

Oxymatrine inhibited cell proliferation by inducing apoptosis in human lung cancer A549 cells¹

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Abstract. To investigate the inhibition effect of oxymatrine induces human lung cancer A549 cells apoptosis. The A549 cells were cultured for 24 h, than the various concentration of oxymatrine (2 mmol/L, 4 mmol/L, 8 mmol/L, 15 mmol/L) were added into different experimental group cells, and 5-fluorouracil were added into the positive control group cells for 12 h, 24 h, 36 h, 48 h respectively. The A549 cells inhibition rate, apoptosis, and the expression of Bcl-2 and Bax were examined by MTT method, Annexin V/PI double staining method, real-time quantitative PCR and western blot, respectively. At same time, the morphological changes of A549 cells were observed with an inverted microscope. In the range of 2 mmol/L~15 mmol/L, oxymatrine had obvious inhibition effects on the proliferation of A549 cells. Compared with the negative control group, it has significantly different ($P<0.01$). There was remarkably the time- and dose-dependent correlation. After A549 cells were treated with 8 mmol/L oxymatrine for 24 h, the morphological change of cell apoptosis was observed and the extent of apoptosis was quantified by flow cytometry. Furthermore, the expression of Bcl-2 was reduced and the expression of Bax was increased remarkably ($P<0.05$). Oxymatrine has significant inhibition effects on the cells proliferation and the effects showed time-dependent and dose-dependent. Oxymatrine can induce apoptosis of the A549 cells by regulating the expression of Bcl-2 and Bax.

Keywords: Oxymatrine, A549 cells, proliferation inhibition, apoptosis, Bcl-2, Bax

1. Introduction

Lung adenocarcinoma is one of the the high incidence and mortality rate of malignant tumors in the world. Despite recent advances in diagnosis and treatment, the mortality rates with an overall 5-year survival of only 15%. Especially the non-small cell lung cancer such as A549 cell lines which constitutes 80% of lung cancer cases, remains an aggressive lung cancer associated with a poor patient survival [1]. Hence, it has become an urgent need to find a low toxicity and efficient plant extract medicine applied to the cancer treatment. Nowadays, growing evidences suggest that natural materials might be a good vehicle to anticancer [2, 3]. Due to these extracts showed low toxicity and high

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biological activities, a lot of herb products have been used in alternative treatments of cancers [4].

Sophora flavescens Ait is a traditional Chinese medicine, and it has been used for the treatment for thousands of years [5, 6]. Oxymatrine (OM), is a kind of alkaloid components found in the roots of sophora species, had been reported to have many pharmacological effects, such as anti-inflammation, anti-virus, anti-fibrotic, anti-allergic and protecting hepatocytes and cardiovascular [7-9]. In previous studies, the effects of oxymatrine on human lung cancer are largely unknown. Considering the extensive effects of oxymatrine, we investigate whether oxymatrine has a role of potently inhibited tumor growth, as well as the mechanisms of action. Some results showed that the mechanisms of cell apoptosis were related with the expressions of Bcl-2 and Bax proteins in human lung cancer A549 cells.

2. Materials and methods

2.1. Reagents

Oxymatrine was purchased from Tianjin Biotechnology Companies, Tianjin, China. Fluorouracil was purchased from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. RPMI 1640 medium was purchased from Hyclone, fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biotechnology Co., China. Thiazolyl blue (MTT) was obtained from Sigma Chemical Corporation, USA. Mouse anti-human monoclonal antibodies to Bcl-2, Bax, β -actin and goat anti-mouse FITC-IgG were purchased from Pharmingen, USA.

2.2. Cell culture and groups

The A549 cells were provided by School of Public Health of Zhengzhou University and were maintained in RPMI 1640 medium with 10% FBS, 1% penicillin and streptomycin in the incubator with a humidified atmosphere (5% CO₂, 37°C). A549 cells were divided into six groups, including oxymatrine various concentrations groups (2 mmol/L, 4 mmol/L, 8 mmol/L, 15 mmol/L), positive control group with an equal volume of 5-FU (15 mmol/L) and negative control group with RPMI 1640 medium.

2.3. Cell growth inhibition assay

A549 cells were seeded on 96-well plates with 2×10^3 cells/well in 200 μ l medium. The cells cultured at 37°C for 24 h, the old medium was sucked out, the fresh 1640 medium matching with different concentrations of oxymatrine (2 mmol/L~15 mmol/L) for 12 h, 24 h, 36 h, 48 h in a final volume of 200 μ L. Each group contains 6 wells. Then 20 μ L MTT (4.8 mmol/L) was added to each well to be detected at different time points, the mixture medium was sucked out after 4 h. Then 150 μ L DMSO was added in each well to dissolve the MTT formazan precipitate, shaken for 10 min and the optical density value (OD value) was detected immediately at 490 nm by Enzyme immunoassay instrument (Opsys MR, Denex Technology, USA). Inhibitory rate (%) = $(1 - \text{OD treatment} / \text{OD control}) \times 100\%$.

2.4. Morphological observation of apoptotic cells

The morphological changes of A549 cells treated with 8 mmol/L oxymatine for 24 h were observed directly with an inverted microscope.

2.5. Annexin V-PI method to measure the apoptotic rate

Based on the instructions shown on the reagent box, the operations are as follows: each group is washed up through PBS, and 0.25% Trypsin is used for digestion for 2 to 3 minutes. Then it is moved to a centrifugal tube with the medium added. After they have been mixed thoroughly, the mixture is spun for 4 minutes at the speed of 1000 r/min. After the supernatant is removed, PBS suspension cells are added. Then, the cell concentration is adjusted to 10^6 /mL. 0.5 mL of the cell suspension above is chosen and spun for 4 minutes at the speed of 1000 r/min. When the supernatant is removed, 0.5 mL of Binding Buffer suspension cells and 1 μ L of fluorescent labeling Annexin V reagent are added and mixed thoroughly. This mixture is cultured for 20 minutes at room temperature without exposure to light. Then, 5 μ L of PI is added and mixed thoroughly, followed by a 5-min culture at the temperature of 4°C in a dark place. Finally, a flow cytometer is adopted to measure the apoptosis rate immediately after 500 μ L of Binding Buffer is added. The experiment is repeated for three times and the software Flow max 2.4 is applied to conduct the data analysis.

2.6. Real-time QPCR system analysis

Primer design: β -actin primer design, upstream primer: 5'-CCGTCTTCCCCTCCATCG-3', downstream primer: 5'-GTCCCAGTTGGTGACGATGC-3', product length 155bp; Bcl-2 series primer, upstream primer: 5'-GTGGAGGAGCTCTTCAGGGA-3', downstream primer: 5'-R-AGGCACCCAGGGTGATGCAA-3', product length 303 bp; Bax series primer, upstream primer: 5'-TGCTTCAGGGTTTCATCCAGG-3', downstream primer: 5-TGGCAAAGTAGAAAAGGGCGA -3', product length 275bp. The total RNA of the cells in each group is extracted and synthesized to cDNA via reverse transcription. PCR reaction system is 10 μ L: SYBR Green I Mixture 5 μ L, upstream primer 0.25 μ L, downstream primer 0.25 μ L, Template cDNA 1 μ L, ddH₂O 3.3 μ L, Rox 0.2 μ L, reaction conditions: 95°C initial denaturation 10min, 95°C denaturation 30 s, 58°C annealing 40 s, 72°C extension 40 s. In total there are 40 cycles. The RNA content of each sample is standardized according to its β -actin content, and 2- $\Delta\Delta$ CT method is used to analyze the relative expression of the Bcl-2 and Bax gene.

2.7. Western blotting

A549 cells (1×10^6) treated with 2 mmol/L~15 mmol/L oxymatine for 48 h were extracted in Cell Lysis Buffer for 50 min on ice, 12000 rpm centrifugation 20 min at 4°C to remove cell debris, collected supernatant fluid. The 30 μ g of protein extra was electrophoresed on 12% SDS-PAGE, then electrotransferred to a PVDF membrane which was blocked with 5% milk for 2 h at room temperature. Respectively, rabbit anti-human Bcl-2 and Bax antibodies were plused and incubated overnight at 4°C. The secondary antibody with HRP-conjugated goat anti-rabbit IgG (1:1000) was incubated at room temperature and on a shaker for 1 h. All PVDF membranes were detected by chemiluminescence (ECL).

2.8. Statistical analyses

Table 1

Inhibitory effects of different concentration of oxymatrine on A549 at different time points ($\bar{x} \pm s$, n = 6)

Group	12 h		24 h		36 h		48 h	
	OD value	Inhibition ratio (%)						
negative control group	0.250±0.015	—	0.643±0.069	—	1.231±0.247	—	1.891±0.130	—
2 mmol/L OM	0.249±0.002	1	0.425±0.039 ^Δ	34	0.591±0.030 ^Δ	52	0.702±0.021 ^Δ	63
4 mmol/L OM	0.240±0.179 [*]	4	0.296±0.226 ^Δ	54	0.336±0.063 ^Δ	73	0.375±0.043 ^Δ	80
8 mmol/L OM	0.202±0.005 ^Δ	19	0.201±0.007 ^Δ	69	0.173±0.022 ^Δ	86	0.294±0.025 ^Δ	91
15mmol/LOM	0.177±0.015 ^Δ	29	0.158±0.009 ^Δ	75	0.172±0.025 ^Δ	88	0.182±0.020 ^Δ	94
positive control group	0.198±0.014 ^Δ	19	0.209±0.005 ^Δ	67	0.238±0.020 ^Δ	81	0.233±0.013 ^Δ	87

Note: Comparison with negative control group; * $P < 0.05$; ^Δ $P < 0.01$.

All statistical analysis was analyzed by the SPSS 13.0 software. The results were described by the format mean \pm standard deviation ($\bar{x} \pm S$) and analyzed by one-way ANOVA. $P < 0.05$ was looked as statistically significant and $\alpha = 0.05$ was significance level.

3. Results

3.1. Effects of oxymatrine on A549 cells proliferation

To verify the effect of oxymatrine on cell proliferation, A549 cells viability was assessed by MTT at different time point. With the extension of time, the cell proliferation inhibition rate gradually increased from 0% to 94% in 2 mmol/L~15 mmol/L concentration range (see Table 1). The oxymatrine inhibited cell proliferation in a dose- and time-dependent manner. Compared with the negative control group, there was a significant statistical significance. The proliferation inhibition rate of two different concentration of oxymatrine 8 mmol/L and 15 mmol/L were higher than the positive control group respectively at the same time.

3.2. Effects of oxymatrine on A549 cells morphology

The significant morphological changes of oxymatrine treated group cells cultured after 24 h were found by an inverted microscope. It showed that the negative control group were adherent in good condition, polygonal, larger cell size, plump cytoplasm, distinct cell borders and oxymatrine treatment cells were round, volume is reduced, but have the integrated cell membrane (see Figure 1).

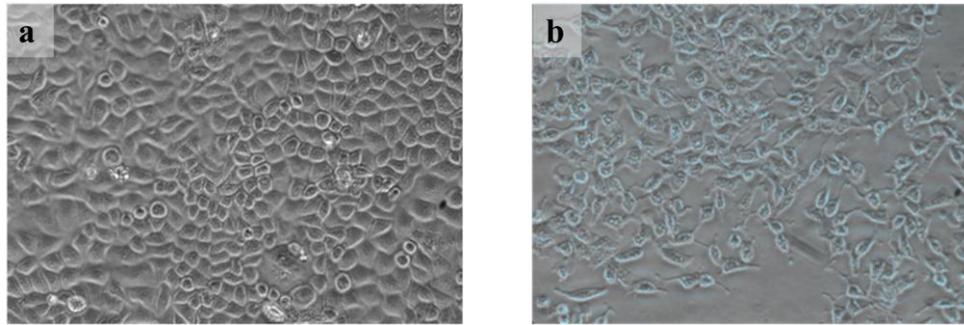


Fig.1. Inverted microscope observation A549 cells apoptosis morphology. (200×) (a) Untreated A549 cells for 24 h; (b) 8 mmol/L oxymatrine-treated A549 cells for 24 h.

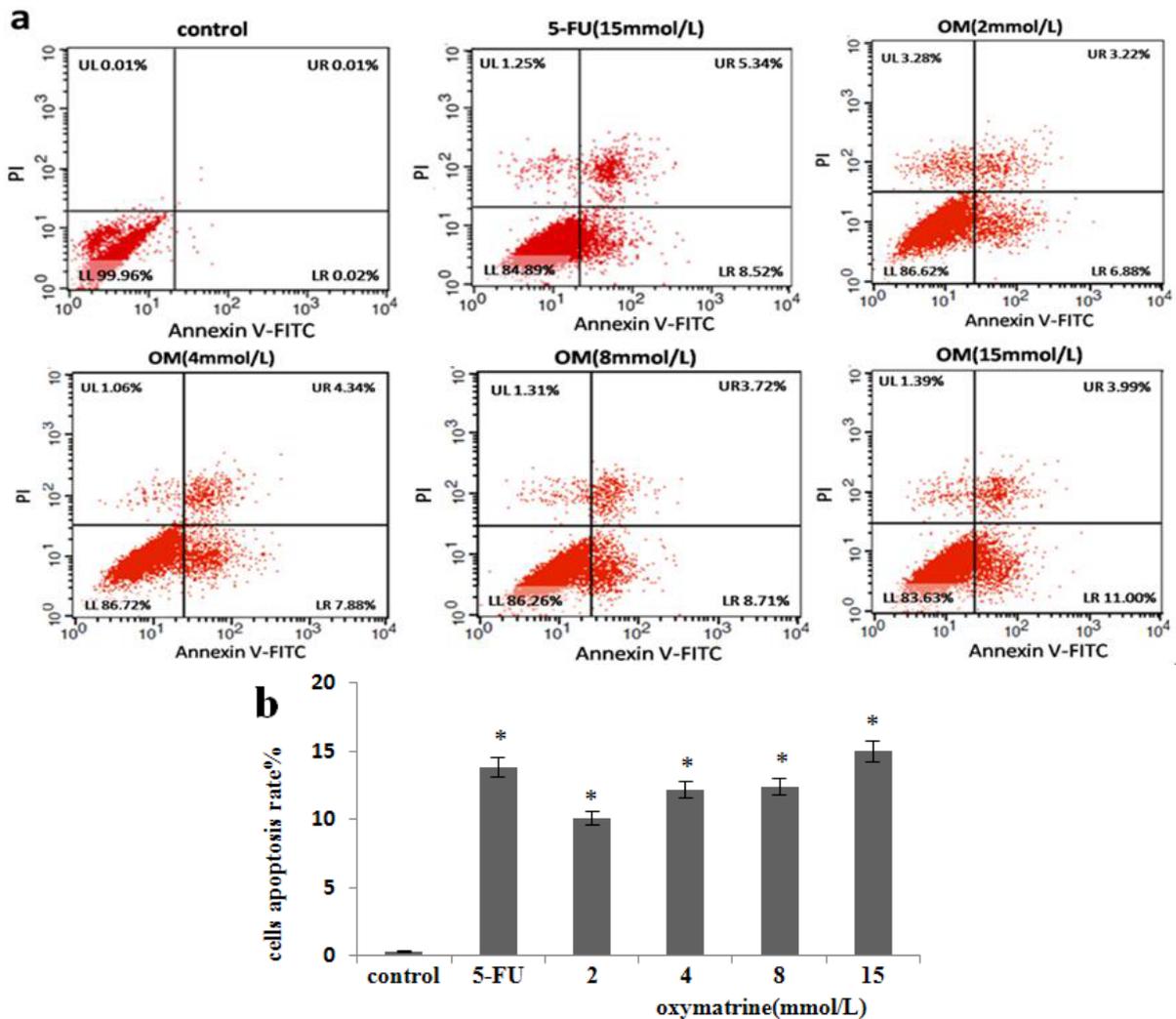


Fig. 2. After treated with 2–15 mmol/L oxymatrine (OM) and 15 mmol/L 5-FU for 48 h, A549 apoptotic cells were double stained with annexin V-FITC and PI and detected by flow cytometric analysis (a, b). Statistical data are shown as mean \pm standard deviation of three independent experiments ($P < 0.01$).

3.3. The examination by Annexin V-FITC/PI flow cytometer

A549 cells treated with 2 mmol/L~15 mmol/L oxymatrine and 15 mmol/L fluorouracil were stained with annexin V-FITC/PI then analyzed by flow cytometer. The result showed that oxymatrine treatment caused a significant increase in apoptosis (see Figure 2(a)). The apoptosis rates of different concentration oxymatrine ranged from $10.44 \pm 0.81\%$ to $15.05 \pm 0.68\%$ was significant compared with the negative control group ($*P < 0.01$) (see Figure 2(b)). Whereas in addition to the 8 mmol/L oxymatrine treatment groups, the other oxymatrine treatment groups have significant difference compared with the positive control group ($P < 0.05$).

3.4. The effect of oxymatrine on the expression of A549 cells Bcl-2 and Bax mRNA

The result of the real-time quantitative PCR shows that oxymatrine at the concentrations of 2 mmol/L ~15 mmol/L and fluorouracil at the concentration 15 mmol/L can both down-regulate the expression of Bcl-2 mRNA and up-regulate the expression of Bax mRNA significantly compared with the negative control group ($P < 0.05$) (see Figure 3). Sametime, the cells which were treated with 2 mmol/L~8 mmol/L oxymatrine resulted in a dose-dependent in the expression of Bcl-2 and Bax mRNA. However, their expressions have no significant difference at the concentration of 8 mmol/L and 15 mmol/L oxymatrine.

3.5. Western blotting

To test the expression of Bcl-2 and Bax of A549 cells in different oxymatrine treatment groups, we determined the protein expression levels of Bcl-2 and Bax by western blot analysis. A549 cells were treated with 2 mmol/L~15 mmol/L oxymatrine for 48 h, we found that the level of Bcl-2 was dramatically decreased and the level of Bax was increased (see Figure 4). At the same time, the cells which were treated with 2 mmol/L~8 mmol/L oxymatrine resulted in a dose-dependent in the expression of Bcl-2. However, the expression of Bax has no obvious dose-dependent.

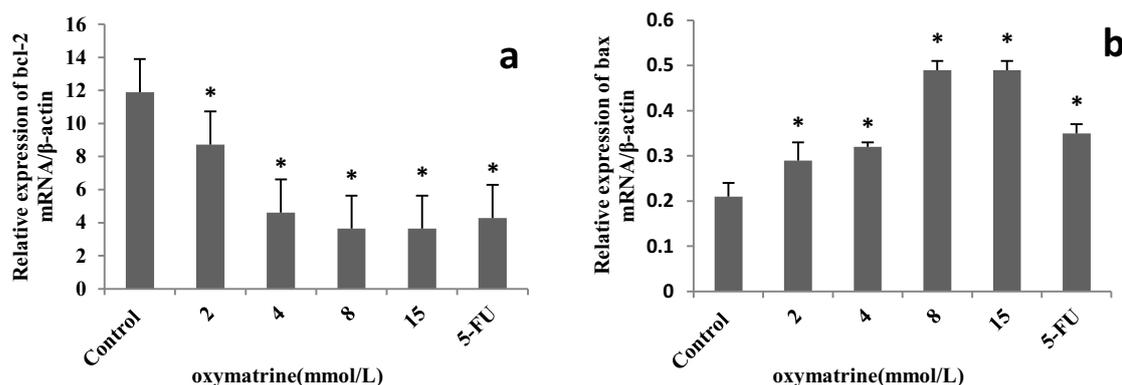


Fig. 3. The effect of oxymatrine on the expression of Bcl-2 and Bax mRNA of human lung cancer A549 cells. A549 cells were treated for 48 h with oxymatrine at concentrations of 2 mmol/L~15 mmol/L. Then the cells were collected and the relative expression level of Bcl-2 (a) and Bax (b) mRNA and β -actin were analyzed by the real-time quantitative PCR. The data was expressed as means \pm SEM, $n=3$ in each group. $*P < 0.05$ compared with the negative control group.

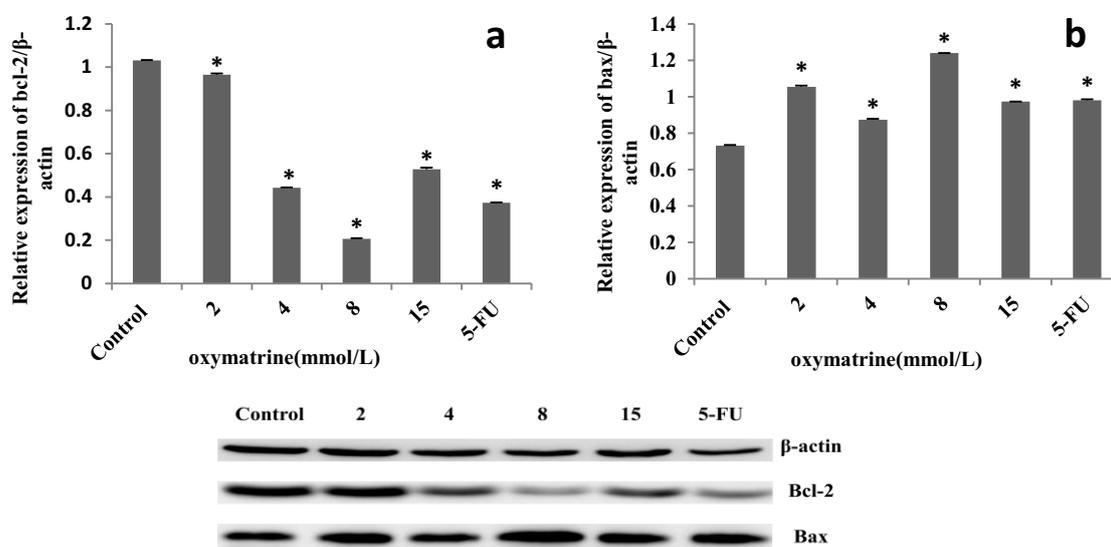


Fig. 4. The effect of oxymatrine on the expression of bcl-2 and bax of human lung cancer A549 cells. A549 cells were treated for 48 h with oxymatrine at concentrations of 2 mmol/L~15 mmol/L. Then the cells were collected and the relative expression level of Bcl-2 (a) and Bax (b) and β -actin were analyzed by western blot. The data was expressed as means \pm SEM, $n=3$ in each group. * $P<0.05$ compared with the negative control group.

4. Discussion

In this study, the oxymatrine significantly inhibited the growth of human lung cancer A549 cells and regulated the expressions of Bcl-2 and Bax mRNA and proteins. As shown in MTT, 2 mmol/L~15 mmol/L oxymatrine inhibited the proliferation of A549 cells in a time-dependent and dose-dependent manner. The inhibition rate at a concentration of 15mmol/L was the highest. A549 cells of oxymatrine treatment were polygon, shrinkage and the apoptosis were detected by flow cytometry. We found that the expression of Bcl-2 and Bax of A549 cells treated with oxymatrine were affected by RT-PCR and western blot analysis.

There are many reports to research on the mechanism of apoptosis, of which Bcl-2 family is the most concerned. Bcl-2 protein can not accelerate cell division in tumor development, but can prolong cell life through resist to various forms of cell death, result in increasing the number of tumor cells, and become a key inhibitory apoptosis regulator [10]. Bax with the role of promotion apoptosis is another member of Bcl-2 family. Bax protein can not only form homodimer itself, also easy to form heterodimers with Bcl-2 protein, thereby inactivating Bcl-2. Bcl-2/Bax heterodimers were increased and eliminated the pro-apoptotic role of Bax along with the Bcl-2 protein expression were increased, so the role of inhibits apoptosis were enhanced. On the other hand, Bax/Bax homodimers were increased when Bax protein expression were increased, the role of pro-apoptotic were enhanced [11, 12]. The results of this study show that after A549 cells treated with 2-15 mmol/L oxymatrine for 48 h, Bax protein expression were increased significantly compared with the negative control; on the contrary, the expression of Bcl-2 were reduced significantly.

All these findings suggest that oxymatrine may significantly inhibit the A549 cell proliferation in time and dose-response relationships, at the same time oxymatrine may induce human lung cancer

cells apoptosis which mechanism may be up-regulation of Bax protein expression and down-regulation of Bcl-2 protein expression. This study suggests that natural materials oxymatrine might be a good vehicle to anticancer. We will further conduct animal experiments in the future, thus provide the experimental basis for the development and utilization of herbal resources.

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