

## Original Article

# The anti-leukemic effect of carnosic acid combined with adriamycin in a K562/A02/SCID leukemia mouse model

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**Abstract:** The effects of carnosic acid (CA) were investigated on the acute myeloid leukemia (AML) cell growth *in vivo*. A NOD/SCID AML mouse model, which was set up by inoculation with K562/A02 cells, was used to study whether tumor growth *in vivo* can be inhibited by CA combined with adriamycin. After being inoculated with K562/A02 cells, the NOD/SCID mice were expressed positive human *mdr1* and *bcr/abl* genes. This result indicates that the K562/A02/SCID leukemia mouse model is successfully established. The mice treated with CA combined with adriamycin exhibit a significant lower number of leukemia cells (20%) than that of untreated animals (32.5%) ( $P < 0.05$ ), in particular with higher percentages of apoptotic cells than the mice treated by single adriamycin (control) group. The median of 95% CI survival time is 19 (10.0-44.2) and 33 (29.4-36.6) days for the control group and the CA-treated group, respectively. The difference is statistically significant ( $P < 0.05$ ). It is illustrated that the natural compound CA, combined with Adriamycin, has high potential to inhibit the growth of malignant cells *in vivo*, and is a promising adjuvant anti-cancer drug. Prospective studies should be conducted to understand the functional mechanism of CA at the molecular level.

**Keywords:** Carnosic acid, K562/A02 cells, leukemia mouse model, adriamycin, *in vivo*

## Introduction

The treatment outcome of acute myeloid leukemia (AML) has achieved great improvement, however it still faces up with a tremendous challenge. The performance of current chemotherapeutic drugs with highly toxic and poorly tolerated is the major obstacle in the treatment, particularly for the treatment of elder patients [1-3]. The drug resistance is also an obstacle in the treatment of cancer, notably for acute leukemia. Previous literature has shown that the resistance may be mediated by the multidrug resistance (MDR-1/P-glycoprotein) gene. A recent review indicates that there is a substantial body of research on P-gp inhibition as a means of improving the efficacy of therapeutic agents that are ATP-binding cassette transporter substrates [4]. Many compounds have been reported to be capable of modulating the MDR phenotype, however, few can be clinically applied due to their unacceptable side

effects or toxicity at the doses required to be effective. Therefore, an alternative approach of developing novel agents with reduced efflux properties may prove to be the most promising way to improve the efficacy of existing agents for AML [4].

Polyphenolic antioxidants are a group of relatively safe compounds that can be used as a component of the diet or herbal medicinal remedies, among a plenty of agents tested in combination with natural plant-derived compounds. Rosemary (*Rosmarinus officinalis* L.) and its extracts such as caffeic acid, rosmarinic acid (RA), ursolic acid (UA), carnosic acid (CA), and carnosol, have been studied for the antitumor or antineoplastic behavior on different types of cancer cell lines/rat or mouse models [5]. The anticancer effects of rosemary extracts were found to be statistically significant in animal models including colonic cancer [6, 7], mammary tumors [8, 9], leukemia [10], and skin

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tumors [11]. In general, the potential anticancer mechanism of rosemary is either the induction of cancer protective markers/factors (apoptosis or carcinogen metabolic enzymes) or the inhibition of tumor promoting events (cell growth and proliferation, DNA adduct/free radical formation, carcinogen activating enzymes, or lipid peroxidation) [12-16].

In our previous work we have demonstrated that CA can reverse MDR of K562/A02 cells *in vitro* by increasing intracellular adriamycin concentration, down-regulating expression of MDR-1, and inhibiting the function of P-gp. CA can decrease viability of the human promyelocytic leukaemia cell line HL-60, in dose- and time-dependent manners [17, 18].

In the article the antileukemic activity of CA combined with adriamycin was investigated *in vivo* by a K562/A02/SCID mouse model of human AML.

### Materials and methods

#### Cell culture

Human leukemic K562/A02 cells (obtained from the laboratory of Immunology of the Institute of Basic Medical, Shandong Academy of Medical Sciences) were cultured in RPMI 1640 medium (Gibco, Los Angeles, CA, USA) supplemented with 10% heat inactivated FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, and maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The cells were sub-cultured twice each week, and the exponentially growing cells were used in all treatments. Before treatment, compound cells were washed with PBS and fresh medium was added. At the time of treatment, working solutions were diluted accordingly in RPMI 1640.

#### Experimental animals

##### *Establishment of K562/A02/SCID mouse model*

Experiments were carried out in the animal facility of Animal Experimental Center of Shandong University in accordance with Shandong University Animal Care Committee approved protocol. Male NOD/SCID (non-obese diabetic/severe combined immune deficient) mice age 4 weeks, were purchased from

Experimental Animal Center of Shanghai Academy of Medical Sciences. Mice were housed in sterilized cages and provided with autoclaved water and a standard powdered rodent diet *ad libitum*. The experimental protocols were initiated following a 7-day acclimatization period.

All NOD/SCID mice were given cyclophosphamide 2 mg/mouse for three days. Using an adjusted K562/A02 cell concentration of 5×10<sup>7</sup>/ml, each mouse was inoculated with 1×10<sup>7</sup>/ml of the cells through their tail vein [19].

##### *K562/A02/SCID leukemia model validation experiments*

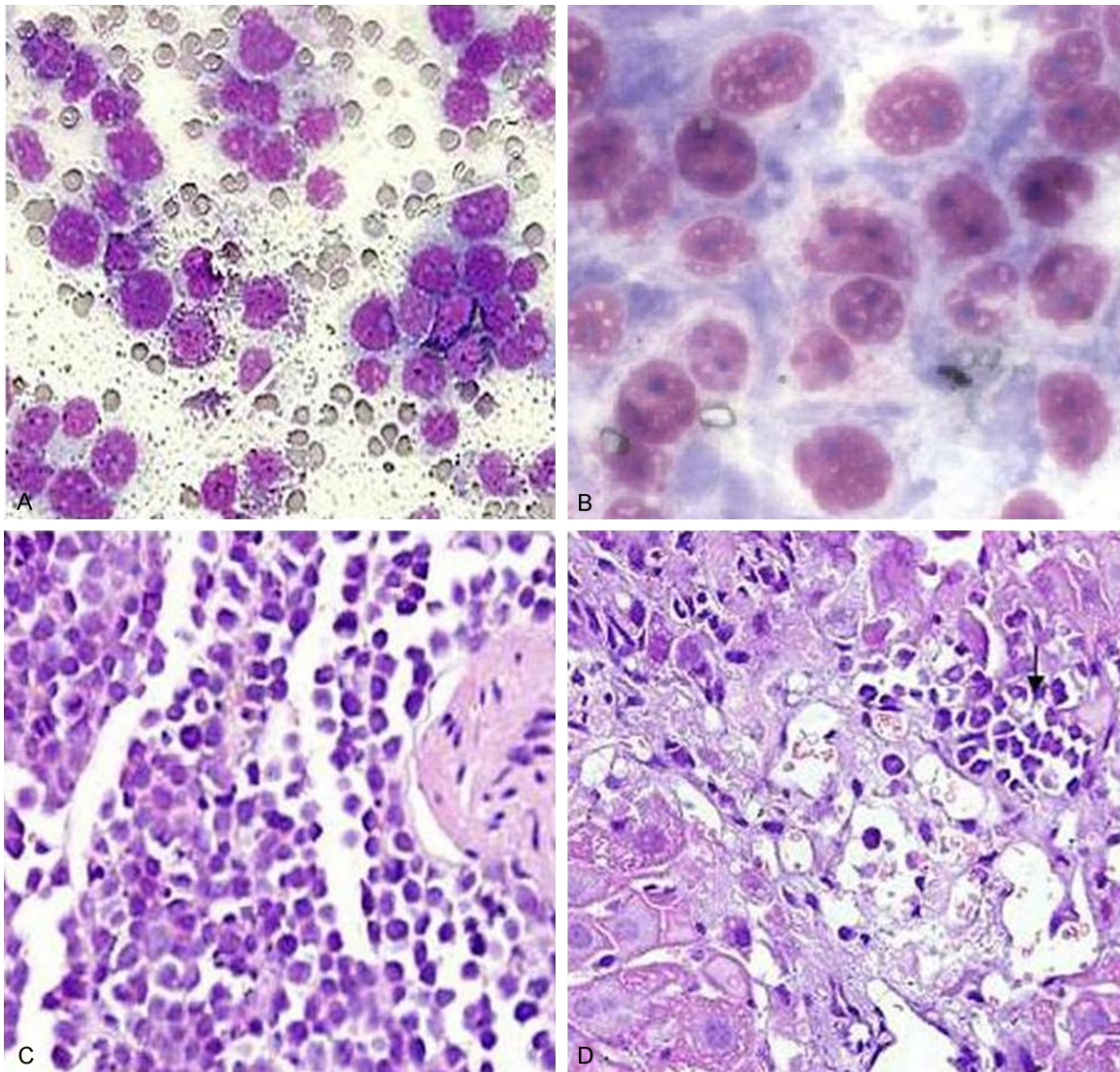
After inoculation of K562/A02 cells in NOD/SCID mice for two weeks, six animals were killed to determinate whether the K562/A02/SCID leukemia model was successful established. White blood cells were separated from the blood. Expressions of human *mdr* and *bcr/abl* mRNA were performed using RT-PCR. The visible leukemia cells were determined from peripheral blood, bone marrow smears, and slices of liver and neck lymph nodes.

##### *Human mdr and bcr/abl mRNA detection by RT-PCR*

*In vitro* total mRNA was extracted from the cells with Trizol reagent (Invitrogen Co. California, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized by reverse transcription from 1 µg of the total RNA using Reverse Transcriptase RNase (RT-PCR) M-MLV (Invitrogen Co. LA, USA) and oligo-dT. The amplification was performed in a final volume of 50 µL, containing 5 µL cDNA, 0.5 µL of each oligonucleotide primer, 1 µL of each dNTP, and 1 unit of Taq DNA polymerase. Amplification was carried out in a Thermal Cycler. The PCR primers and expected product size were as follows:

*mdr1*, forward: 5'-CCCATCATTGCAATAGCAGG-3' and reverse: 3'-ACTCCTCGTCTTCAAATTG-3' (157 bp); *bcr/abl*, forward: 5'-AGCATTCCGCTG-ACCATCA-3' and reverse: 5'-GCGTGATGTAG-TTGCTTGGGAC-3' (200 bp); β-actin, forward: 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse: 5'-CTCCTTAATGTCACGCACGATTTC-3' (539 bp).

Circulating conditions were 94°C one min, 57°C one min, 72°C one min, 30 cycles, and



**Figure 1.** The human K562/A02 cells of mice tissue biopsies. Note: A: Peripheral blood smear; B: Bone marrow smear ( $\times 1000$ ); C: Lymph node; D: Liver (Same magnification in A, C and D,  $\times 400$ ).

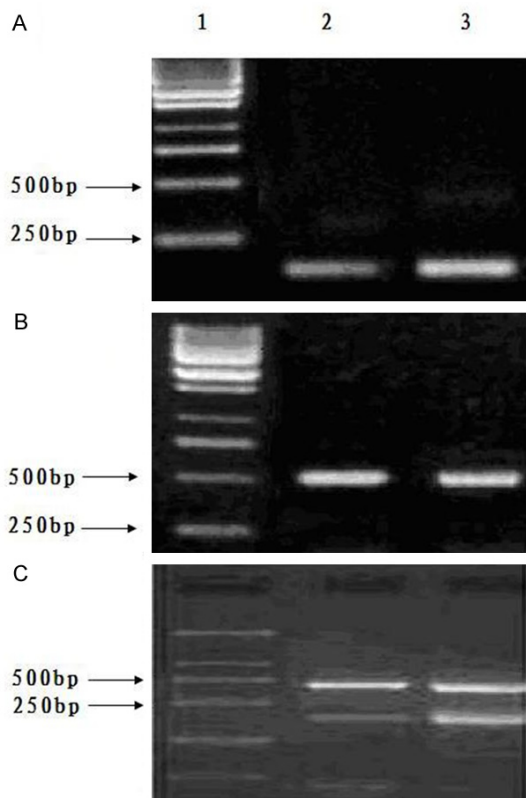
72°C for 10 min. The end products were identified by electrophoresis using 1.5% agarose gel. The positive control was P210 *bcr/abl* cDNA, and the negative control was the reverse transcription product of total RNA in normal human peripheral blood cells.

*The increasing curative effect treatment with CA combined with adriamycin in the K562/A02/SCID leukemia model*

Twenty K562/A02/SCID male were randomly divided into 2 groups, and each group had ten mice. The experimental group was fed with 1% (v/v) CA powder (purity  $\geq 91\%$ , Sigma, USA) feed

sterilized by Co-60 radiation. After 2 weeks of feeding, a solution of 1 mg/kg adriamycin (the purity higher than 98.0 (HPLC, Sigma, USA) was injected by intraperitoneal injection, with an interval time of two days, and a total of 3 injections for the animals in the two groups. Clinical manifestations of all the animals were observed and recorded daily. After feeding for 4 weeks, tail vein blood was collected, the number of the leukemia cells was calculated in the blood smears, and expressions of human *mdr* and *bcr/abl* mRNA were determined by RT-PCR (the same method as above). Protein expressions of the *mdr* and *bcr/abl* genes were also detected by Western Blot. The percentages of apoptotic





**Figure 2.** Expressions of *mdr1*, *bcr/abl* mRNA, and  $\beta$ -actin. A: *mdr1*; B:  $\beta$ -actin; C:  $\beta$ -actin and *bcr/abl*; 1. Marker; 2. Experimental model group; 3. Positive control group. Note: Expressions of *mdr1* and *bcr/abl* mRNA were at the same basic levels for the experimental model group and the positive control group.

cells were determined for the two groups (the mice treated with adriamycin group and the mice treated with CA combined with adriamycin group) by flow cytometry analysis. Each dead animal's organs including lung, liver, kidney and bone marrow were fixed using 10% formalin and histopathologically detected. Overall animal survival was estimated by Kaplan-Meier analysis.

#### Statistical analysis

Quantitative data in the study fit the normal distribution. Continuous variables were expressed as mean  $\pm$  SD (standard deviation). Comparing with those in the control group, the difference in mean of apoptosis cells in the experiment group was analyzed by Student's t test. The percent of leukemia cells in peripheral blood smears between the two groups tested by Chi-square. A log-rank (Wilcoxon) test was used to

determine significant differences between survival curves generated by the Kaplan-Meier method. *P* values of  $<0.05$  (two-sided) were considered statistically significant. All data analysis was performed using SPSS19.0 (SPSS Inc., USA).

#### Results

##### Determination of the established K562/A02/SCID leukemia mouse model

Two weeks after inoculation of K562/A02 cells in the NOD/SCID mice, a large number of infiltrated K562/A02 cells were found in the peripheral blood, bone marrow smears, and slices of neck lymph nodes and liver of mice (**Figure 1**).

Expressions of human *mdr1* and *bcr/abl* mRNA in the experimental animals were nearly at the same levels as in the positive control (**Figure 2**). These specific markers of human leukemia cells indicated that human K562/A02 leukemia cells grew in the mouse model.

##### Animal assay of increasing curative effect of leukemia with treatment with CA combined with adriamycin

Much more leukemia cells were in the peripheral blood and bone marrow smears. In peripheral blood smears the percentages of visible leukemia cells in 200 white cell counts were 20.0% and 32.5% for the CA-treated and the control groups respectively, and the difference was statistically significant.

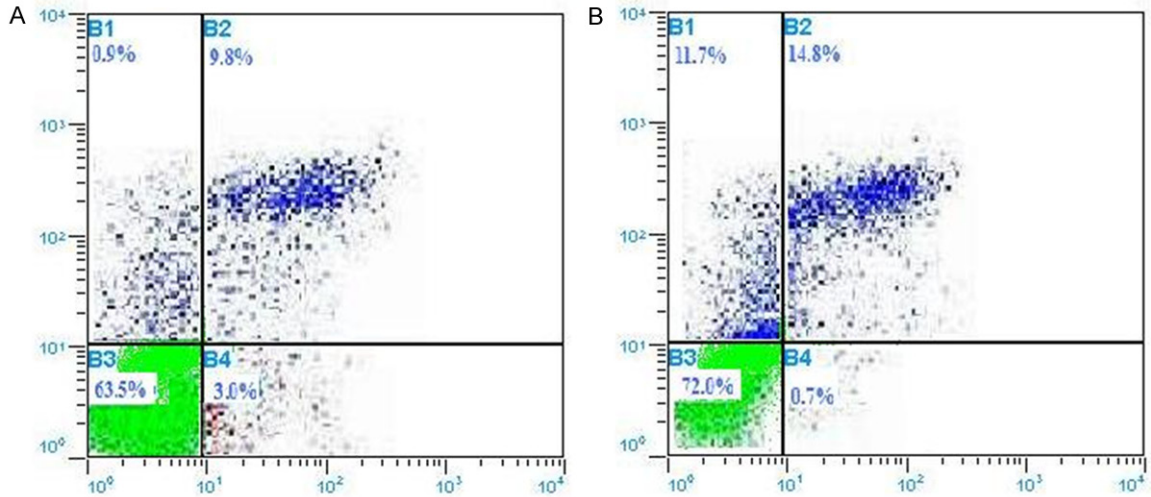
The percentages of apoptotic cells of mice treated with single adriamycin ( $15.5 \pm 1.56$ ) and the mice treated with CA combined with adriamycin ( $11.2 \pm 1.80$ ) have a significant difference ( $P < 0.05$ ) (**Figure 3**).

Expressions levels of *mdr1* and *bcr-abl* mRNA and protein in the experimental animals were significantly lower than that of the control animals (**Figures 4 and 5**).

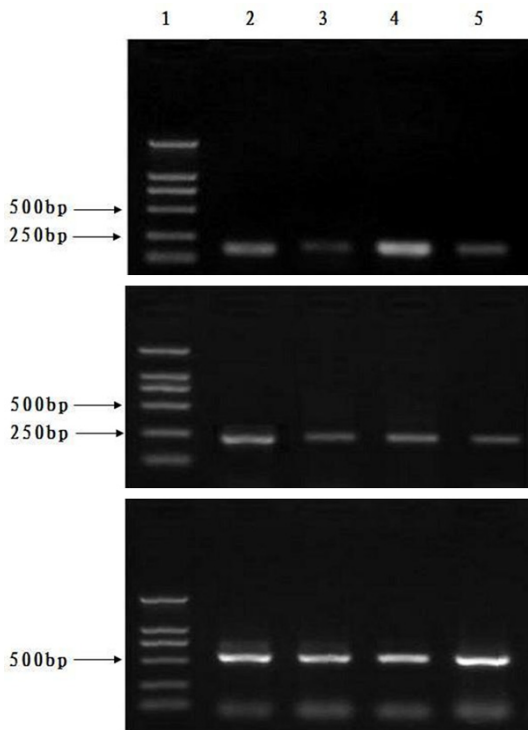
In the slices of lung, liver, kidney and bone marrow, the pathological changes have no significant difference, but micro-vessels show much more leukemia cells in the control animals than those in the CA-treated animals (**Figure 6**).

The median of 95% CI survival time is 19 (10.0-44.2) and 33 (29.4-36.6) days for the control

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**Figure 3.** The distribution of percentages of apoptosis (A) for mice treated with adriamycin (control group); (B) for the mice treated with CA combined with adriamycin (experimental group) tested by flow cytometry. The percentages of apoptotic cells of A group ( $15.5 \pm 1.56$ ) and B group ( $11.2 \pm 1.80$ ) has a significant difference ( $P < 0.05$ ).



**Figure 4.** Expressions levels of *mdr1* and *bcr-abl* mRNA in the experimental animals were significantly lower than in the control animals. A: *mdr1*; B: *bcr/abl*; C:  $\beta$ -actin; line 1, Marker; line 2 and 4, control animal group; line 3 and 5, treated animal group. Note: Compared with the mice treated with adriamycin (control group), expression levels of human *mdr1* and *bcr/abl* mRNA declined in mice treated with CA combined with adriamycin (experimental group).

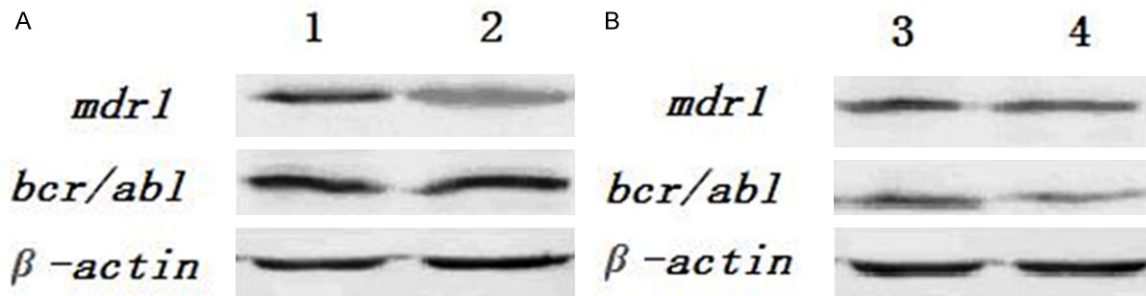
group and the CA-treated group, respectively. The difference is statistically significant ( $P < 0.05$ ) (Table 1; Figure 7).

### Discussion

The K562 cell line is of human chronic myeloid leukemia cells with positive expression of the *bcr-abl* gene. The multidrug-resistance (MDR) leukemia cell line K562/A02 is established through *in vitro* selection of K562 cells with an increasing concentration of adriamycin. The cells with the characteristic of over-expressing the *mdr1* gene are a tool for *in vitro* research of MDR [19]. In this work the K562/A02/SCID mouse model is established on the basis of the previous method [20]. Positive expression of *mdr* and *bcr/abl* mRNA and the outcome of pathological tests confirm that the animal model is successfully established.

CA is an extract from rosemary (*Rosmarinus officinalis* L.). Its antitumor or antineoplastic activities on different types of cancer cell lines/rat or mice models have been studied [5]. However, the anticancer mechanism of CA is not clear yet. From research of outcomes of all rosemary's extracts, the mechanism involves probably the apoptosis, carcinogen metabolic enzymes, or inhibition of tumor promoting events [15, 16]. In our previous work, we found that *in vitro* assay CA decreased the viability of

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**Figure 5.** Protein expression of *mdr1*, *bcr/abl*, and  $\beta$ -actin genes by Western blot determination in the experimental animals were significantly lower than in the control animals. Note: A: Line 1 for the established animal model, line 2 for the positive control; B: Line 3 for mice treated by adriamycin (control group), line 4 for mice treated by CA combined with adriamycin (experimental group).

the human promyelocytic leukemia cell line HL-60, in dose- and time-dependent manners, and induced G1 arrest and apoptosis. And these effects of CA were augmented when induced by a low (physiological) concentration of As203, which was associated with the up-regulation of p27 and activation of caspase-9. The mechanism was found to be mediated by the induction of phosphatase and tensin homologue (PTEN) expression [18]. The results of *in vivo* study in this work are consistent with the previous *in vitro* experimental results. Therefore, it is reasonable to conclude from both *in vitro* and *in vivo* results that CA is a promising adjuvant chemical drug candidate in AML therapy.

MDR is mainly caused by a cancer cell membrane of P-glycoprotein (P-gp), which can pump the anti-cancer drug out of the cell, and therefore reduce the intracellular drug concentration and trigger MDR. The basic strategy to reverse MDR is adopting the low toxicity or non-toxic compounds to bind P-gp and block its transport function [21].

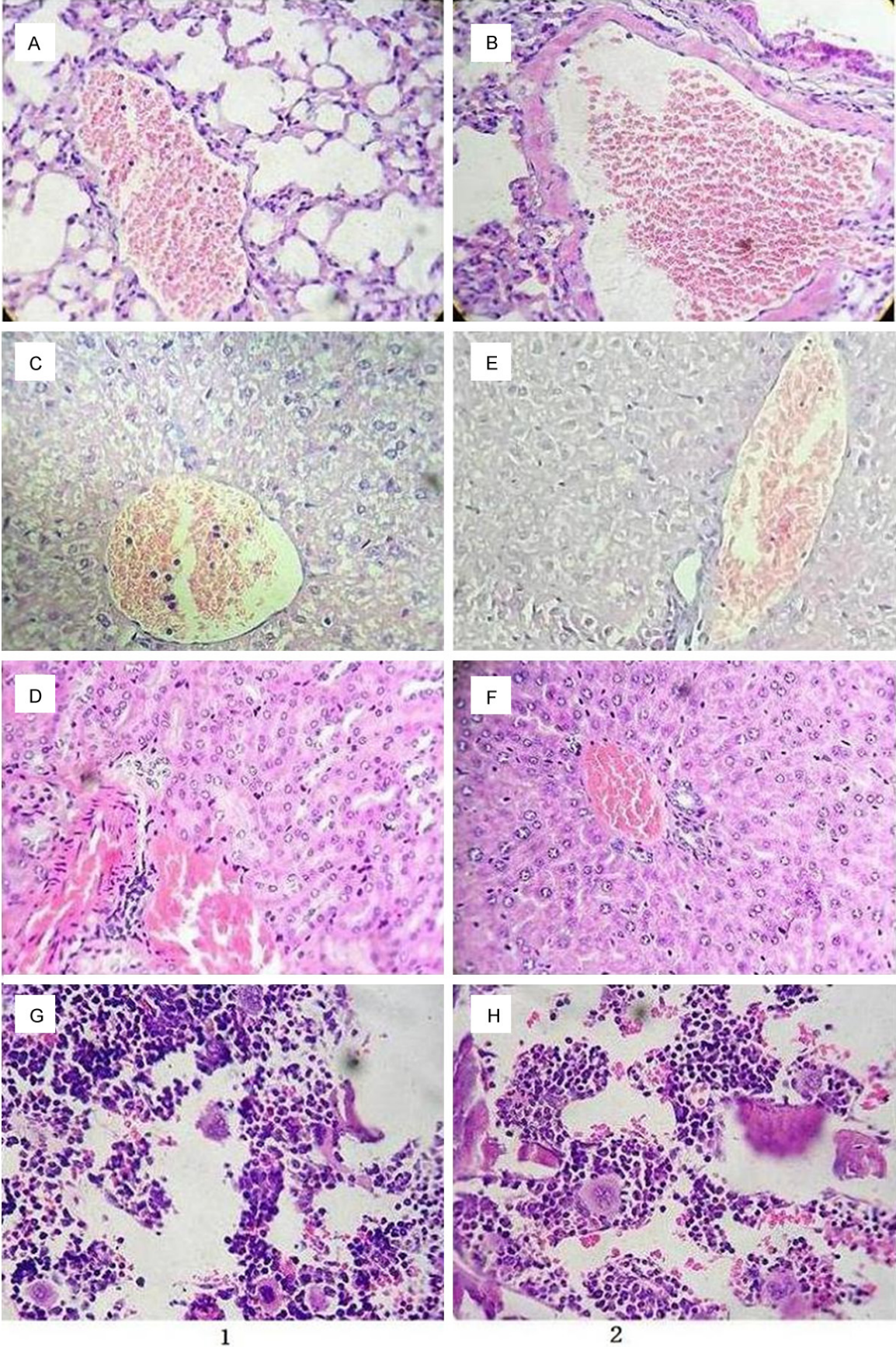
Increasing interest in CA is due to its pharmacological properties. However, no report was found on its safety as a medical drug. In our previous work we evaluated the medical security of CA. The results showed that the oral lethal dose (LD50) for mice was 7100 mg/kg of body weight in the acute toxicity study. Histopathological changes were observed in the heart and liver of all surviving mice treated with single-dose CA, and in the kidney with inflammatory cell infiltration for the surviving mice treated with doses larger than 7500 mg/kg and over of CA. For the sub-chronic toxicity

study, CA was administered for 30 days to produce slight reductions in the weight gain pattern, which did not reach a significant level comparing with the control values. For blood and serum biochemistry tests and histopathological examination of the animal organs, the effects of low-dose CA exposure on the experimental rats have no observable adverseness. The results suggest that the clinical dose of CA has relatively very low toxicity [22].

The results of the present study indicate that CA can prevent the growth of tumors with minor side effects. It is demonstrated that rosemary extracts inhibit the glycolysis and ATP synthesis in tumor cells in culture, except normal cells [23]. The molecular mechanism of this effect is not understood completely yet. In fact, the study of the mechanism on how CA differentially affects the normal and tumor cell energy metabolism may help to explain the basic differences, and suggest a potential new pathway to fight cancers without negative effects on normal cells [24]. CA can improve a protective immune reaction. Natural killer (NK) cells and type I interferons (IFN $\alpha$ ), tumor necrosis factor (TNF), IL-12, IL-10, and other cytokines play a major role in the innate control of immune-system replication. Notably, enhancing induction of inflammation factor over-expression is via a nuclear factor-kappa B (NF- $\kappa$ B) dependent mechanism [25]. Recently, studies have found that CA inhibits the prostaglandin E (2) (PGE (2)), an inflammatory and tumorigenic agent, formation [26]. Our previous work determines that the strong synergistic effect of inhibiting leukemia cell activities in CA and As203 combined treatment was associated with high



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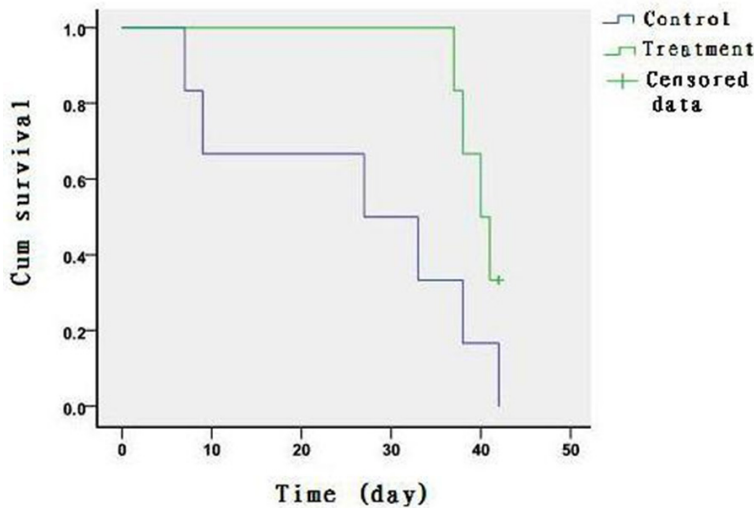
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**Figure 6.** The outcome of pathological slices examination for the treatment and control groups. Note: Line 1 for the mice treated with adriamycin (control group); A: Lung; C: Liver; D: Kidney; G: Bone marrow; Line 2 for the mice treated with CA combined with adriamycin (experimental group); B: Lung; E: Liver; F: Kidney; H: Bone marrow (Same magnificence in A-H,  $\times 400$ ).

**Table 1.** Survival analysis for the treatment and control groups\*

Group	Mean (day)	95% CI	Median (day)	95% CI
Control	19.5	9.3-29.6	19.0	10.0-44.2
Treatment	33.0	31.5-34.5	33.0	29.4-36.6

\*: CI, confidence interval; Generalized Wilcoxon test: Breslow=5.60,  $P=0.018$ .



**Figure 7.** Kaplan-Meier curves of the survival time (day) in the mice treated with adriamycin or CA combined with adriamycin.

expressions of cleaved caspase-3, PTEN, and p27 gene proteins [17, 18].

As compared with all chemotherapy drugs, adriamycin is highly toxic and poorly tolerated by patients. The present work indicates that CA combined with the current chemotherapeutic drugs could reverse MDR and reduce the dose of clinical AML therapy. However, the limitation of evidence does not fit the preclinical study. Further research on the mechanism for CA combined with other chemical drugs in the treatment of leukemia is necessary.

## Conclusion

In the present work we confirmed that CA combined with adriamycin can significantly extend the survival time in the K562/A02 leukemia animal model. CA is a promising adjuvant

chemical drug candidate for clinical AML therapy.

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## Disclosure of conflict of interest

None.

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