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Evaluation of In Vitro Bio-Activities Effects of WST (Wushanshencha)

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Abstract: As a traditional Chinese drink, tea is favored for its rich flavor and its medicinal functionality. In this study, the in vitro bioactivities of Wushanshencha (WST; a local tea from Chongqing, China), which is processed mainly from the leaves of the wild *Malus hupehensis* (Pamp.) Rehd.). We assessed the scavenging capacity of tea extracts on 1, 1-diphenyl-2-picrylhydrazyl (DPPH); 2, 2′-azino-bis (3-ethylbenzthiazoline-6- sulphonic acid) diammonium salt (ABTS); and hydroxyl (OH) free radicals, and demonstrate the high antioxidant activity and dose-dependent relationship of these extracts. We also detail the anti-mutagenic effect of these tea extracts against the *Salmonella typhimurium* TA98 strain induced by the 2, 7-diaminofluorene (2, 7-AF) mutagen and the TA100 strain induced by the N-methyl-N′-nitro- N- nitrosoguanidine (MNNG) mutagen at concentrations of 1.25 and 2.50 mg/plate, respectively, with the high-dose groups showing better results. We investigated the anticancer mechanisms of WST extracts (40, 100, and 160 μg/mL) in HepG2 human hepatoma cells via 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay and quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR). The results showed that the proliferation of HepG2 cells was significantly inhibited in a dose-dependent manner by the tea extracts. Moreover, apoptosis in HepG2 cells was induced via upregulation of Caspase-3, Caspase-7, Caspase-8, Caspase-9, p21, p53, and Bax as well as downregulation of Bcl-2 apoptosis-associated factors, as assessed via mRNA expression levels after treating with WST extracts. The expression of inflammation-related factors, e.g., NF-κB, and Cox-2, was significantly downregulated by the WST extracts, demonstrating its inflammatory properties. Together, these observations indicated that WST extracts have anti-inflammatory and anti-cancer properties. In addition, high-performance liquid chromatography (HPLC) analysis showed that WST extracts contained chlorogenic acid, 4-hydroxycinnamic acid, isoquercitrin, taxifolin, quercitrin, rosmarinic acid, myricetin, baicalin, neosperidin dihydrochalcone, and quercetin. As such, WST appears to be an effectively functional drink, due to its rich functional components and anti-cancer activity.

Keywords: Wushanshencha; anti-oxidant; anti-mutagenic; anti-cancer; high-performance liquid chromatography

1. Introduction

WST comes from the Three Gorges Basin of the Yangtze River in Wushan, Chongqing, China. This tea is mainly made from the young leaves of native wild *Malus hupehensis* (Pamp.) Rehd., and its leaves grow on the top of cliffs in a primeval, pollution-free forest environment. These raw leaves are dried at the appropriate temperature and humidity, and with their own fermentation and a series of complex chemical changes, they form a unique flavor and mouthfeel. This tea is also known as "longevity tea" by the local people [1]. The raw leaves used for processing this tea was reported to contain a variety of trace elements beneficial to the human body as well as multiple amino acids and flavonoids, and it has demonstrated many health functions, including weight loss, lowering of blood pressure, soothing of nerves, strengthening of teeth, bacteriostasis, cancer prevention, anti-aging, and improved immunity [2,3]. However, the functional application scope of WST is still relatively unclear; there is little research about its extracts on functional effect, and even less research on its specific active compounds. This research is the first to study the functions and potential active compounds of WST.

The aerobic metabolism of the human body is inseparable from energy conversion, and free radicals serve as carriers during energy transfer. Free radicals are independent chemical substances with unpaired electrons, and when the amount of free radicals exceeds the range of the antioxidant protection system, excessive oxidation occurs, promoting free radicals to attack the cells of the body to acquire electrons in order to maintain their own stability, causing damage to the conformation and function of cells and triggering inflammation, molecular degradation, and other types of diseases, such as cardiovascular disease, neurologic disease, and cancer [4,5]. In addition to the body's own resistance system (such as antioxidant enzymes, proteins, and other molecules), antioxidants can also capture and stabilize free radicals, such as ascorbic acid, vitamin E, various polyphenols, and other exogenous small molecules [6], thus protecting the cells from free radical damage. As a traditional Chinese drink, this tea appears to be rich in tea polyphenols and can provide antioxidants to alleviate the damage caused by free radicals [7].

Prevention of chromosomal aberration and gene mutation is important for preventing tumorigenesis [8]. Environmental pollution is a principal mutagen in the pathogenesis of tumors, though it is difficult to eliminate the causes of such contamination [9]. As an important segment of natural products, tea extracts are available in a wide range of types and present a broad range of efficacies and research values [10], especially in regard to anti-mutagen activities.

Hepatocellular carcinoma (HCC) is a common malignant disease with a high morbidity and mortality around the world, and the morbidity and mortality of HCC in China account for about half of that occurring throughout the world [11]. At present, there is no effective treatment for liver cancer, and its mortality rate can only be reduced by early detection, diagnosis, and treatment. The bioactive components in traditional Chinese medicine and some foods have been shown to prevent and treat different types of cancer by improving the body's immunity. For example, drinking tea can benefit liver cancer patients in regard to surgery, chemotherapy, and radiotherapy outcomes [12].

In this context, WST extracts were used as a raw material to explore their effects regarding anti-oxidant and anti-mutagenic activities. Additionally, in vitro anti-cancer experiments on human hepatoma HepG2 cells utilizing these extracts were assessed, and their potential bioactive compounds were investigated via HPLC.

2. Materials and Methods

2.1. Preparation of WST Extracts

WST was purchased from the Xiajiang Tea Co., Ltd. (Xiajiang Tea Co., Ltd., Wushan, Chongqing, China), freeze-dried, and subsequently ground into separate fine powders. A ten-fold volume of 70% ethanol was added to the powdered samples and extracted twice at 60 °C for 2 h. The two extracts were then merged and filtered using a FL-3 macroporous resin. The extract was then evaporated to remove the water and ethanol using a rotary evaporator (N-1100; Eyela; Tokyo, Japan) and then freeze-dried and stored at 4 °C until required.

2.2. DPPH Free-Radical Scavenging Activity

A total of 1.0 mL of different concentrations of WST extract (0.0, 0.2, 0.6, 1.0 mg/mL) was added to 2.0 mL of DPPH ethanol solution (0.63 mM). Afterwards, the solution was mixed gently and left in the dark at room temperature for 30 min. The absorbance of the solution was determined at 517 nm using a UV-visible spectrophotometer (BioMate 3S; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sample absorbance and background absorbance were then deducted. Ascorbic acid (0.2 mg/mL) was used as positive control. The measurements were performed three times and averaged [13].

2.3. OH Free-Radical Scavenging Activity

The phenanthroline colorimetry method was adopted [14] for use in this study. The reaction system consisted of 2.0 mL of PBS solution (pH 7.4, 0.01 M), 1.0 mL of phenanthroline reagent (7.50 M), 1.0 mL of ferrous sulfate solution (7.50 M), 3.0 mL of WST extract (0.0, 0.2, 0.6, 1.0 mg/mL), and 2.0 mL of hydrogen peroxide solution (0.06%), which were added in the above order. The mixed solution was bathed at 37 °C for 60 min, and the absorbance was measured at 536 nm. The sample absorbance and background absorbance were deducted. Ascorbic acid (0.2 mg/mL) was used as positive control. These experimental procedures were repeated three times.

2.4. ABTS Free-Radical Scavenging Activity

The ABTS reagent (6.83 mM) was mixed (volume, 1:1) with $K_2S_2O_8$ (2.47 mM), oxidized for 12 h at room temperature in the dark, and diluted with absolute ethanol to an absorbance of 0.7 ± 0.02 (734 nm). A total of 1.0 mL of different concentrations of the WST extract (0.0, 0.2, 0.6, 1.0 mg/mL) was added to 2.0 mL of the above ABTS working solution, mixed, and placed in a dark environment for 10 min at room temperature. Absorption was measured at 734 nm. The sample absorbance and background absorbance were deducted. Ascorbic acid (0.2 mg/mL) was used as positive control. These experimental procedures were repeated three times [15].

2.5. Anti-Mutagenic Experiments

The Ames test is widely recognized as a short-term biological test for detecting mutagenesis [16]. *Salmonella typhimurium* strains TA98 (0.80 μg/plate of the 2, 7-AF mutagen) and TA100 (0.40 μg/plate of the MNNG mutagen) were purchased from the Beijing Institute of Beina Chuanglian Biotechnology (BNCC, Beijing, China) and used as the experimental strains. The routine identification of these bacteria was qualified. In brief, 0.5 mL phosphate buffer (0.2 mol/L, pH 7.4), 0.1 mL overnight culture (1-2 × 10^9 cells/ml), 0.1 mL sample solution at different concentrations (1.25, 2.5 mg/plate), 0.1 mL direct mutagen, and 2.0 mL top agar culture medium (containing L-histidine, D-biotin and pre-warmed at 45 °C) were added to sterilized capped tubes and mixed for 5 s. Mutagens were not added to the spontaneous mutation experiments. The mixture was evenly distributed on the bottom agar medium. The plates were then turned over after solidification and incubated at 37 °C for 48 h. The total number of bacteria on each plate was counted. There were three plates in each group [16].

2.6. Cell Culture Preparation

Human hepatoma HepG2 cells obtained from the Shanghai Institute of Biochemistry and Cell Biology (SIBCB, Shanghai, China) were used for the anti-cancer tests. The cancer cells were distributed in RPMI-1640 medium (containing 10% fetal bovine serum and 1% penicillin-streptomycin bi-antibody solution) and cultured in a saturated moist environment of 37 °C and 5% carbon dioxide (model 311 S/N29035; Forma, Waltham, MA, USA). The medium was replaced two to three times a week.

2.7. MTT Assays

A suspension of human hepatoma HepG2 cells (180 μL, 1×10^4 cells/mL) was added to 96-well plates and incubated for 24 h to allow the cells to adhere. Different concentrations of WST solution (0–220 μg/mL) were added to the suspension and incubated at 37 °C in 5% CO_2 for 48 h. A total of 20 μL of MTT (Ekear; Shanghai, China) reagent with a concentration of 5 mg/mL was added to each well, and the cells were then cultured for 4 h under the same conditions. The supernatant was discarded, and 150 μL of dimethyl sulfoxide (DMSO) reagent was added to each well and mixed for 30 min in dark conditions at a constant temperature of 40 °C. The absorbance of each well was detected using an enzyme-linked immunosorbent assay reader (model 680; Bio-Rad, Hercules, CA, USA) at 490 nm and processed to calculate the cell viability [17,18].

2.8. RT-qPCR Assays

Human hepatoma HepG2 cells (treated by WST extracts for 48 h) were inoculated into six-well plates (1×10^5 cells/well) and treated with different concentrations of WST extracts. Total RNA from the HepG2 cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, and the concentration and purity of the RNA were determined at 260 nm using a micro ultraviolet visible spectrophotometer. The total RNA concentration of each group was adjusted to the same level. According to the manufacturer's suggestion (RevertAid First Strand cDNA Synthesis Kit; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 μL of oligo (dT)18 primer (500 ng) and 1.0 μL of total RNA (1.0 μg) were added to 10.0 μL of nuclease-free water and heated at 65 °C for 5 min on a gradient PCR instrument. Then, the mixed reagent containing 4.0 μL of 5× Reaction Buffer, 1.0 μL of Ribolock RNase Inhibitor (20 U), 2.0 μL of 10 mM dNTP Mix, and 1.0 μL of ReverAid Reverse Transcriptase (200 u/μL) were added into the mentioned total RNA system, respectively, and reverse transcribed into cDNA under the condition of 60 min at 42 °C and 5 min at 70 °C. The total reaction system (20 μL) consisted of 1.0 μL of cDNA, 1 μL of forward and reverse primers (10 μM), respectively, 10.0 μL of premix (SYBR®Select Master Mix; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 7.0 μL of sterilized double-steamed water, which was mixed and reacted on an automatic thermocycler (StepOnePlus; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The amplification conditions were as follows: denatured at 95 °C for 3 min, annealed at 60 °C for 30 s, and extended at 95°C for 1 min, which was cycled 40 times. The expression levels of Caspase-3, -7, -8, -9, Bax, Bcl-2, NF-κB, COX-2, p21, and p53 were respectively assessed by RT-qPCR. The sequences of the related primers are shown in Table 1. The cDNA samples for each gene were amplified three times in parallel, and the mean value of Ct was taken. The housekeeping gene GAPDH was used as an internal reference gene, and the levels of the related genes were calculated according to $2^{-\Delta\Delta CT}$ [19].

Table 1. The sequences of reverse transcription-polymerase chain reaction primers used in this study.

Accession Number	Gene Name	Sequence
NM_004346.3	*Caspase-3*	Forward: 5′- CAT GGA AGC GAA TCA ATG GAC T-3′ Reverse: 5′-CTG TAC CAG ACC GAG ATG TCA-3′
NM_033339.4	*Caspase-7*	Forward: 5′-CGG TCC TCG TTT GTA CCG TC-3′ Reverse: 5′-CGC CCA TAC CTG TCA CTT TAT CA-3′
NM_001228.4	*Caspase-8*	Forward: 5′-ATT TTG AGA TCA AGC CCC ACG-3′ Reverse: 5′-GGA TAC AGC AGA TGA AGC AGT CC-3′
NM_032996.3	*Caspase-9*	Forward: 5′-CTC AGA CCA GAG ATT CGC AAA C-3′ Reverse: 5′-GCA TTT CCC CTC AAA CTC TCA A-3′
NM_078467.2	*P21*	Forward: 5′-TGT CCG TCA GAA CCC ATG C-3′ Reverse: 5′-AAA GTC GAA GTT CCA TCG CTC-3′
NM_000546.5	*P53*	Forward: 5′-CTT TGA GGT GCG TGT TTG TGC-3′ Reverse: 5′-GGT TTC TTC TTT GGC TGG GGA-3′
NM_004324.3	*Bax*	Forward: 5′- TCC ACC AAG AAG CTG AGC GAG-3′ Reverse: 5′- GTC CAG CCC ATG ATG GTT CT -3′
NM_000633.2	*Bcl-2*	Forward: 5′- ATG TGT GTG GAG AGC GTC AAC C-3′ Reverse: 5′- CAG AGA CAG CCA GGA GAA ATC AA-3′
NM_003998.3	*NF-κB*	Forward: 5′- GAA GCA CGA ATG ACA GAG GC-3′ Reverse: 5′- GCT TGG CGG ATT AGC TCT TTT-3′
NM_000963.3	*COX-2*	Forward: 5′- CTG GCG CTC AGC CAT ACA G-3′ Reverse: 5′- CGC ACT TAT ACT GGT CAA ATC CC-3′
NM_002046.7	*GAPDH*	Forward: 5′- TCA AGA AGG TGG TGA AGC AGG-3′ Reverse: 5′- AGC GTC AAA GGT GGA GGA GTG-3′

p21, cyclin-dependent kinase inhibitor 1A; p53, cellular tumor antigen p53; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; NF-κB, nuclear factor-kappa-light-chain-enhancer of activated B cells; COX-2, cyclooxygenase-2.

2.9. Chemical Standards

Chlorogenic acid, 4-hydroxycinnamic acid, and rosmarinic acid were obtained from Fluka Chemical Co. (Buchs, Switzerland). Myricetin and baicalin were purchased from Solarbio Technology Co. (Beijing, China). Isoquercitrin, taxifolin, quercitrin, neosperidin dihydrochalcone, and quercetin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.10. HPLC Analysis

The WST extract was dissolved in DMSO to obtain a solution with a concentration of 10 mg/mL and diluted with 50% methanol to produce a final concentration of 2.5 mg/mL. The sample was passed through a 0.22-μm organic filter before testing. Ten microliters of the diluted WST sample solution was analyzed using an UltiMate3000 HPLC System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and separated using an Accucore C18 column (2.6 μm, 4.6 × 150 mm; Thermo, America). Mobile phase A was water containing 0.5% acetic acid, and mobile phase B was acetonitrile. The flow rate was 0.5 mL/min with a column temperature of 30 °C, and the detection wavelength was 359 nm. The gradient elution conditions were as follows: 0–30 min with 12–45% B (linear gradient), 30–35 min with 45–100% B (linear gradient), and 35–40 min with 100% B (isocratic).

2.11. Statistical Analysis

The SPSS version 20.0 statistical software (SPSS Inc, Chicago, Illinois, USA) was used to analyze the experimental data. The results were analyzed via one-way analysis of variance (ANOVA) with Duncan's multiple range tests, and $p < 0.05$ was considered statistically significant. All experiments were repeated three times, and the data were presented as the mean ± standard deviation (SD).

3. Results

3.1. Anti-Oxidant Activities of WST Extracts

The scavenging ability of the WST extracts on DPPH radicals (Figure 1A) were assessed at concentrations of 0.2, 0.6, and 1.0 mg/mL, demonstrating radical scavenging activity values of 25.9%, 53.5%, and 78.6%, respectively. This indicated that the extracts had a dose-dependent relationship with the scavenging capacity towards DPPH free radicals. The scavenging ability of the WST extracts to hydroxyl free radical was determined using the phenanthroline-Fe^{2+} method (Figure 1B). The scavenging rate of 1.0 mg/ml extracts towards hydroxyl radicals was 56.1%, higher than that at 0.2 mg/mL (9.9%) or 0.6 mg/mL (30.8%). ABTS can be oxidized by active oxygen to form the blue-green cation ABTS+. After adding an antioxidant-active substance, the antioxidant reacts with ABTS+ to discolor the system. The ABTS radical scavenging ability (Figure 1C) of the WST extracts in the concentration range of 0.2-1.0 mg/mL ranged from 25.5% to 65.6%, which was significantly different from the controls ($p < 0.05$).

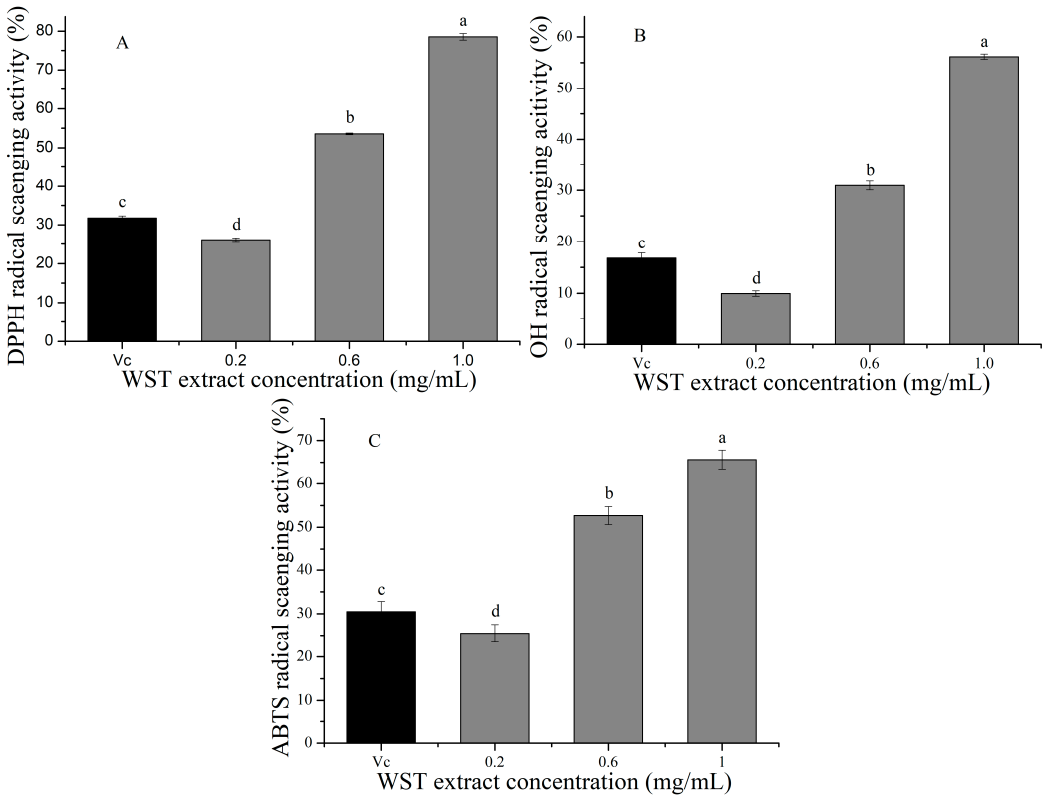

Figure 1. Scavenging activity of Wushanshencha (WST) extracts towards 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), hydroxyl radicals (OH), and 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt radicals (ABTS). (**A**): DPPH radical; (**B**): OH radical; (**C**): ABTS radical. Ascorbic acid was used as positive control in anti-oxidant experiments. $^{a–d}$ Mean values with different letters in the same bar graph are significantly different ($p < 0.05$) according to Duncan's multiple range test.

3.2. Anti-Mutagenic Effect of WST Extracts

The WST extracts showed inhibitory effects towards spontaneous mutations in the *Salmonella typhimurium* TA98 strain and TA100 strain (Table 2). In the previous cytotoxicity test, the *Salmonella typhimurium* cells were treated with WST extracts at a concentration of 1.25 and 2.5 mg/plate, and the survival rate were all over 90%. In the spontaneous mutation inhibition test using TA98, at 1.25 mg/plate, the inhibitory rate was 37.6%; at 2.5 mg/plate, the inhibitory rate was 65.4%. These results indicated that WST had a certain inhibitory effect on the occurrence of spontaneous

mutations. In the spontaneous mutation inhibition test using TA100, at 1.25 mg/plate, the inhibitory rate was 51.7%. When the concentration of WST was increased to 2.5 mg/plate, the inhibition rate was 80.8%.

Table 2. Effect of WST extracts on the spontaneous mutagenicity of the *Salmonella typhimurium* TA98 and TA100 strain.

Treatment (level of sample, mg/plate)		Revertants/plate	
		1.25	2.5
TA98	Spontaneous	68 ± 5^{aA}	
	WST [B]	43 ± 3^{b} (37.6) [C]	24 ± 2^{c} (65.4)
TA100	Spontaneous	127 ± 7^{aA}	
	WST [B]	61 ± 5^{b} (51.7) [C]	24 ± 2^{c} (80.8)

[a–c] Mean values with different letters in the same column are significantly different ($p < 0.05$) according to Duncan's multiple range test. [A] Values are mean ± SD of revertants/plate. [B] Water solution of WST extracts. [C] The values in parentheses are the inhibition rates (%): inhibition rate = [(spontaneous − WST)/spontaneous] × 100%.

The WST extract exhibited an anti-mutagenic effect in the *Salmonella typhimurium* TA98 strain induced by 2, 7-AF and the TA100 strain induced by MNNG (Table 3). In the anti-mutation experiment using *Salmonella typhimurium* TA98, when the concentration of the WST extract was 1.25 mg/plate, its mutagenic inhibitory rate was 33.6%. After increasing the concentration to 2.5 mg/plate, the anti-mutagenic effect was increased significantly, with an inhibitory rate of 60.5%. In the anti-mutation experiment using TA100, at 1.25 mg/plate, the inhibitory rate was 46.5%; at 2.5 mg/plate, the inhibitory rate was 77.1%. The inhibitory effect of the WST extracts towards the mutagenic *Salmonella typhimurium* TA100 was substantially better than that towards TA98.

Table 3. Effect of WST extracts on the mutagenicity induced by 2, 7-AF (0.8ug/plate) in the *Salmonella typhimurium* TA98 strain, and induced by MNNG (0.4ug/plate) in the *Salmonella typhimurium* TA100 strain.

Treatment (level of sample, mg/plate)		Revertants/plate	
		1.25	2.5
TA98	Spontaneous	68 ± 5^{A}	
	2,7-AF [B] (control)	512 ± 5^{a}	
	WST [C]	363 ± 9^{b} (33.6) [D]	244 ± 13^{c} (60.5)
TA100	Spontaneous	127 ± 7^{A}	
	MNNG [B] (control)	1199 ± 15^{a}	
	WST [C]	701 ± 16^{b} (46.5) [D]	372 ± 11^{c} (77.1)

[a–c] Mean values with different letters in the same column are significantly different ($p < 0.05$) according to Duncan's multiple range test. [A] Values are mean ± SD of revertants/plate. [B] 2, 7-AF: 2, 7-Diaminofluorene. [C] Water solution of WST extracts. [D] The values in parentheses are the inhibition rates (%): inhibition rate = [(2,7-AF or MNNG − WST)/(2,7-AF or MNNG − spontaneous)] × 100%.

3.3. Growth Inhibition Effect (MTT method) of WST Extracts on Human Hepatoma HepG2 Cells

As shown in Figure 2, at different WST extract dosages (0, 10, 20, 40, 70, 100, 130, 160, 190, 220 μg/mL), the growth inhibition rate towards human hepatoma HepG2 cells was 2.5 ± 1.1, 10.2 ± 0.6, 24.0 ± 0.7, 33.2 ± 1.4, 48.6 ± 0.7, 66.5 ± 1.3, 81.1 ± 0.6, 91.0 ± 0.5, and 94.9 ± 0.2, respectively. The WST extracts had a significant and dose-dependent growth inhibitory effect towards HepG2 cells. When the extract dosage was 190 μg/mL, the cell inhibition rate exceeded 90%, so the 40, 100, and 160 μg/mL concentrations were selected for use in the follow-up experiments.

3.4. Effects of WST Extracts on the Gene Expression of Caspase-3, -7, -8, and -9 in Human Hepatoma HepG2 Cells

Effects on gene expression of Caspase-3, -7, -8, and -9 in human hepatoma HepG2 cells, as assessed by RT-qPCR assays, after 48 h of treatment with 40, 100, or 160 μg/mL of WST extract. As demonstrated

in Figure 3A–D, compared with the low expression level of the control group, the expression of the apoptotic genes Caspase-3 (1.2, 1.7 and 2.1 fold), -7 (1.1, 1.3 and 1.6 fold), -8 (1.3, 2.5 and 3.6 fold), and -9 (1.5, 1.9 and 2.5 fold) was significantly increased in the extract-treated cells at concentrations of 40, 100, and 160 μg/mL, respectively.

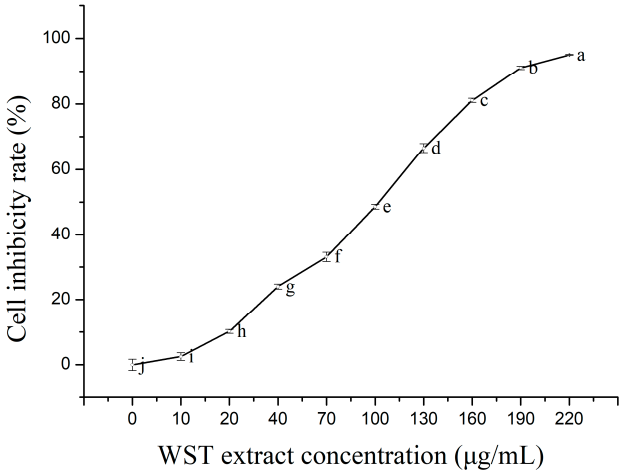

Figure 2. Growth inhibition of human hepatoma HepG2 cells treated with WST extract as evaluated using MTT assays. [a–j] Mean values with different letters in the same bar graph are significantly different ($p < 0.05$) according to Duncan's multiple range test.

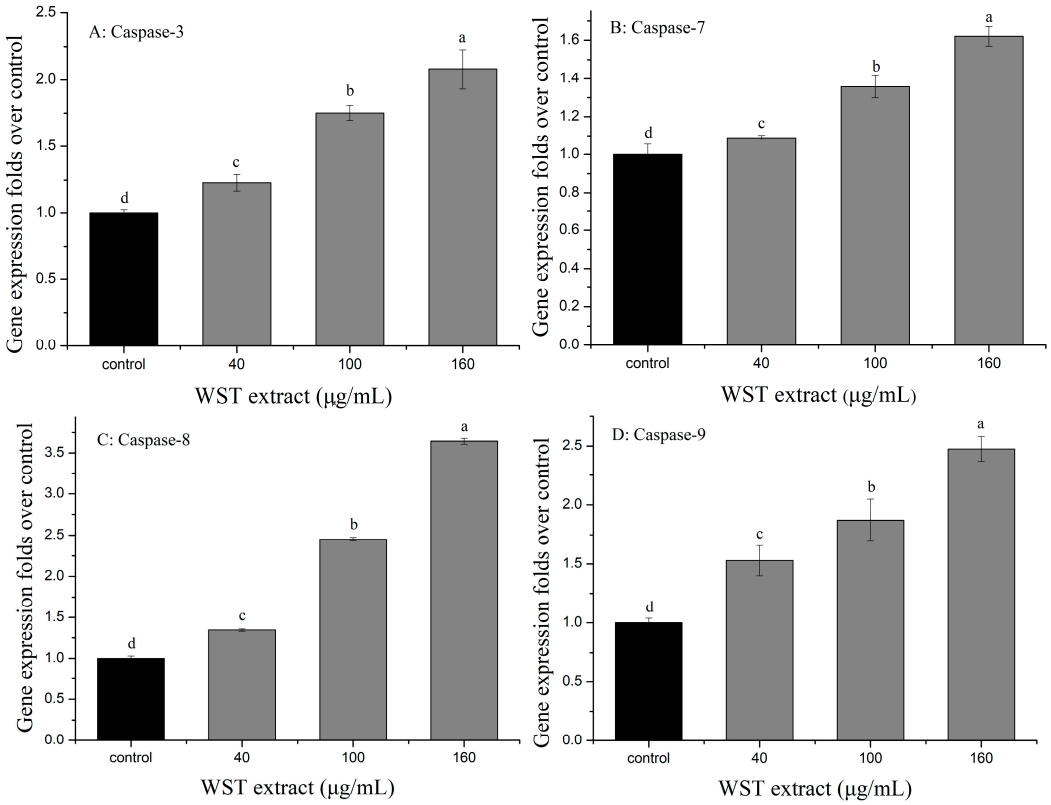

Figure 3. Effects of WST extracts on the gene expression of Capase-3 (**A**), -7 (**B**), -8 (**C**), and -9 (**D**) in human hepatoma HepG2 cells. The fold increase of Capases-3 (**A**), -7 (**B**), -8 (**C**), and -9 (**D**) mRNA expression relative to the control. The calculation formula is as follows: gene expression/GAPDH × control numerical value (control fold ratio: 1). [a–d] Mean values with different letters in the same bar graph are significantly different ($p < 0.05$) according to Duncan's multiple range test.

3.5. Effects of WST Extracts on the Gene Expression of the Apoptosis-Related p21 and p53 Genes in Human Hepatoma HepG2 Cells

The mRNA expression levels of p21 and p53 in HepG2 cells were significantly increased following WST extract treatment compared with the control group (Figure 4A,B). The highest dose of WST extract (160 μg/mL) significantly increased the mRNA levels of p21 (2.9 fold) and p53 (3.8 fold) compared to the untreated control group. The 40 and 100 μg/mL concentrations of WST extract also increased the mRNA expression of p21 (1.2 and 1.9 fold, respectively) and p53 (2.2 and 2.8 fold, respectively) in HepG2 cells.

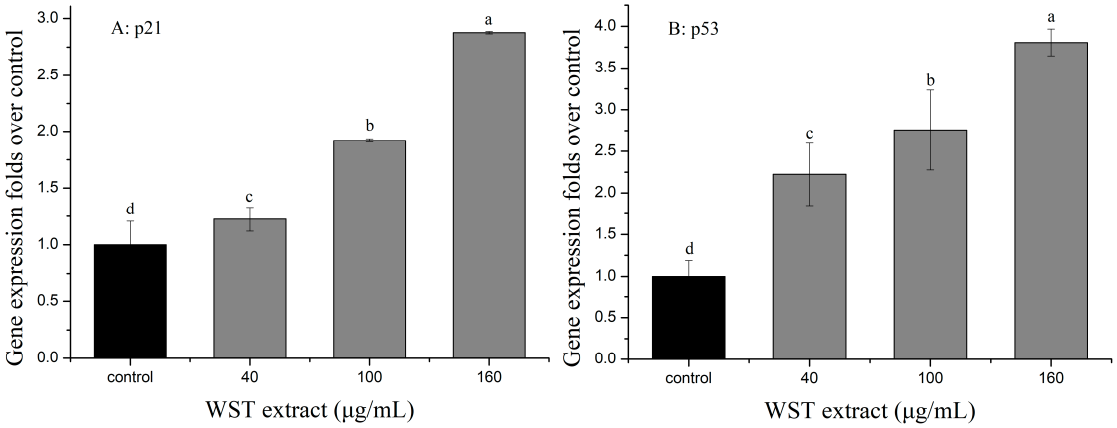

Figure 4. Effects of WST extracts on the gene expression of p21 (**A**) and p53 (**B**) in human hepatoma HepG2 cells. Fold ratio of p21 (**A**) and p53 (**B**) mRNA expression over the control. The calculation formula is as follows: gene expression/GAPDH × control numerical value (control fold ratio: 1). [a–d] Mean values with different letters in the same bar graph are significantly different ($p < 0.05$) according to Duncan's multiple range test.

3.6. Effects of WST Extracts on the Gene Expression of the Apoptosis-Related Bax and Bcl-2 Genes in Human Hepatoma HepG2 Cells

Treatment with WST extracts significantly increased the mRNA expression of Bax (Figure 5A), and decreased the mRNA expression of Bcl-2 (Figure 5B). At concentrations of 40, 100, and 160 μg/mL, WST extracts significantly increased the mRNA levels of Bax to 2.1, 3.5, and 5.4 fold, respectively, compared with untreated HepG2 cells. In contrast, the different concentrations (40, 100, and 160 μg/mL) of WST extracts could also reduce the mRNA expression of Bcl-2 (72.5, 64.6, and 55.6%, respectively) compared with the control group.

3.7. Effects of WST Extracts on the Gene Expression of the Related NF-κB and COX-2 Genes in Human Hepatoma HepG2 Cells

Compared with the control group, the WST-extract treatment groups effectively reduced the mRNA expression of NF-κB and COX-2 (Figure 6A,B). At a high dose of 160 μg/mL, the mRNA level of NF-κB was reduced to 60.1% and the mRNA level of COX-2 was reduced to 37.7% relative to the control group. At other concentrations (40 and 100 μg/ml), the mRNA levels of NF-κB (77.7 and 77.2% compared with control, respectively) and COX-2 (79.4 and 53.6% compared with control, respectively) were also significantly reduced.

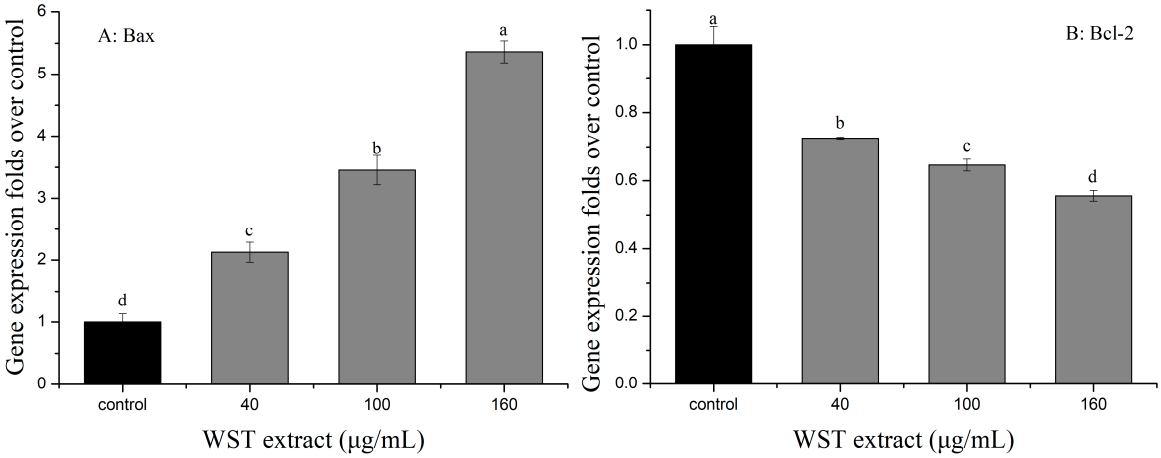

Figure 5. Effects of WST extracts on the gene expression of Bax (**A**) and Bcl-2 (**B**) in human hepatoma HepG2 cells. Fold ratio of Bax (**A**) and Bcl-2 (**B**) mRNA expression over the control. The calculation formula is as follows: gene expression/GAPDH × control numerical value (control fold ratio: 1). [a–d] Mean values with different letters in the same bar graph are significantly different ($p < 0.05$) according to Duncan's multiple range test.

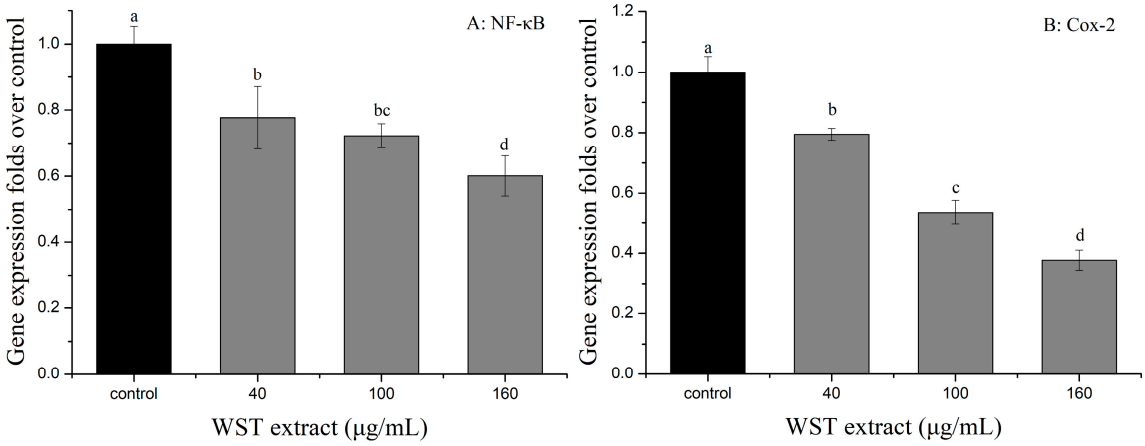

Figure 6. Effects of WST extracts on the gene expression of NF-κB (**A**) and Cox-2 (**B**) in human hepatoma HepG2 cells. Fold ratio of NF-κB (**A**) and Cox-2 (**B**) mRNA expression over the control. The calculation formula is as follows: gene expression/GAPDH × control numerical value (control fold ratio: 1). [a–d] Mean values with different letters in the same bar graph are significantly different ($p < 0.05$) according to Duncan's multiple range test.

3.8. Analysis of the Chemical Composition of WST Extracts

The HPLC analysis of the WST extracts is shown in Figure 7A. Compared with the retention time of the chemical standards (Figure 7B) and related literature, ten peaks were identified from the WST extracts, including chlorogenic acid (peak 1, 4.88 min), 4-hydroxycinnamic acid (peak 2, 10.05 min), isoquercitrin (peak 3, 11.21 min), taxifolin (peak 4, 12.03 min), quercitrin (peak 5, 13.31 min), rosmarinic acid (peak 6, 14.54 min), myricetin (peak 7, 15.17 min), baicalin (peak 8, 16.30 min), neosperidin dihydrochalcone (peak 9, 17.90 min), and quercetin (peak 10, 19.75 min).

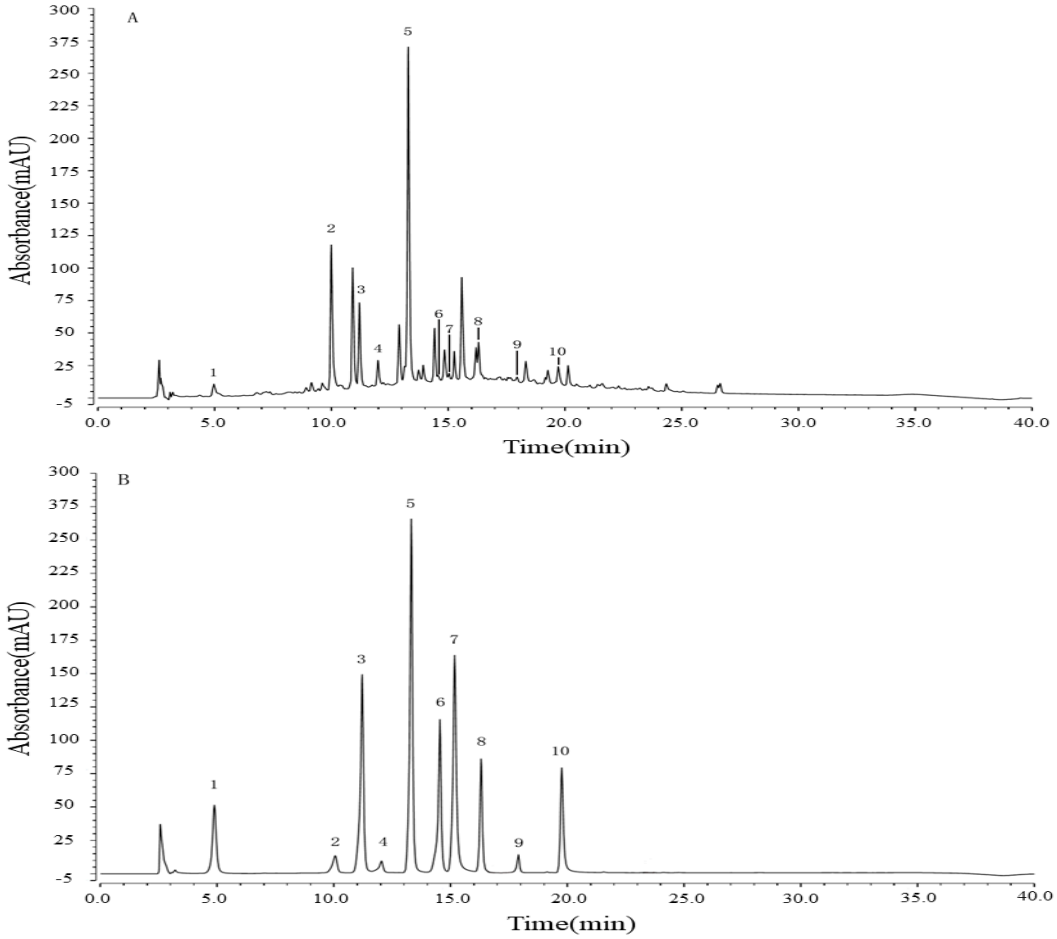

Figure 7. Analysis of the constituents of WST extracts via HPLC assay. (**A**): WST chromatogram; (**B**): standard chromatograms.

4. Discussion

At this point in time, more than two-thirds of the world's population consumes tea. Tea trees native to the Bayu region of southern China were first used as a medicinal beverage in China. According to different processing methods, tea can be divided into non-fermented (such as green tea), semi-fermented (such as oolong tea), and full-fermented teas (such as black tea), and their bioactive ingredients are quite different. The active ingredients in tea mainly include polyphenols, flavonoids, amino acids, vitamins, and other compounds [20]. WST belongs to the non-camellia tea group, and the young leaves used to make WST are rich in polyphenols, flavonoids, amino acids, vitamins, and trace mineral elements. These compounds have many pharmacological effects, including anti-bacterial, anti-inflammatory, anti-hypoxia, anti-fatigue, hypoglycemic, hypolipidemic, and physique enhancement [4]. Tea polyphenols are chemopreventive agents against malignant tumors and have strong free radical scavenging and reducing activities. These compounds capture and detoxify the free radicals of various promoters of carcinogenesis as well as the free radicals produced by exposure to radiation and light. These compounds also inhibit the production of nitroso compounds, which possess potent carcinogenic activities, by reducing bisulfite in the digestive organs. Because tea polyphenols inactivate enzymes and inhibit viral activity, they are effective against the carcinogenic effects of certain viruses [21]. Isoquercitrin, a flavonol glycoside compound that is readily soluble in organic solvents, has anti-inflammatory, anti-oxidant, anti-viral, anti-tumor, anti-atherosclerotic, neuroprotective, blood pressure lowering, and other biological activities [22]. Quercitrin is widely found in plants, and studies have shown that quercetin can lower blood fat and blood sugar and has anti-oxidation and anti-virus effects [23]. Quercetin has been shown to be an inhibitor of cancer in

many tissues, including the colon, lungs, and intestines [24]. Taxifolin has remarkable effects, including anti-tumor, anti-inflammatory, and anti-radiation, and is non-toxic, non-teratogenic, and non-mutating, but it is difficult to dissolve in water, resulting in a low bioavailability [25]. Myricetin can inhibit the proliferation and migration of many types of tumor cells [26], and the pharmacological effects of Baicalin include anti-atherosclerosis, protection against myocardial ischemia-reperfusion injury, protection of cardiomyocytes and endothelial cells, inhibition of myocardial remodeling, and other properties [27]. Neohesperidin dihydrochalcone, a flavonoid derivative (vitamin P), has the effect of protecting the liver and inhibiting gastric acid secretion [28]. Others, such as chlorogenic acid, p-coumaric acid, and rosmarinic acid, also have significant pharmacological effects regarding immune regulation, inhibition of tumors, and prevention of cardiovascular diseases [29–31]. Similar to many other studies on tea, the presence of these bioactive chemicals in WST extracts may be the main cause of their biological activities.

The oxidation phenomenon caused by free radicals is the main inducement of aging in the human body [32]. In order to protect the body from free radical damage, more and more antioxidant substances have been investigated and applied, but to evaluate the level of antioxidant activity of any compound requires an accurate and scientific method. Compared with in vivo antioxidant evaluation methods, the in vitro antioxidant evaluation method is widely used because of its rapidity and convenience. DPPH is a stable free radical with a single electron that accepts an electron or hydrogen radical and becomes a stable yellow diamagnetic molecule. The reaction mechanism between antioxidants and DPPH depends on the structural conformation of the antioxidants. At present, DPPH free-radical scavenging experiments are widely used to quantitatively determine the antioxidant capacity of biological samples, phenolic substances, and foods [33]. Excessive OH radicals can lead to oxidative damage towards DNA, proteins, and lipids, which may induce cancer, mutagenesis, and/or cytotoxicity. Therefore, the potential oxidative damage towards DNA can be prevented by capturing OH free radicals [34]. ABTS is a chemical free-radical initiator and also a color developer. ABTS is oxidized by active oxygen to form the stable blue-green cation $ABTS^+$. After being added to a substance having antioxidant activity, the antioxidant reacts with $ABTS^+$ to decolorize the reaction system. This reaction is a typical single electron transfer pathway. After the antioxidant is oxidized, a single electron is transferred from the antioxidant molecule to the oxidant molecule. The absorbance of the antioxidant or oxidant is measured using an ultraviolet-visible spectrophotometer, and the ability of the antioxidant to scavenge free radicals is evaluated by the change in absorbance [35]. Antioxidant substances, antioxidant activity, and antioxidant capacity evaluation methods are related to each other, and the determination of antioxidant activity requires a systematic evaluation system. The application of one method alone can't completely and comprehensively evaluate the activity of antioxidants or foods containing antioxidants, as each method has its own application scope and characteristics. In order to comprehensively and scientifically evaluate the antioxidant activity of antioxidants, at least two methods need to be employed simultaneously. In this study, the scavenging ability of WST extracts on DPPH, OH, and ABTS free radicals was simultaneously determined, and significant effects were obtained.

At present, the detection of the anti-mutagenic activity of a substance is mainly based on the results of short-term genetic toxicology tests. The basic principle is that the known mutagens and the tested exogenous compounds act together on a certain biological system, and the influence of the exogenous subjects on the mutagenicity of the mutagens is then monitored. If the compound exhibits significant inhibition, it is considered that it has a potential anti-mutagenic activity. The Ames test is a short-term biological testing method for the detection of chemical carcinogens, which is fast, convenient, and sensitive. The improved Ames assay can react to a mutagenic effect on the test substance at the level of point mutations (including frameshift mutations and base substitutions) [36]. Chemical mutagens are a class of chemicals that act on DNA, alter its structure, and cause genetic variation, and these include base analogs, alkylating agents, frameshift mutants, and others. A mutagen only works on a certain part of a gene, thus conferring its specific characteristics. As a frameshift mutagen, 2, 7-AF can act on sensitive points of bacterial DNA molecules to increase or delete nucleotides, causing changes in

gene cryptography and induce frameshift mutations. In contrast, MNNG, an alkylating agent, can cause base pairing errors to form base substitution mutations during DNA molecular replication [37]. So far, there are many different explanations for the mechanism of action of natural products against mutagenesis. Most scholars believe that the protection mechanism conferred by anti-mutagenic agents is mainly through one of two ways: one way is to act outside the cell membrane, preventing the uptake and formation of the mutagen, directly inactivating the pre-mutant and mutagenic agents. Another way is to function within the cell, preventing the activation of the mutagen, preventing the mutagen from reaching the targeting site, or preventing the interaction of the mutagen with the targeting as well as enhancing the activity of an associated detoxification enzyme, eliminating the presence of mutagenic free radicals, or promoting DNA repair [38]. In this context, WST is likely to exert its anti-mutagenic effects via intracellular, extracellular, or synergistic interactions, thus playing a role in preventing base-pair substitutions and shift mutations.

Cellular apoptosis is a spontaneous process that presents as the autonomous, orderly death of cells controlled by specific genes. Different from cell necrosis, apoptosis is an active process involving the activation, expression, and regulation of a series of genes. It is not a phenomenon of autologous injury under pathological conditions, but a death process that is actively induced for adapting to the living environment and maintaining internal environmental stability. This system is capable of removing defective or damaged cells before they are abnormally divided and replicated during the early stages of cancer development. Therefore, it is an effective mechanism for preventing cancer [39]. Liver cancer is one of the most common malignant tumors, with a high incidence and mortality, and there is currently no effective treatment. Some ingredients in food have been shown to prevent liver cancer, and these active ingredients prevent cancer by inducing apoptosis and death of cancer cells by stimulating apoptosis [40]. As typical apoptosis-related factors, the bcl-2 family of proteins play an important role in the regulation of apoptosis and includes the anti-apoptotic factor bcl-2 and the pro-apoptotic factor bax. Bcl-2 inhibits apoptosis by preventing cells from releasing cytochrome C (Cyt-C) from the mitochondria and limiting its entry into the cytosol [41]. In contrast, bax has an inducing effect on apoptosis, and the depolarization of the mitochondrial inner membrane potential leads to the release of Cyt-C and apoptotic protease-activating factor-1 (Apaf-1) into the cytosol, which is combined with the caspase-9 zymogen to form the apoptosome. The latter interaction promotes the activation of the caspase-9 zymogen and ultimately activates the apoptotic effectors caspase-3 and caspase-7, leading to apoptosis [42]. The proportional balance between anti-apoptotic factors and pro-apoptotic factors affects the occurrence of apoptosis. In this study, WST extracts were shown to increase the expression of bax, reduce the expression of bcl-2, especially enhancing the expression level of proapoptotic factor bax, and subsequently increase the mRNA levels of caspase-9, caspase-3, and caspase-7, thus inducing apoptosis of liver cancer cells. These results indicated that WST has a strong anticancer activity against liver cancer cells.

The caspase signaling cascade is a key event in endogenous and exogenous apoptosis, and caspase-3, -7, -8, and -9 are the main proteases involved in apoptosis [43]. Caspase-8 and -9 are the starting caspase enzymes, located upstream of the apoptotic pathway. After activation by different pathways, they are responsible for activating other caspases, such as caspase-3 and caspase-7, downstream of the apoptotic pathway. As an executor of apoptosis, this cascade induces apoptosis [44]. During this process, proteins such as calpain, cathepsin, and endonuclease perform programmed cell death and enhance cancer cell apoptosis through their interactions with caspases [45]. WST extracts enhanced the expression of caspase-3, -7, -8, and -9 mRNA levels by activating endogenous and exogenous apoptotic factors, thus inducing apoptosis in HepG2 cancer cells.

The tumor suppressor p53 monitors and regulates the DNA damage repair process in the G1 phase of the cell cycle, and it serves to induce multiple pathways to initiate apoptosis if the repair process fails [46]. When cells are exposed to various stresses, such as hypoxia and DNA damage, p53 can initiate the exogenous apoptotic pathway by inducing the expression of FAS and FASL, and it can also interact with members of the Bcl-2 family to promote the expression of bax, which ultimately alters

the permeability of the mitochondrial membrane, activates the caspase effect in the cytoplasm, and induces cells to enter the mitochondria-mediated apoptotic pathway (i.e., the endogenous apoptotic pathway) [47]. In addition, caspase-7 is a response gene of p53. Increased expression of the p53 transcription factor allows the caspase-7 gene to be further transcribed and activated, with its protein expression showing a time-dependent increase with increasing p53 expression [48]. Under certain conditions, the expression of p21 is closely related to the apoptotic precursor protein bax and can function to enhance the expression of bax. WST can induce apoptosis by increasing the expression levels of both p53 and p21.

NF-κB is an important negative regulator of tumor cell apoptosis and is involved in important processes, such as the immune response, the inflammatory response, and apoptosis. Activated NF-κB can interact with other inflammatory signaling pathways and promote the expression of inflammatory factors, adhesion molecules, and inflammation-related enzymes. Upon induction of multiple factors, NF-κB is released from IκB and transferred to the nucleus where it binds to the κB binding site of the target gene promoter region [49]. Cox-2 has an inflammatory effect, and its overexpression is an important factor in the transition from inflammation to the occurrence of malignant tumors [50]. After treatment with WST, the mRNA levels of NF-κB and Cox-2 in liver cancer cells were significantly reduced, increasing their anti-inflammatory activity.

In addition, on the basis of previous related experiments, we found the optimal concentrations range of WST extracts in the anti-oxidant (0.2, 0.6, 1.0 mg/mL), anti-mutation (1.25, 2.5 mg/plate) and anti-cancer (40, 100, 160 μg/mL) research project, respectively, and obtained good biological activity. However, we have not further studied the Western blot and the structural analysis of specific compounds, and we will further improve them in future experiments.

5. Conclusions

In summary, we evaluated the anti-oxidant, anti-mutagenic, and anti-cancer effects of WST extracts via in vitro experiments, and conducted a preliminary analysis of potential bioactive substances contained in these extracts. The results showed that the WST extracts had significant scavenging activity towards DPPH, OH, and ABTS free radicals. WST could also reduce the occurrence of spontaneous mutations in *Salmonella typhimurium* trophic deficiency TA98 and TA100 stains as well as the mutagenic effects induced by 2,7-AF and MNNG. The anti-hepatocarcinoma experiment also showed that the extracts of WST could inhibit the growth of cancer cells within a certain concentration range and could stimulate this anti-cancer effect by regulating the expression of Bcl-2, bax, Caspase-3, -7, -8, -9, p21, p53, NF-κB, and Cox-2. In addition, these biological activities of WST were likely conferred by its chemical composition, which included flavonoids. Ten compounds were detected by high-performance liquid chromatography, and these compounds have previously been shown to have many biological activities, including anti-oxidant, anti-cancer, anti-angiogenesis, and others. This study, however, was only a preliminary examination of the biological activity and function of WST; the specific biological activities, anti-cancer effects, and mechanisms of the various biologically active compounds in the extracts will be the topics of future studies.

Author Contributions: C.L. (Chong Li) and C.L. (Chaomin Liu) performed the majority of the experiments and wrote the manuscript; J.Z., H.-L., Y.Z., and Y.L. contributed to the data analysis; X.Z. and W.L. designed and supervised the study and read the final manuscript.

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