%.

Preparation of the intermediates is shown in **Scheme 2**: The aniline is subjected to diazotization to obtain a phenyl azide compound, which is then reacted with propynylamine under the catalysis of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate to obtain intermediates **1a-1h**. After reacting a mixture of benzaldehyde, diethyl phosphite and amine acetate overnight, HCl was adjusted to pH = 1-4, and NaOH was adjusted to pH = 10, and extracted with dichloromethane to obtain intermediates **2a-2e**. Characterization of all target compounds by ^{1}H-NMR spectroscopy before biological evaluation.

3.2 Evaluation of anti-T. gondii activity in vitro and andstructure-activity relationship (SAR) studies

588 The IC_{50} in *T. gondii*-infected HeLa cells and IC_{50} in HeLa cells were determined by the MTT
589 assay and the selectivity index was calculated (**Table 1**). *In vitro*, the selectivity index was
590 calculated using formula: the ratio of the CC_{50} value for host cells not infected with *T. gondii* to the
591 IC_{50} for *T. gondii* cultivated in host cells (SI= CC_{50} / IC_{50}) [50]. It is generally believed that the
592 larger the SI, the stronger the activity against *T. gondii* and the lower the cytotoxicity [47]. To
593 provide references for the subsequent *in vivo* experiments. In this paper, the lead compound used
594 (+)-usnic acid, positive control drugs used spi, pyrimethamine and sulfadiazine. Can be seen from
595 **Table 1**., the cytotoxicity of (+)-usnic acid was lower than spi, and the selectivity index was also
596 higher which indicated a strong anti-*T. gondii* potential. Compound **A** series introduced different
597 substituted aminotriazoles into the lead (+)-usnic acid, and the sequence of selectivity index was as
598 follows: *p*-Br> *p*-CF_3> *p*-F> *o*-Cl> *o*-OCH_3> *p*-OCH_3> *p*-H> *p*-Cl. To some extent, it suggests that
599 for the anti-*T. gondii* activity,it may be beneficial to introducing strong electron withdrawing groups
600 in the para position of the benzene ringl. Among these compounds, the cytotoxicity of the
601 compounds **A3**, **A4**, **A5**, **A6** and **A8** were lower than (+)-usnic acid, and the selectivity index was
602 higher, which indicated more anti-*T. gondii* activity. The compound **B** series introduced different
603 substituted 2-aminoethyl secondary amines into the lead (+)-usnic acid. These compounds showed a
604 significant increased cytotoxicity compared to the lead (+)-usnic acid, and only the compound **B2**
605 which the R group was piperidine substituted had the higher selectivity index than that of (+)-usnic
606 acid. The preliminary structure-activity relationship showd that the nitrogen atoms increasing
607 reduced the anti-*T. gondii activity*, while increasing in carbon atoms seemed to increase the activity.
608 Compound **C** series introduced different substituted phosphoramidates in the lead (+)-usnic acid.
609 In this series, **C1** showd the strongest cytotoxicity, which indicated that the introduction of
610 substituents on the benzene ring was beneficial for cytotoxicity reduction. The **D** series linked four
611 different amino acid methyl esters on the (+)-usnic acid molecule. Among them, the compound **D3**
612 had the highest selectivity index under the premise of low cytotoxicity, The selective index value
613 was greater than 2.77, anti-*T. gondii* activity was significantly better than the three positive control

drugs or the lead, which indicated its value in further research. To some extent, the R group was the most active when it was a fat-soluble benzyl group, and the activity of the R group which contained a secondary amine or a phenolic hydroxyl group was weakened. The compound **E** series linked different substituted benzoquinones on the (+)-usnic acid. The preliminary structure-activity relationship indicated that for anti-*T. gondii* activity, the introduction of an electron withdrawing group at the meta position of the benzene ring may enhance it. Compound **F** series linked different substituted aminoquinolines on the (+)-usnic acid. Among them, compound **F3** had relatively low cytotoxicity and high selectivity index, which indicated its activity was better than the lead or the three positive control drugs.

3.3 Number of tachyzoites in vivo

Assessment of the amount of tachyzoites in the mice peritoneal cavity to verify whether (+)-usnic acid derivatives **D3** and **F3** show the effect against *T. gondii in vivo*. Can be seen from **Table 2** and **Figure 2**, untreated KM mice had 2.25×10^6 tachyzoites in the peritoneum. After 100 mg/kg of spi, (+)-usnic acid, **D3** and **F3** treatment, the tachyzoite inhibition rate in the peritoneal cavity of mice reached to 55.2% ($p < 0.01$), 58.3% ($p < 0.01$), 76.0% ($p < 0.001$) and 64.6% ($p < 0.001$), and the tachyzoites number was significantly reduced. At the same concentration, (+) -usnic acid, **D3** and **F3** inhibited tachyzoites much more effectively than the positive control spi. Moreover, compounds **D3** and **F3** had better anti-*T. gondii* activity than the natural product (+)-usnic acid. These results showed that compounds **D3** and **F3** are effective in inhibiting *T. gondii in vivo*, especially **D3**.

3.4 Liver and spleen indices

Liver has been shown to be the main part of tissue pathology during acute, lethal toxoplasmosis in mice [51], and it is the spleen that plays an irreplaceable role in coordinating

innate immune responses and adaptive [52]. Therefore, liver and spleen indices were used to assess the protective action of drugs on internal organs. Calculate the liver and spleen indices by following formula: viscera index = viscera weight/body weight. Can be seen from **Figure 3**, There were no significant differences in the data between each group in the liver index experiment, and it was not statistically significant. The spleen index of the normal group was significantly lower than the *T. gondii* infection group, it was shown that splenomegaly of organs related to the immune system was caused by acute *T. gondii* infection ($p < 0.05$). After administration in mice infected with *T. gondii*, splenomegaly was reduced than the normal group, especially in the (+)-usnic acid-treated group ($p < 0.05$) and the **D3**-treated group ($p < 0.01$). These indicate that splenomegaly caused by *T. gondii* infection can be effectively alleviated by compound **D3**.

3.5 ALT and AST

The largest gland organ in the human body is the liver. In addition to detoxifying various metabolites and synthesizing proteins and biochemicals necessary for digestion, it also has a central role in the pathophysiology of parasitic infection [53]. The level of serum ALT and AST activity are very sensitive indicators to evaluate liver injury, and their elevation roughly reflects the degree of the damage. ALT and AST levels in KM mice serum were measured to further investigate the toxicity of these compounds (**Figure 4**). Compared with the normal group, mice serum ALT levels in the the *T. gondii* infection group were significantly higher ($p < 0.05$). Serum ALT levels were significantly lower ($p < 0.01$) in the **D3** or **F3** treatment group and were superior to the (+)-usnic acid treatment group. Simultaneously, compared with the normal group, serum AST levels in mice infected with *T. gondii* was significantly higher ($p < 0.001$). Serum AST levels were significantly lower in the **D3** treatment group ($p < 0.01$), and superior to the spi treatment group ($p < 0.01$). These results indicate that compound **D3** can reduce hepatotoxicity while resisting *T. gondii.*

3.6 MDA and GSH

GSH is a special substance for detoxification. It is an important free radical scavenger and antioxidant for the person, combining with heavy metals and free radicals to convert harmful toxins in the body into harmless substances and excrete them out of the body [54]. *In vivo*, free radicals act on lipids to enact a peroxidation reaction producing MDA, which can cause the cross-linking polymerization of other living macromolecules, nucleic acids and proteins, and is cytotoxic. Can be seen from **Figure 5**, the GSH content of the *T. gondii* infection group was significantly lower than the normal group($p < 0.05$). Compared with the *T. gondii* infection group, the GSH content of the compounds **F3** and **D3** treatment group was significantly increased ($p < 0.001$), and was also better than the normal group ($p < 0.05$), and the (+)-usnic acid treatment group ($p < 0.01$, $p < 0.001$). *T. gondii* infection caused a significant increase in MDA levels ($p < 0.01$). Compared with the *T. gondii* infection group, compound **D3** could significantly decrease the MDA content ($p < 0.01$), which was the same as spi treatment. These results indicate that compound **D3** exerts a strong antioxidant effect against *T. gondii* infection.

3.7 Histological Analysis

To determine the effect of the compounds on liver, we observed the general changes of the liver. The color of the livers from the normal group were dark red, while the livers from infected mouse displayed a white surface. The livers from the groups under treatment showed different degrees of whitening, and (+)-usnic acid group showed the least significant effect. It indicated that the (+)-usnic might have potentials on liver protection during acute infection (data not shown). Furthermore, pathological analysis of sections of livers of mice infected with *T. gondii* to compare the anti-*T. gondii* effect of (+)-usnic acid and its derivatives with the spi-treated positive group (**Figure 6.**, **Table 3**.). Staining tissue samples from mice infected with *T. gondii* with H&E. Liver specimens from the negative control group showed a moderate level of granuloma; the amount of

granuloma were 4/10 HPF (High Power Field), and 1 cyst/50 HPF. In the spi treatment group, granuloma levels were mild: the amount of granuloma were 2/10 HPF, and 0 cysts/50 HPF. In the (+)-usnic acid treatment group, granuloma levels were negative: 0/10 HPF, and 0 cysts/50 HPF. This was better than the spi group, which showed mild levels of granuloma. In the **D3** group, granuloma levels were mild: 1/10 HPF, and 0 cysts/50 HPF. In the **F3** group, granuloma levels were mild: 2/10 HPF, and 0 cysts/50 HPF. These results suggested that (+)-usnic acid and its derivatives had the same inhibitory effect as spi on cyst development in the liver, and displayed a significant anti-granuloma effects on *T. gondii*-infected mice.

3.8 Weight Increment

The weight increment was calculated in the experiment to assess the effect of the compounds on body weight. Can be seen from **Figure 7.**, for the *T. gondii* infection group, there is no significant difference in weight gain compared with normal group, indicated that *T. gondii* infection had no significant effect on the change of body weights increment. But, compared with normal group and *T. gondii* infect group, spi group could be significantly reduce the mouse weight increment ($p < 0.001$), indicates that spi could have a significant impact on weight increment. Spi may inhibit lipogenesis and effectively reduce HFD-induced obesity and hepatic steatosis。 It should be noted that spi could inhibit adipogenesis and hepatic steatosis and effectively attenuates HFD-induced obesity [55]. For the **D3** treatment group, the body weight of the mice was significantly lower than the normal group ($p < 0.001$), and the body weight was also significantly lower than that of the Toxoplasma infected group ($p < 0.01$). For the **F3** treatment group, the body weight of the **F3** group was significantly lower than the normal group ($p < 0.01$). However, the body weight of the mice was increased to some extent than the spi group ($p < 0.05$). For the (+)-usnic acid-treated group, (+)-usnic acid showed a significant increase in body weight compared

713 with the spi group ($p < 0.01$). Moreover, compared with the normal group, there was no significant
714 difference in body weight, indicating that (+)-usnic acid has good research value.

715

716 In the present study, In order to change the structure of (+)-usnic acid, six different structural
717 fragments were used, and synthesize 30 unique (+)-usnic acid derivatives. ^{1}H-NMR, ^{13}C-NMR, and
718 high-resolution mass spectrometry were used to identify their structures. Finally, the anti-*T. gondii*
719 effects of each compound were evaluated. *In vitro* studies, most of the compounds exhibited
720 stronger anti-*T. gondii* activity and lower cytotoxicity than that of (+)-usnic acid. In particular, *in*
721 *vitro* and *in vivo* studies, compound **D3** shown the most notable anti-*T. gondii* activity and lower
722 cytotoxicity and was superior to (+)-usnic acid and spi. Hence, these (+)-usnic acid-derived
723 compounds are likely to be used as anti-parasitic drugs and are for further study.

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Author Contributions

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Notes

The authors declare no competing financial interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 21662036, 81160409).

The authors are grateful to Dr Longzhe Han(Yanbian Hospital) for his help.

Abbreviations used

T. gondii, Toxoplasma gondii; SI, selectivity index; TLC, thin-layer chromatography; $CDCl_3$, deuterated chloroform; OD, optical density; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, glutathione; MDA, malonaldehyde; spi, spiramycin; H&E, hematoxylin and eosin; HPF, High Power Field; MTT, methyl thiazolyl tetrazolium; SD, standard deviation;

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Table 1. The selectivity of compounds in HeLa cells.

Compd	IC_{50}[a] in Hela cells (μM)	IC_{50}[b] in *T. gondii*-infected HeLa cells (μM)	SI[c]
A1	173.9±2.7	425.9±5.3	0.41
A2	175.3±2.4	>1000	-
A3	358.1±11.2	301.7±2.6	1.19
A4	445.5±7.5	364.2±5.8	1.22
A5	349.8±8.3	261.0±3.4	1.34
A6	290.5±13.2	233.2±0.3	1.25
A7	312.8±5.1	396.1±18.2	0.79
A8	408.0±2.3	359.5±17.9	1.13
B1	104.4±2.9	149.4±24.4	0.70
B2	198.0±2.7	190.9±1.0	1.04
B3	83.1±0.5	156.2±27.7	0.53
B4	31.8±1.5	40.0±5.1	0.79
B5	169.1±2.0	195.8±28.4	0.86
C1	191.7±2.8	266.8±8.4	0.72
C2	793.9±4.7	648.9±0.4	1.22

C3	366.1±7.6	>1000	-
C4	684.1±6.2	518.9±9.0	1.32
C5	395.8±5.2	909.6±0.3	0.44
D1	172.3±3.5	229.6±6.8	0.75
D2	265.2±5.8	395.4±18.2	0.67
D3	**>1000**	**358.8±6.6**	**>2.77**
D4	253.6±6.4	297.7±19.8	0.85
E1	237.2±1.1	290.6±23.0	0.82
E2	233.2±2.8	231.9±19.6	1.01
E3	242.6±2.9	271.1±2.3	0.89
E4	309.0±1.1	315.7±0.3	0.98
F1	216.9±0.5	481.3±21.4	0.45
F2	234.5±1.8	213.1±11.1	1.10
F3	**708.9±6.5**	**573.1±4.1**	**1.24**
F4	474.9±3.5	470.8±26.1	1.01
Spiramycin	189.0±1.5	262.2±7.5	0.72
sulfadiazine	368.0±4.2	318.7±2.6	1.15
pyrimethamine	760.2±1.8	850.5±4.3	0.89

(+)-usnic acid	216.5±3.8	225.7±3.8	0.96

Each value is expressed as the mean ± SD (n = 3)

a: IC_{50} in HeLa cells = Concentration required to reduce HeLa cells growth by 50%..

b: IC_{50} in *T. gondii*-infected HeLa cells = Concentration required to reduce *T. gondii*-infected HeLa cells growth by 50%.

c: SI = Selectivity index, a measure of efficacy, calculated by IC_{50} in HeLa cells/IC_{50} in *T. gondii*-infected HeLa cells.

Table 2. *In vivo* anti-*T. gondii* activity

	Spi[b]	(+)-usnic acid	D3	F3
Inhibition rate[a]	55.2% ± 4.7%	58.3% ± 3.1%	76.0% ± 3.9%	64.6% ± 1.5%

Each value is expressed as the mean ± SD (n = 6)

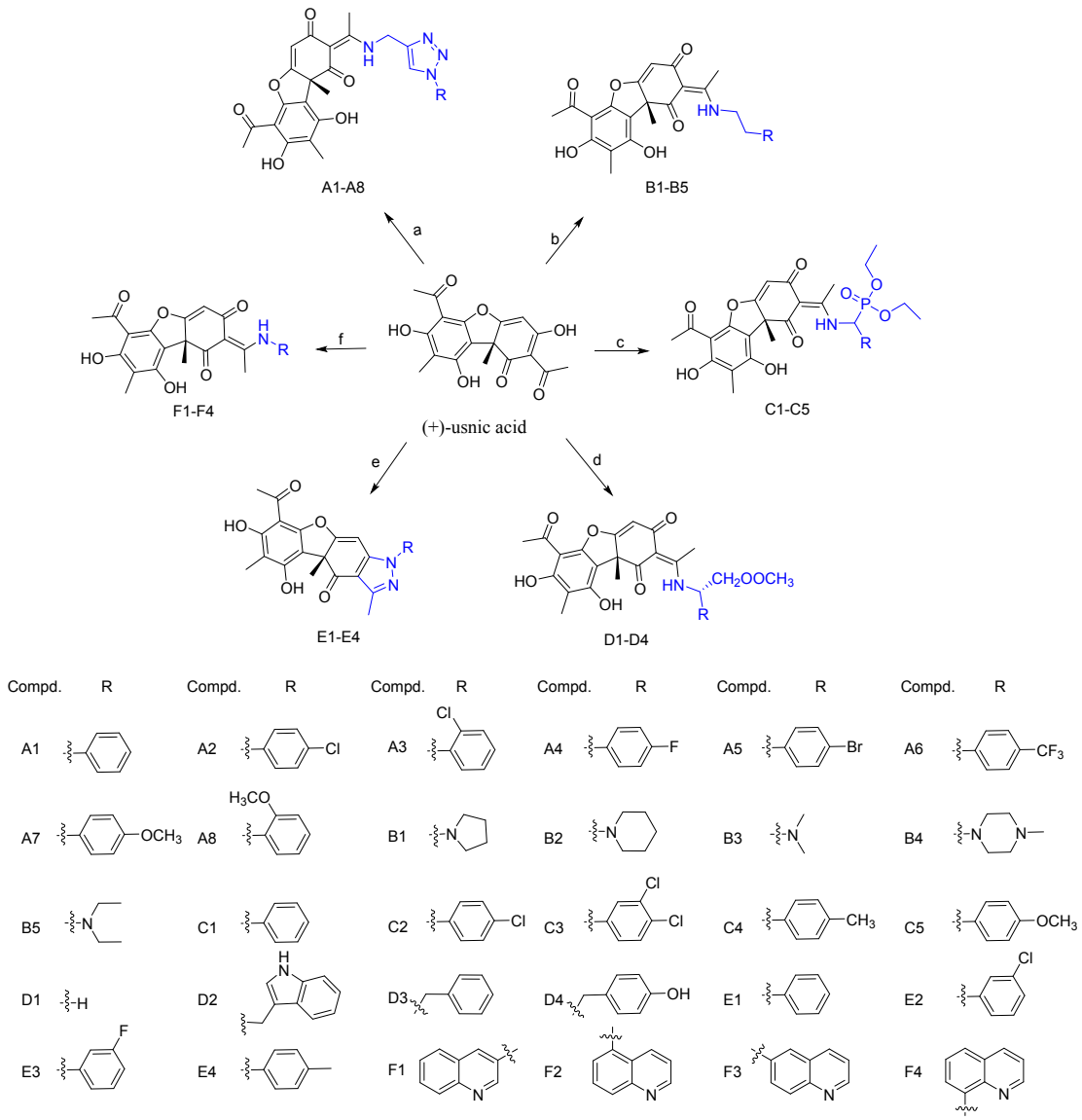
a: Mice peritoneal *T. gondii* inhibition rate, calculated by (untreated group - treated group) / untreated group × 100%

b：Spiramycin

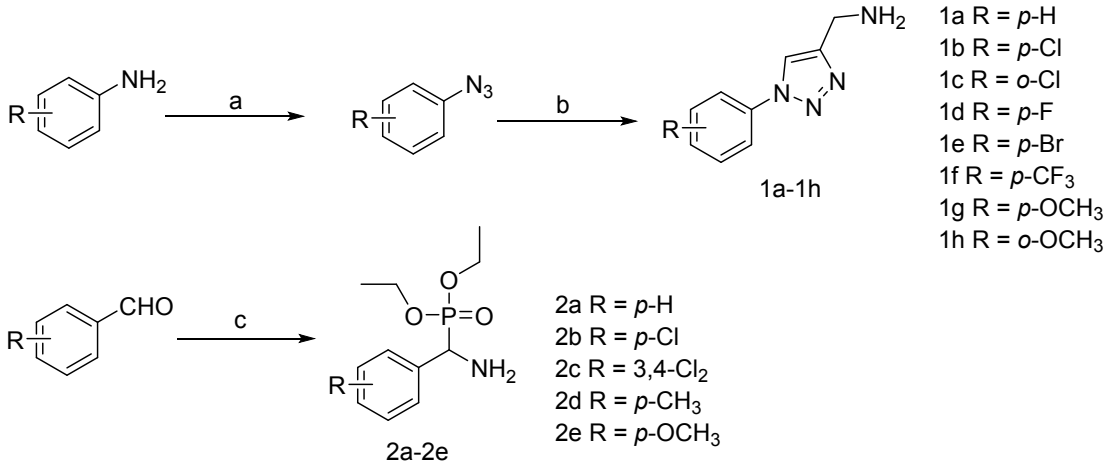
Table 3. Histological Analysis in *T. gondii*-Inoculated Mice

Group	Liver	
	Amount of Gr. (/10HPF)	Amount of cyst (/50HPF)
Normal	Negative/0	0
T.G.+D.W.	Moderate/4	1
T.G.+Spi	Mild/2	0
T.G.+(+)-usnic acid	Negative/0	0
T.G.+D3	Mild/1	0
T.G.+F3	Mild/2	0

Degree of granuloma: negative: none/10HPF; mild: 1-3/10HPF; moderate: 4-6/10HPF; severe: > 6/10HPF; HPF: high power fifield. Gr.: granuloma; T.G.: *T. gondii*; D.W.: distilled water; spi: spiramycin; D3: (+)-usnic acid derivative; F3: (+)-usnic acid derivative.



Scheme 1. Reagents and conditions: (a) **1a-1h**, CH_3CH_2OH, 78℃, 2-3 h. (b) different 2-aminoethyl secondary amine, CH_3CH_2OH, 78℃, 2-3 h. (c) **2a-2e**, CH_3CH_2OH, 78℃, 2-3 h. (d) different L-amino acid methyl ester, Et_3N, CH_3CH_2OH/H_2O, 78℃, 3-5 h. (e) different phenylhydrazine hydrochloride, Et_3N, CH_3CH_2OH, 78℃, 3-5 h. (f) different aminoquinoline, CH_3COOH, CH_3CH_2OH, 78℃, 12 h.



Scheme 2. Reagents and conditions: (a) $NaNO_2$, NaN_3, 10%HCl, rt, 2 h. (b) propynylamine, $CuSO_4 \bullet 5H_2O$, sodium ascorbate, t-BuOH/H_2O (1:1), 30℃, overnight. (c) diethyl phosphite, CH_3COONH_4, 90℃, overnight.

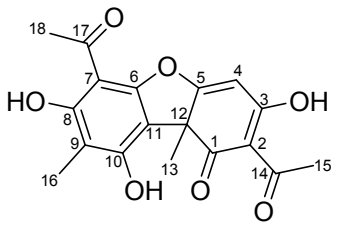


Figure 1. The chemical structure and atom number of usnic acid

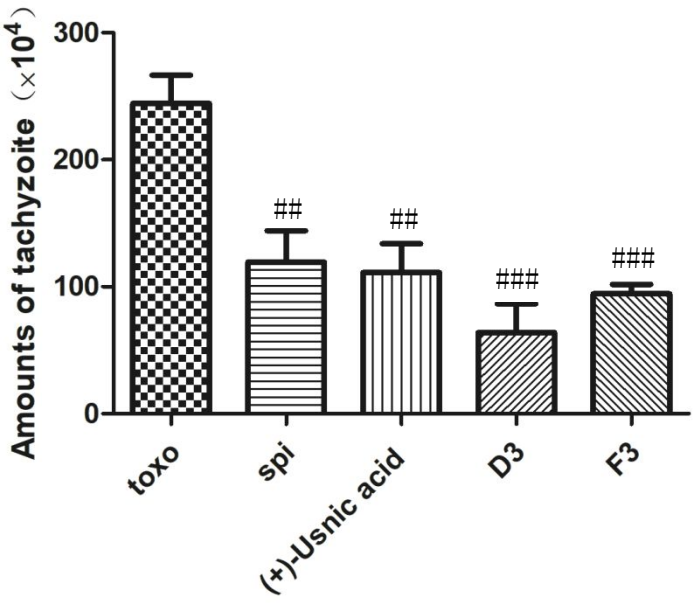


Figure 2. Effect of compounds on the number of tachyzoites in mice, ##$p < 0.01$ compared with toxo group; ###$p < 0.001$ compared with toxo group.

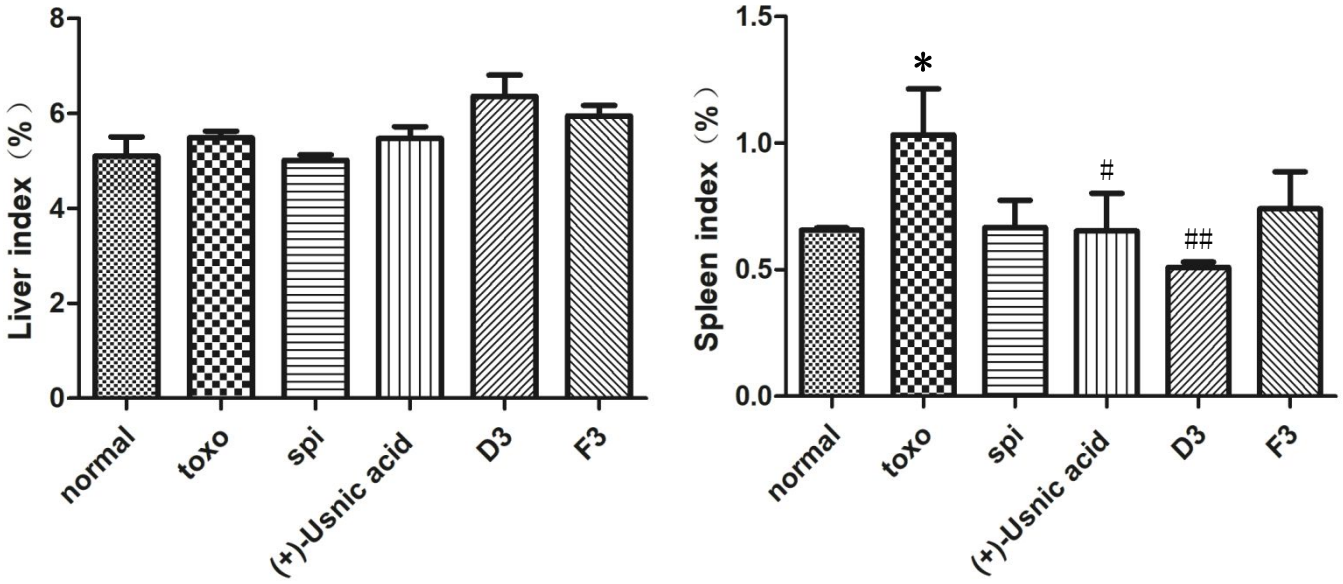


Figure 3. Effect of compounds on spleenand liver weights in *T.gondii*-infected KM mice, *$p < 0.05$ compared with normal group; #$p < 0.05$ compared with toxo group; ##$p < 0.01$ compared with toxo group

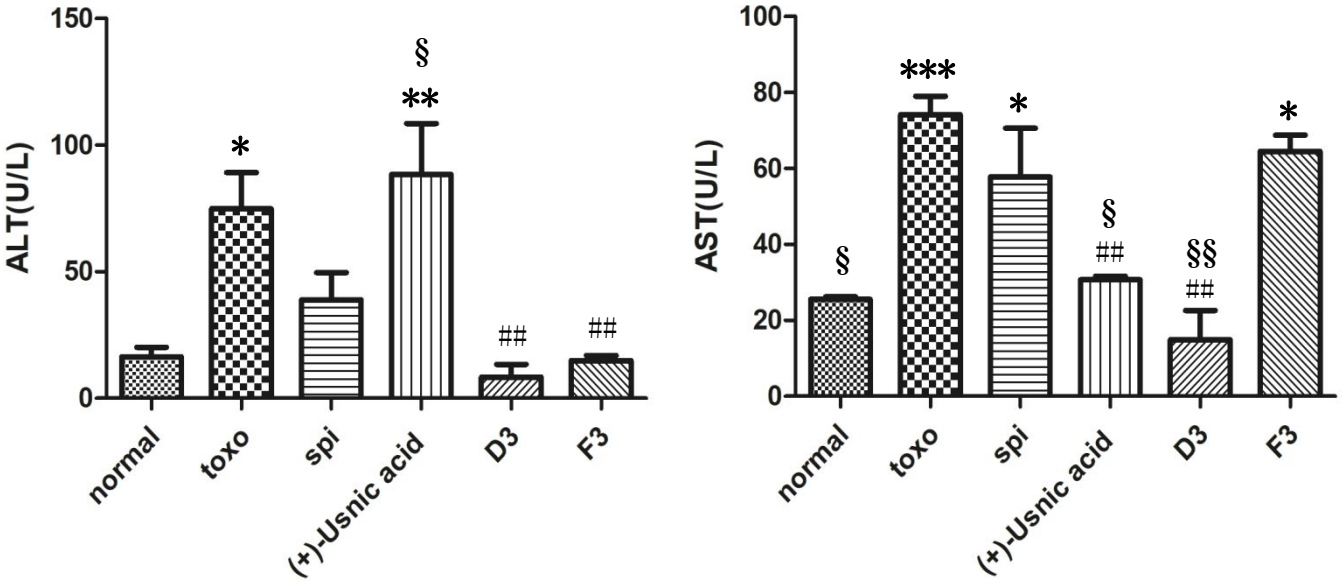


Figure 4. Effect of compounds on ALT and AST levels in *T.gondii*-infected KM mice, *$p < 0.05$ compared with normal group; **$p < 0.01$ compared with normal group; ***$p < 0.001$ compared with normal group; #$p < 0.05$ compared with toxo group; ##$p < 0.01$ compared with toxo group; ###$p < 0.001$ compared with toxo group; §$p < 0.05$ compared with spi group; §§$p < 0.01$ compared with spi group.

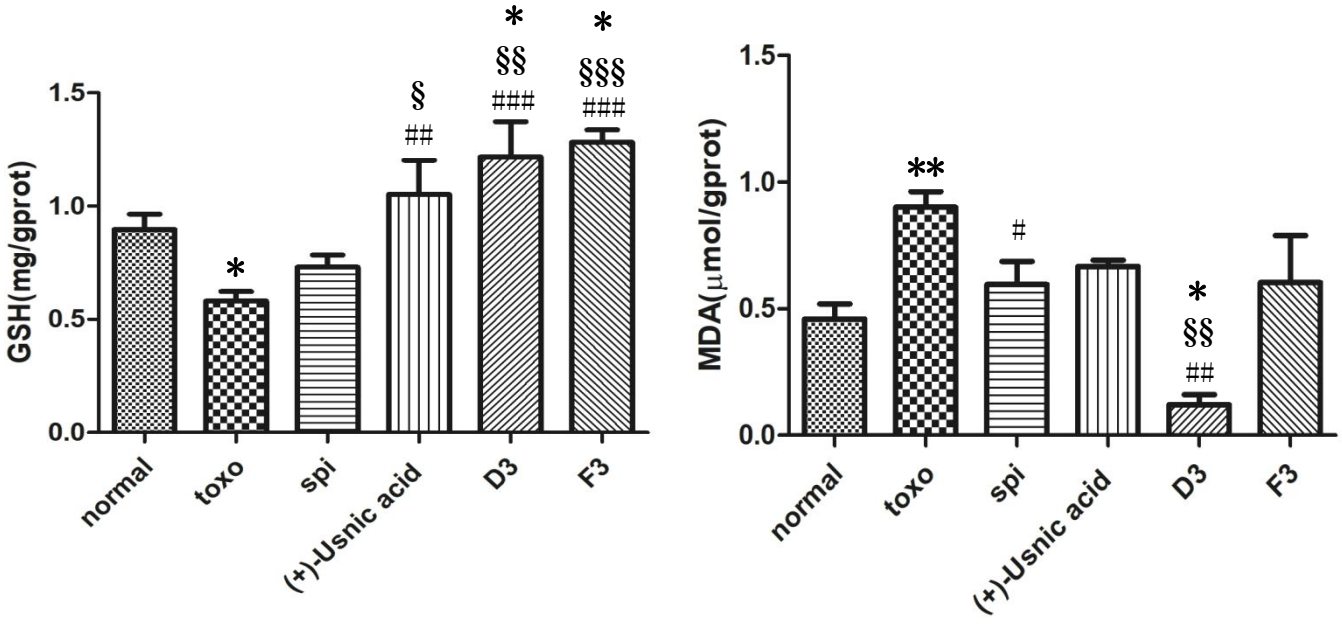


Figure 5. Effect of compounds on GSH and MDA levels in *T.gondii*-infected KM mice,*$p < 0.05$ compared with normal group; **$p < 0.01$ compared with normal group; #$p < 0.05$ compared with toxo group; ##$p < 0.01$ compared with toxo group; ###$p < 0.001$ compared with toxo group; §$p < 0.05$ compared with spi group; §§$p < 0.01$ compared with spi group; §§§$p < 0.001$ compared with spi group.

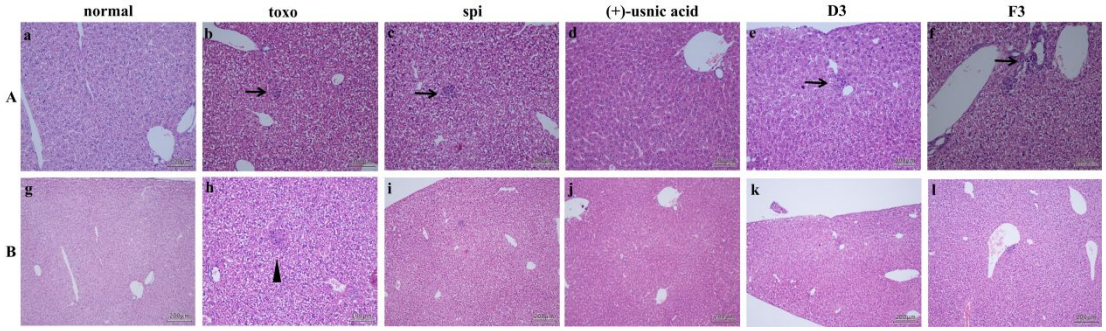


Figure 6. Histopathological Photos of Mice Livers 4 d after Treatment with Chemicals (H&E Stain)

A: H&E Stain photograph of granulomatou; B: H&E Stain photograph of cyst. a and g: Normal liver parenchyma without granulomas or cysts in the control group. b and h: *T. gondii*-infected group shows moderate degree of granulomatous inflammation with a cyst (2/50 HPF) in the liver; c and i: spi-treated group shows a decrease in the degree of granulomatous inflammation in the liver; d and g: The (+)-usnic acid-treated group also shows a decrease in the degree of granulomatous Inflammation in the liver, and to a greater extent than the spi-treated group; e and k: The **D3**-treated group shows a decrease in granulomatous inflammation in the liver; f and l: The **F3**-treated group shows a decrease in granulomatous inflammation in the liver, and to a same extent than the spi-treated group. g, i, j, k and l are × 100; others are × 200. Arrow indicates granulomatous and triangle indicates cyst. HPF: High Power Field.

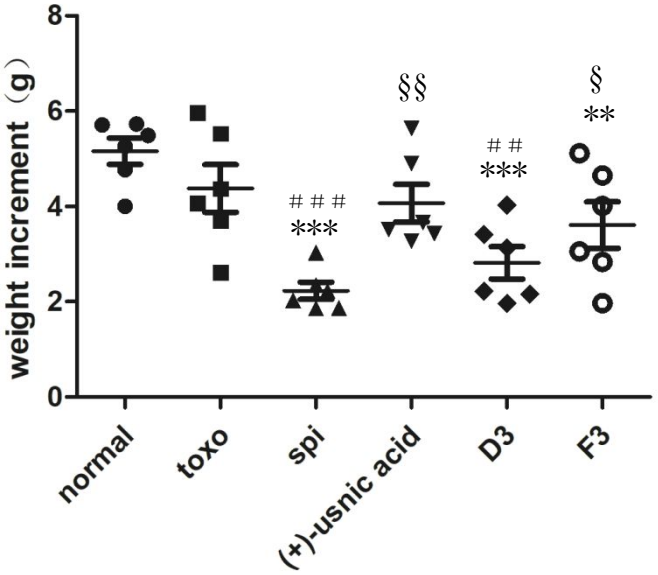
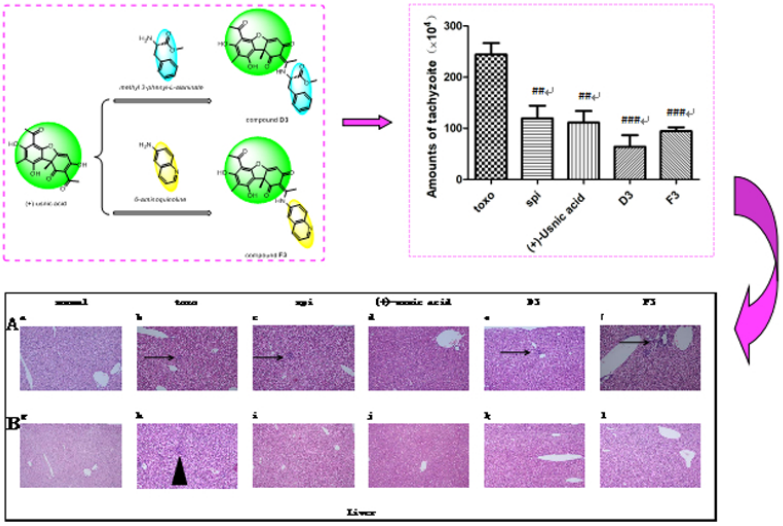


Figure 7. Effect of compounds on weight increment in mice, $^{**}p < 0.01$ compared with normal group; $^{***}p < 0.001$ compared with normal group; $\#\#p < 0.01$ compared with toxo group; $\#\#\#p < 0.001$ compared with toxo group; $\S p < 0.05$ compared with spi group; $\S\S p < 0.01$ compared with spi group.



82x44mm (300 x 300 DPI)