Combined efficacy of *Cinnamomum zeylanicum* and doxorubicin against leukemia through regulation of TRAIL and NF-kappa B pathways in rat model

Sidra Bukhari1 · Muhammad Hamid Siddique1 · Anum Naeem1 · InamUllah Khan1 · Zain Ali1 · Asiya Essa1 · Falak Fazal1 · Riffat Aysha Anis2 · Lukas Moran3 · Aneesa Sultan1 · Iram Murtaza1 · Mariam Anees1

Received: 29 April 2021 / Accepted: 14 April 2022 / Published online: 17 May 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract

**Background**  Recent discoveries in cancer therapeutics have proven combination therapies more effective than individual drugs. This study describes the efficacy of the combination of *Cinnamomum zeylanicum* and doxorubicin against benzene-induced leukemia.

**Methods and results**  Brine shrimp assay was used to assess the cytotoxicity of *C. zeylanicum*, doxorubicin and their combination. After AML induction in Sprague Dawley rats, the same drugs were given to rat groups. Changes in organ weight, haematological profile, and hepatic enzymes were determined. Real-time PCR was used to elucidate the effect on the expression of STMN1, GAPDH, P53 and various TRAIL and NF-kappaB components. *C. zeylanicum* reduced the cytotoxicity of doxorubicin. The combination treatment showed better anti-leukemic results than any of the individual drugs as evident from STMN1 expression (p < 0.001). It was particularly effective in reducing total white blood cell counts and recovering lymphocytes, monocytes and eosinophils along with hepatic enzymes ALT and AST (p < 0.001). All doses recovered relative organ weights and improved blood parameters. The combination therapy was particularly effective in inducing apoptosis, inhibition of proliferation marker GAPDH (p < 0.001) and NF-kappaB pathway components Rel-A (p < 0.001) and Rel-B (p < 0.01). Expressions of TRAIL components c-FLIP (p < 0.001), TRAIL ligand (p < 0.001) and caspase 8 (p < 0.01) were also altered.

**Conclusion**  *Cinnamomum zeylanicum* in combination with doxorubicin helps to counter benzene-induced cellular and hepatic toxicity and improves haematological profile. The anti-leukemic effects are potentially due to inhibition of GAPDH and NF-kappa B pathway, and through regulation of TRAIL pathway. Our data suggests the use of *C. zeylanicum* with doxorubicin to improve anti-leukemic therapeutic regimes.

**Keywords**  *Cinnamomum zeylanicum* · Doxorubicin · Acute myeloid leukemia · TRAIL · NF-kappa B · Rat model

Introduction

Leukemia is a malignancy of white blood cells (WBCs) with 437 thousand estimated cases per year worldwide [1]. It is the uncontrolled division of abnormal WBCs produced from hematopoietic stem cells in the bone marrow [2]. Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells and proliferation of leucoplasts along with a decrease in red blood cells (RBCs) and platelets [3]. AML occurs mainly in adults under the age of 60 [4]. Different factors including radiation, chemical injuries, chromosomal abnormalities, and viral infections are known to cause leukemia, but exposure of benzene is most common.
Benzene induces the cancer of the myeloid cells by its secondary metabolites like phenol, catechol and hydroquinone [5]. When benzene is metabolized in the liver, a large number of reactive oxygen species (ROS) are produced which causes cytotoxicity that leads to leukemia [6, 7].

Doxorubicin is a widely used chemotherapeutic drug against AML. It induces apoptosis by DNA damage through intercalation [8]. Moreover, it also inhibits the DNA repair mechanism. Doxorubicin, however, not only targets the cancer cells but also affects normal growing cells of the body leading to unavoidable side effects including ROS production, double stranded DNA breaks and apoptosis of normal cells [9]. Combinations of different chemotherapeutic drugs are being tested by oncologists to increase the effectiveness of the therapeutic regimes with lower doses of individual drugs. Various phytochemical compounds including natural plant extracts have also been combined with chemotherapy and indicated promising results. *Cinnamomum zeylanicum* is a plant with known antioxidant and anticancer potential and has been used as natural medicine for centuries [10]. In addition to anti-cancer potential, it has also exhibited anti-microbial, anti-diabetic, anti-oxidant, anti-inflammatory and wound healing properties [11]. It has also shown anti-proliferative efficacy in AML [12]. The aim of this study was to evaluate the effect of *C. zeylanicum* in conjugation with doxorubicin and to investigate the regulation of underlying molecular machinery in AML using a rat model.

Materials and methods

Preparation of aqueous *Cinnamomum zeylanicum* extract (CE)

Commercially available powder of *C. zeylanicum* bark (1000 mg) was dissolved in 100 mL distilled water by heating at 100°C for 3 h to make a stock of 10 mg/mL. The volume was retained at 100 mL through addition of distilled water to compensate for evaporative loss. The solution was centrifuged for 10 min at 10,000 rpm and the supernatant extract was filtered and stored at 4°C. Standard *C. zeylanicum* extract (CE) dose of 30 mg/kg was administered to rats. The weight of rats in our experiment ranged from 150 to 200 g. For preparing dose, a simple unitary method was applied. For example, for a 150 g rat the calculations were done as following:

1000 g weighing rat needs *Cinnamomum zeylanicum* = 30 mg
1 g weighing rat needs *Cinnamomum zeylanicum* = 30/1000
150 g weighing rat needs *Cinnamomum zeylanicum* = 30/1000 × 150 = 4.5 mg

The stock solution was 10 mg/mL so 450 μL CE stock was mixed with 550 μL distilled water to make up the volume to 1 mL which was then administered orally to the rat through a feeding tube.

Cytotoxicity assay

For determination of the optimum dose of drug and chemotherapy, the Brine shrimp assay was performed. Brine Shrimp eggs were incubated in sea salt solution for 24 h under fluorescent light. Dilutions of *C. zeylanicum*, doxorubicin and their combinations (1:1 v/v) were prepared. 10 brine shrimps were added in each dilution of *C. zeylanicum* i.e., 7 mg/mL, 3.5 mg/mL, 1.75 mg/mL and 0.875 mg/mL. Similarly, Brine shrimps were added in different dilution of doxorubicin i.e. 2 mg/mL, 1 mg/mL, 0.5 mg/mL and 0.25 mg/mL as described in a previous study [13]. The same dilutions were used for combination of *C. zeylanicum* and doxorubicin. After 24 h, the lethal concentration (LC₅₀) was calculated by counting number of alive vs dead shrimps. The value of LC₅₀ is inversely proportional to the toxicity of compound. The standard values for cytotoxicity are non-toxic > 1000 μg/mL, weak toxic ≥ 500 μg/mL to ≤ 1000 μg/mL, toxic < 500 μg/mL.

Animals

Five week old Sprague Dawley female rats (n = 25), weighing between 150 and 200 g were used in this study. All rats were obtained from the National Institute of Health (NIH), Islamabad. All experimental protocols were approved by the Bioethical Committee of Quaid-i-Azam University through letter No. #BEC-FBS-QAU2020-221. Rats were housed in pairs, in laboratory cages which were made of polypropylene with stainless-steel covers (standard size of cage was 8 × 12 × 5 inches). These rats were fed with standard rodent chow and water in Primate Facility of Quaid-i-Azam University. Photoperiods equaled 12 h of light and 12 h of darkness daily, with the environmental temperature and humidity maintained at 25°C ± 2 and 42% ± 5, respectively.

Experimental design

Rats were distributed into five equal groups (5 rats/group). Animals were allowed to acclimatize for a period of 7 days. Leukemia was induced by 0.2 mL of intravenous benzene (Sigma-Aldrich Cat. No. 71-43-2) injections diluted in saline solution in a ratio 0.5:1 for 14 alternate days. Group A (control) was the control group which was given...
intravenous injections of normal saline. Group B (Benz) was given 0.2 mL intravenous benzene injections for 2 weeks. In group C (Cinn), each rat was given aqueous CE 30 mg/kg orally on alternate days for 2 weeks after completion of benzene injections as described previously [14, 15]. In group D (Doxo), each rat was given intravenous injections of doxorubicin (Actavis, Italy Cat. No. 25316-40-9) 3.2 mg/kg as described previously [13] for 2 weeks after completion of benzene administration. In group E (Cinn+Doxo), each rat was given both C. zeylanicum orally and doxorubicin intravenously for 2 weeks after completion of benzene injections.

**Morphological study and blood parameters**

After dissections of the rats were performed, slides were prepared from fresh blood and liver tissues for morphological analysis. Blood slides were air dried, stained with 100% Giemsa for 5 min, washed with water and then dried for microscopic analysis. Paraffin embedded tissue slides were processed for histology using standard H&E staining protocol. AML induction was confirmed by morphological changes in blood smear and tissue histology expressing increase in immature blast cell numbers, less cytoplasm to nucleus ratio in WBCs and erythro-phagocytosis. Blood CP (complete picture) for total cell counts was performed on an automated instrument in National Agricultural Research Centre (NARC), Islamabad. Weights of the organs were measured after dissection using a standard laboratory balance. Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using biochemical diagnostic kits (Sigma-Aldrich Cat. No. AP0100, Cat. No. MA052 and Cat. No. MA055, respectively).

**Superoxide dismutase assay (SOD)**

Samples were prepared by homogenizing 100 mg of preserved rat liver in 1 mL potassium phosphate buffer (100 mM) and 1 mM EDTA (pH 7.4). Mixture was centrifuged at 10,000 rpm for 10 min (4 °C). Reaction mixture was prepared from 1200 μL sodium pyrophosphate buffer (0.052 mM; pH 7.0). 100 μL phenazine methosulphate (186 μM), and 300 μL of supernatants. 0.2 mL NADH (780 μM) was used to start enzyme activity. Absorbance was determined at 560 nm.

**Real time PCR analysis**

For relative mRNA expression analysis, RNA was extracted from freshly resected liver of rats using protocol described by Chomczynski [16]. cDNA was synthesized from mRNA using VIVANTIS kit (Cat. No. cDSK01-050). For relative gene expression MIC qPCR by Bio Molecular Sciences (BMS) was used. The PCR-amplification was performed in a final volume of 20 μL containing 4 μL of 5x EvaGreen® qPCR Mix Plus (ROX), Solis Bio Dyne, 2 μL of forward and reverse primers, 1 μL of cDNA and made up to the final volume with nuclease free water. After an initial heat denaturation step at 95 °C for 15 min, 40 cycles were programmed as follows: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Subsequently, a melting curve analysis was programmed so that its slope formed from 72 to 95 °C by raising 1 °C each step. The program was designed to wait for 118 s of pre-melt conditioning on first step and for 5 s for each step afterwards. Beta-actin was used as the housekeeping gene. Sequences of the primers used for screening target genes were as follows:

| Gene          | Forward primer 5′-3′ | Reverse primer 5′-3′ |
|---------------|---------------------|---------------------|
| STMN1         | F-5′TTGCCAGTGATTGTGTAGAG3′ | R-5′TTCCTTGTATGCGGCTAG3′ |
| GAPDH         | F-5′TCCAGTATGCTTACCCACG3′   | R-5′CACGATCAGTCCGACGAG3′ |
| P53           | F-5′TCCCCACTATACACTTCACTAC3′ | R-5′GCACAAACACGCACTCCAAAG3′ |
| Rel-A         | F-5′CTACGAGACCTTCAAGAGCATC3′    | R-5′GATGTGAAAGGGCAGATGG3′ |
| Rel-B         | F-5′CTTCTCAGCTGACGTGC3′       | R-5′AGATCTCCAAGGGCCTGATG3′ |
| c-FLIP        | F-5′AGAGGCCCCTACCTTGTTC3′     | R-5′CTCTCCAGTGGCTACCTTG3′ |
| DR5           | F-5′TCAACCTGCTGCCAATCC3′      | R-5′ATGAACCTCTCCAGGTG3′ |
| TRAIL ligand  | F-5′CACATTACC GGATCATCG3′     | R-5′AGCTCTCGTTTCTCAAGTG3′ |
| Caspase 8     | F-5′GAGCTGCCAGTCTTGTGTTC3′    | R-5′GGTGAAGACAGATCGTACC3′ |

**Flow cytometry for apoptosis measurement**

Cells were washed twice with cold BioLegends Cell Staining Buffer. Next, Annexin V Binding Buffer was used to re-suspend the cells at a concentration of 0.25–1 × 10^7 cells/mL. 100 μL of cell suspension was transferred to a 5 mL test tube. 5 μL of FITC Annexin V was then added. Then, 10 μL of propidium iodide solution was added to it. The cells were vortexed gently and then they were incubated at 25 °C for 15 min in the dark. Subsequently, in each tube, 400 μL of Annexin V Binding Buffer was added and analyzed on the flow cytometer. Results were obtained using standard operating protocols of the flow cytometer.

**Data and statistical analysis**

Relative Expression Software Tool (REST-384, version 2) was used for analysis of the relative gene expression. Descriptive statistics were computed using Graph Pad Prism8 software and results were described as
mean ± standard deviation (SD). Inter-group comparisons were made using one-way analysis of variance (ANOVA). Groups were further separated using Tukey’s post-hoc test. Statistical difference with a p value < 0.05 was taken as significant and p < 0.001 as highly significant.

Results

*Cinnamomum zeylanicum* reduced the cytotoxicity of doxorubicin

The cytotoxicity assay results revealed that *C. zeylanicum* was non-toxic (LC$_{50}$ = 38980 μg/mL) while doxorubicin caused significant toxicity (LC$_{50}$ = 228.5 μg/mL) to the Brine shrimps. However, using the combination of doxorubicin with *C. zeylanicum* decreased the cytotoxicity of doxorubicin to LC$_{50}$ value of 11,694.1 μg/mL rendering it non-toxic. Generally, compounds with LC$_{50}$ of > 1000 μg/mL are considered as non-toxic, those having LC$_{50}$ between 1000 and 500 μg/mL weakly toxic and those having LC$_{50}$ of < 500 μg/mL considered toxic. In this way, we can conclude that *C. zeylanicum* masked the toxicity of doxorubicin.

Recovery of whole-body and relative organ weights in treated groups

Mean whole-body weight was reduced in leukemic rats by 1.3-fold (p < 0.05). *C. zeylanicum* and doxorubicin helped in recovery of whole-body weight to some extent. However, the combination treatment of *C. zeylanicum* with doxorubicin was most effective in weight recovery by 1.2-fold as depicted in Fig. 1A. The relative organ weights of heart, kidney and liver were increased in leukemic rats after benzene administration potentially, due to the hypertrophy of tissues. The treatment groups showed significant decline (p < 0.05) in the comparison of (mean ± SD) weights of liver, kidney and heart, respectively. The intergroup comparisons were made using ANOVA with Tukey’s post hoc test. Statistical difference of p < 0.05 was taken as significant and represented as asterisk (*)

![Fig. 1 Comparison of body and relative organ weights. Panel A depicts mean ± SD body weight of Sprague Dawley rats on alternate days of treatment. There were 5 rats in each experimental group. Combined treatment of doxorubicin with cinnamon significantly recovered whole body weight back to normal. Panels B–D describe the comparison of (mean ± SD) weights of liver, kidney and heart, respectively. The intergroup comparisons were made using ANOVA with Tukey’s post hoc test. Statistical difference of p < 0.05 was taken as significant and represented as asterisk (*)](image-url)
organ weights. For liver, the combination treatment showed the lowest (1.6-fold, p < 0.01) reduction in organ weight as shown in Fig. 1B. *C. zeylanicum* alone was as effective as the combination therapy in reducing kidney and heart weight (Fig. 1C, D).

**Restoration of blood cell counts, morphological and histological features**

The level of WBCs was increased by 1.8-fold while the level of RBCs and platelets were remarkably reduced by 2.4- and 2.3-fold respectively in leukemic rats (p < 0.05) as depicted in Fig. 2. The WBC counts were improved by 1.2-fold in combination of doxorubicin and *C. zeylanicum* (p < 0.05). Levels of RBCs and platelets were significantly recovered in all treatment groups (p < 0.001). Hemoglobin levels were decreased upon benzene administration by 1.2-fold (p > 0.05) but were not affected by any of the treatment regimens. Morphological analysis of Giemsa stained blood slides showed decreased concentration of erythrocytes and the presence of abnormal leucocytes in benzene administered leukemic rats. Likewise, increased nucleus-to-cytoplasm ratio and leukocytosis was also observed as shown in Fig. 2E (middle panel) along with a significantly increased number of blast cells. Similar observations were made in H&E stained tissue slides as blast cells appeared in benzene treated rats (Fig. 2F, column 2). However, treatment with combination therapy restored the normal morphology of leucocytes in treated rats along with a reduction in blast cells as shown in Fig. 2F.

**Immunomodulatory effects of combination therapy**

The populations of neutrophils, monocytes and eosinophils were increased by 1.2-, 1.5- and 2.3-fold respectively while the lymphocytes were decreased by 1.1-fold in response to benzene administration as shown in Fig. 3. The combination of *C. zeylanicum* and doxorubicin was most effective (p < 0.05) in recovering lymphocyte (increasing 1.1-fold), monocyte (decreasing 1.4-fold) and eosinophil (decreasing 1.7-fold) counts. Neutrophil counts were not recovered by any of the treatments. Eosinophil population was improved by all treatment regimens (p < 0.05).

**Recovery of hepatic enzyme levels and SOD upon treatment**

Hepatic biomarkers ALT and AST both showed a marked increase while ALP showed substantial decrease upon leukemia induction in rats (2.2-fold, p < 0.01). It was observed that combination therapy was most effective for reducing ALT and AST levels (2.2- and 4.3-fold respectively, p < 0.05). ALP levels however, were improved by individual drugs. The results are shown in Fig. 4. The level of SOD were slightly increased after the benzene administration. *C. zeylanicum* alone and combination of *C. zeylanicum* and doxorubicin showed noticeable increase in levels of SOD enzyme (as shown in Fig. 4D).

**Confirmation of acute myeloid leukemia by STMN1 expression**

The genetic marker STMN1 was used for the confirmation of AML induction [17, 18]. STMN1 expression increased significantly upon benzene treatment confirming leukemia in our rats (20.7-fold, p < 0.001). *C. zeylanicum* treatment did not have any marked effect on its expression. Doxorubicin, being the classical chemotherapy, reduced STMN1 expression by 2.4-fold (p < 0.001). However, the combination therapy was the most effective in lowering the STMN1 expression close to normal levels (6.3-fold, p < 0.001) signifying the importance of our therapeutic regime as shown in Fig. 5A.

**Restoration of GAPDH and P53 gene expression**

GAPDH is a marker of increased metabolism and proliferation in transformed cells. Its expression was significantly increased in the benzene group (5.2-fold, p < 0.001). All the treatment groups significantly lowered its levels (p < 0.001) but the most reduction was seen in the combination group (17-fold, Fig. 5B). The expression of tumor suppressor gene p53 was significantly decreased upon leukemia induction (threefold, Fig. 5C). The levels improved significantly upon treatment with *C. zeylanicum* alone (2.7-fold, p < 0.001). However, the combination did not prove to be effective potentially due to masking of *C. zeylanicum* effects by chemotherapy.

**Synergistic increase in apoptotic potential of combination therapy**

An Annexin-V/Propidium Iodide binding assay revealed significant increase in apoptotic potential of cinnamon and its combination with doxorubicin as compared to control group (as shown in Supplementary Fig. 1). Percentage apoptosis in control group was 0.34%, in leukemic group it was nearly 8%, in doxorubicin treated group it was 14.5%, in *C. zeylanicum* treated group it was 31% and in the combination group it reached as high as 67% showing a synergistic effect. This suggests some cross-linking between *C. zeylanicum* and doxorubicin against cancerous cells, rendering their combination an effective remedy against AML.
Inhibition of canonical and non-canonical NF-kappa B pathways

The NF-kappa B pathway is involved in the proliferation of cancer cells. The expression of NF-kappa B genes Rel-A (from canonical pathway) and Rel-B (from non-canonical pathway) was increased (35.58-fold, $p < 0.001$ and 3.72-fold, $p < 0.01$ respectively) in response to benzene induced leukemia depicting higher proliferation levels (Fig. 5D, E). C. zeylanicum alone and the combination therapy both reduced the expression of both Rel-A and Rel-B genes ($p < 0.05$), thus showing the anti-proliferative potential.

Regulation of TRAIL pathway by various treatment regimes

TRAIL is one of the important pathways which induces apoptosis in transformed cells and can be regulated by intrinsic and extrinsic pathways. C. zeylanicum and combination therapy showed promising results in modulating TRAIL pathway. The expression of c-FLIP, which is an inhibitor of apoptosis, was increased (2.2-fold) in response to leukemia induction but most effectively reduced by the combination of C. zeylanicum with doxorubicin (11.99-fold, $p < 0.001$) as given in Fig. 6A. DR5 expression which is known to induce apoptosis was increased by C. zeylanicum (twofold, $p < 0.01$); however, its combination with doxorubicin diminished its effects (Fig. 6B). TRAIL ligand expression was increased by benzene (5.6-fold, $p < 0.001$) but the combination therapy reduced the levels (4.0-fold) back to normal (Fig. 6C). In addition, the combination therapy significantly increased the expression of caspase 8 (1.4-fold, $p < 0.01$) which plays a central role in extrinsic TRAIL pathway (Fig. 6D). C. zeylanicum also showed some improvement in the expression levels of Bax, caspase 7 and 9 but did not show any effects in combination with doxorubicin (data not significant).

Discussion

Leukemia is a hematopoietic malignancy of non-epithelial cells [19] that is divided into four types including AML which could also be artificially induced through benzene exposure [20]. The most common way to treat leukemia is chemotherapy [21]. However, due to the cytotoxicity induced by the chemotherapeutic drugs in normal cells, most patients suffer during treatment [22]. Currently, focus has shifted towards herbal medicines extracted from medicinal plants to augment chemotherapy [11]. C. zeylanicum being a source of several beneficial compounds has proven to be an important herbal drug against leukemia. Cinnamaldehyde, one of its active ingredients, induces apoptosis in human leukemic cells through ROS generation leading to loss of mitochondrial membrane potential, thereby releasing cytochrome c into their cytosol. Evidence also suggests involvement of mitochondria-dependent cytochrome c-Apaf-1-procaspase-9 pathway in cinnamaldehyde induced apoptosis [23]. Cinnamon also has a potent chemotherapeutic sensitizer, as it inhibits STAT3 and AKT pathways signaling to overcome drug resistance in chemo-resistant cancer cells [24]. Similarly, eugenol, which accounts for 2.77% of cinnamon bark suppresses NF kappaB activation pathway in AML resulting in cancer cell growth arrest [25]. It also targets the Notch-Hes1 signaling pathway to eliminate resistant ovarian cancer stem cells [26]. This study revealed the anticancer efficacy of combination treatment of a C. zeylanicum bark aqueous extract with doxorubicin in benzene administered leukemic rats.

Cytotoxicity assay showed the non-toxic nature of C. zeylanicum as reported previously [12, 15]. Our data suggests that the toxicity of doxorubicin was reduced by its combination with C. zeylanicum as supported by literature [27, 28]. The induction of AML was confirmed by using a STMN1 genetic marker (Fig. 5A) [29] as well as morphological changes in blood smears (Fig. 2E) [30].

The increase in organ weights during in-vivo experiments indicated increased proliferation of cells leading to hypertrophy in these organs (Fig. 1B–D). The hypertrophy observed is due to infiltration of malignant leukemic cells into the liver, spleen, and other vital organs. Therefore, these organs may enlarge and increase in weight causing hypertrophy in leukemia [31]. However, the reduction of organ weights back to normal by the treatment regimens provides significant evidence of the protective effect of C. zeylanicum against benzene induced hypertrophy. However, loss in overall body weight was observed in leukemic rats (Fig. 1A) because the rapidly dividing leukemic cells take up most of the energy that otherwise would have been utilized or stored as fat. Combination of C. zeylanicum and doxorubicin helped in recovery of body weight to a considerable extent.

Improvement in differential WBC counts (Fig. 2A) by C. zeylanicum and its combination with chemotherapy may be attributed to the immunomodulatory effects of C. zeylanicum [32]. Decrease in RBC and platelet counts observed in leukemic rats, reverted to normal in treated groups. The reason may be that C. zeylanicum has a potential to stimulate the
process of protein synthesis and thus helps in the recovery of normal morphology of erythrocytes. The active ingredients in cinnamon are known to elevate the levels of thyroid hormones in animals, which play a direct role in increasing the metabolic rate in the body, as well as having an indirect role in controlling the blood cells production in bone marrow, thymus and other organs through increased protein synthesis. This leads to induced erythropoiesis causing increment of RBCs [33].

When given alongside chemotherapy, *C. zeylanicum* counters the toxicity caused by the drug and shows renal and hepato-protective potential [34, 35]. In leukemic rats, benzene deregulates the levels of ALP, ALT and AST due to toxicity of ROS produced by its metabolites (Fig. 4) [36]. Normally these enzymes help in detoxification of compounds. Changes in their levels indicate toxicity of liver [37]. This was significantly minimized by *C. zeylanicum* treatment, probably due to its antioxidant, free radical-scavenging properties and reduction in lipid peroxidation [32]. As SOD enzymes scavenge ROS species by dismantling them into peroxides, SOD enzymes activity directly relates with concentration of ROS produced. This suggests that in combination therapy increased SOD activity reflects increased ROS production leading to ROS mediated cell death in leukemic cells [38].

STMN1 being the marker of AML, showed elevated expressions (Fig. 5A) after benzene administration [29]. This effect of benzene was minimized by the combination therapy. The upregulation of STMN1 expression promote cell proliferation, invasion and migration [17, 18].

The expression of GAPDH was significantly reduced by all the treatment groups (Fig. 5B) suggesting the decrease using ANOVA with Tukey’s post hoc test. Statistical difference of p<0.05 was taken as significant and represented as asterisk (*)

![Fig. 3](image-url) Differential counts of **A** lymphocytes, **B** neutrophils **C** monocytes and **D** eosinophils in various experimental groups. Each bar represents mean ± SD value. The intergroup comparisons were made using ANOVA with Tukey’s post hoc test. Statistical difference of p<0.05 was taken as significant and represented as asterisk (*)
in the rate of glycolysis by cancer cells which further suggests reduced proliferation of the transformed cells [39, 40]. Expression of P53 was enhanced by *C. zeylanicum* which was downregulated in case of leukemia (Fig. 5C). Loss of p53 is the common genetic alteration mostly observed in cancers [41, 42]. Doxorubicin causes apoptosis through p53 dependent or p53 independent pathway by ROS production [43, 44]. Our results suggested that *C. zeylanicum* alone has a potential to enhance the expression of this critical tumor suppressor gene and so has the ability to counter cellular damage; however, the combination did not show any promise in this regard. *C. zeylanicum* and combination therapy inhibit the NF-kappa B pathway by downregulation of canonical pathway component Rel-A (Fig. 5D) and non-canonical pathway component Rel-B (Fig. 5E) which suggests that along with the apoptosis inducing capability, *C. zeylanicum* also has anti-proliferative potential [45].

Our results predicted that *C. zeylanicum* has anti-cancer activity potentially through its metabolites. Its combination with doxorubicin modulates the TRAIL pathway by increasing the expression of DR5 (Fig. 6B) and decreasing the expression of inhibitory protein c-FLIP (Fig. 6A). Doxorubicin is known to induce apoptosis through p53 dependent and independent pathways [46]. At low doses, doxorubicin displayed promising results in increasing DR5 and decreasing c-FLIP expression in human prostate cell lines [47, 48]. It is important to mention that although TRAIL is known to induce apoptosis however, high expression of TRAIL

**Fig. 4** Comparison of hepatic markers **A** ALT, **B** ALP and **C** AST in blood serum of rats. **D** Comparison of super oxide dismutase (SOD) levels in liver samples of rats. Combination therapy significantly increased the levels of SOD. The values are shown as mean ± SD.

The intergroup comparisons were made using ANOVA with Tukey’s post hoc test. Statistical difference of *p* < 0.05 was taken as significant and represented as asterisk (*)
Fig. 5 Gene expression analysis of molecular markers A STMN1, B GAPDH, C P53, D Rel-A and E Rel-B by real time PCR in various experimental groups. The intergroup comparisons were made using ANOVA with Tukey’s post hoc test. Statistical difference of $p < 0.05$ was taken as significant and represented as asterisk ($^*$)
is usually seen in transformed cells (Fig. 6C) [49]. TRAIL expression is increased as a first line of defense against tumorigenesis. However, its presence does not necessarily induce apoptosis as numerous other regulators are also involved in the process including the death and decoy receptors and the presence or absence of inhibitory proteins [49]. The combination therapy was highly effective in increasing caspase 8 expression in our study (Fig. 6D).

Conclusion

*Cinnamomum zeylanicum* increases the anti-cancer and therapeutic potential of doxorubicin. It also shows remarkable ability of decreasing the cytotoxicity caused by doxorubicin. The combination treatment exhibits excellent anti-leukemic potential and improves the blood parameters, reduces organ hypertrophy and restores the hepatic enzyme levels. The
anticancer potential is mediated through GAPDH, TRAIL and NF-kappa B pathways. Hence CE can be administered/combined with doxorubicin to function as an excellent chemotherapeutic agent against AML.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07478-y.

**Acknowledgements** We acknowledge the technical assistance provided by Alpha Genomics in sample processing, National Training and Research Laboratory for providing cDNA synthesis kits, National Institute of Health for providing rats and NARC for facilitation in hematological profiling. We also extend our gratitude to Jonathan Baker, Research Fellow, Faculty of Medicine & Health Sciences, University of Nottingham, UK, for proofreading our manuscript.

**Author contributions** Conceptualization: MA, AN; Methodology: SB, MHS, AN, IU, K, AE, FF; Software: SB, MHS; Validation: AS, IM, MA; Formal analysis: AS, IM, RAA; Investigation: SB, MHS, AN, IU, K; Data curation: MHS, AE, ZA; Original draft preparation: MA, SB; Review and editing: MA, SB, PV, LM.

**Funding** The work was financially supported by University Research Fund (URF) of Quaid-i-Azam University, Islamabad, Pakistan.

**Data availability** All data recorded during experiments is made available in the manuscript in transparent manner. Further details can be provided upon request.

**Code availability** Not Applicable.

**Declarations**

**Conflict of interest** All authors declare no conflict of interests.

**Ethical approval** The study was approved by the Bioethical Committee of Quaid-i-Azam University, Islamabad, Pakistan through letter No. #BEC-FBS-QAU2020-221.

**Consent to participate** NA.

**Consent for publication** All authors submit their consent for publication of this manuscript in Molecular Biology Reports Journal.

**References**

1. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin D, Piñeros M, Znaor A, Bray F (2019) Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer 144(8):1941–1953.
2. Dean A, Ferguson J, Marvanr E (2003) Acute leukaemia presenting as oral ulceration to a dental emergency service. Aust Dent J 48(3):195–197.
3. Lowenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. N Engl J Med 341(14):1051–1062.
4. Shipley JL, Buteña JN (2009) Acute myelogenous leukemia. Exp Hematol 37(6):649–658.
5. Karaulov AV, Mikhailova IV, Smolyagin AI, Boev VM, Kalogeraki A, Tsatsakis AM, Engin A B (2017) The immunotoxicological pattern of subchronic and chronic benzene exposure in rats. Toxicol Lett 275:1–5.
6. Aksoy M (1989) Hematotoxicity and carcinogenicity of benzene. Environ Health Perspect 82:193–197.
7. Atkinson TJ (2009) A review of the role of benzene metabolites and mechanisms in malignant transformation: summative evidence for a lack of research in nonmyelogenous cancer types. Int J Hyg Environ Health 212(1):1–10.
8. Ashley N, Poulton J (2009) Mitochondrial DNA is a direct target of anti-cancer anthracycline drugs. Biochem Biophys Res Commun 378(3):450–455.
9. Carvalho C, Santos RX, Cardoso S, Oliveira PJ, Santos MS, Moreira PI (2009) Doxorubicin: the good, the bad and the ugly effect. Curr Med Chem 16(25):3267–3285.
10. Marongiu B, Piras A, Porseida S, Tuveri E, Sanjust E, Meli M, Sollai F, Zucca P, Rescigno A (2007) Supercritical CO2 extract of *Cinnamomum zeylanicum*: chemical characterization and anti-tyrosinase activity. J Agric Food Chem 55(24):10022–10027.
11. Ranasinghe P, Piga S, Premakumara GS, Galappaththy P, Constantine GR, Katulanda P (2013) Medicinal properties of ‘true’ cinnamon (*Cinnamomum zeylanicum*): a review. BMC Complement Altern Med 13(1):275.
12. Assadiollahi V, Parivar K, Roudbari NH, Khalaftabary AR, Motamed M, Ezatpour B, Dashiti GR (2013) The effect of aqueous cinnamon extract on the apoptotic process in acute myeloid leukemia HL-60 cells. Adv Biomed Res. https://doi.org/10.4103/2277-9175.108001.
13. Golla K, Cherukuvada B, Ahmed F, Kondapi AK (2012) Efficacy, safety and anticancer activity of protein nanoparticle-based delivery of doxorubicin through intravenous administration in rats. PLoS ONE 7(12):e51960.
14. Qin B, Nagasaki M, Ren M, Bajotto G, Oshida Y, Sato Y (2003) Cinnamon extract (traditional herb) potentiates in vivo insulin-regulated glucose utilization via enhancing insulin signaling in rats. Diabetes Res Clin Pract 62(3):139–148.
15. Hong JW, Yang GE, Kim YB, Eom SH, Lew JH, Kang H (2012) Anti-inflammatory activity of cinnamon water extract in vivo and in vitro LPS-induced models. BMC Complement Altern Med 12(1):237.
16. Chomczynski P (2010) Reagents and methods for isolation of purified RNA. Google Patents.
17. Ni PZ, He JZ, Wu ZY, Ji X, Chen LQ, Xu XE, Liao LD, Wu JY, Li EM, Xu LY (2017) Overexpression of Stathmin 1 correlates with poor prognosis and promotes cell migration and proliferation in oesophageal squamous cell carcinoma. Oncol Rep 38(6):3608–3618.
18. Serachi FdO, Marie SKN, Oba-Shinjo SM (2017) Relevant coexpression of STMN1, MELK and FOXM1 in glioblastoma and review of the impact of STMN1 in cancer biology. Med Express. https://doi.org/10.5935/MedicalExpress.2017.05.07.
19. Weinberg RA (2013) The biology of cancer. Garland Science, New York.
20. Collins J, Ireland B, Buckley C, Shepperdy D (2003) Lymphohematopoietic cancer mortality among workers with benzene exposure. Occup Environ Med 60(9):676–679.
21. Carney D, Westerman D, Tam C, Milner A, Prince H, Kenealy M, Wolf M, Januszewicz E, Ritchie D, Came N (2010) Therapy-related myelodysplastic syndrome and acute myeloid leukemia following Fludarabine combination chemotherapy. Leukemia 24(12):2056.
22. Chatterjee K, Zhang J, Honbo N, Karliner JS (2010) Doxorubicin cardiomyopathy. Cardiology 115(2):155–162.
23. Ka H, Park HJ, Jung HJ, Choi JW, Cho KS, Ha J, Lee KT (2003) Cinnamonaldehyde induces apoptosis by ROS-mediated mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells. Cancer Lett 196(2):143–152.
24. Xi J, Yun M, Lee D, Park MN, Kim EO, Sohn EJ, Kwon BM (2015) Cinnamaldehyde derivative (CB-PIC) sensitizes chemoresistant cancer cells to drug-induced apoptosis via suppression of MDR1 and its upstream STAT3 and AKT signalling. Cell Physiol Biochem 35(5):1821–1830

25. Aggarwal BB, Shishodia S (2004) Suppression of the nuclear factor-kB activation pathway by spice-derived phytochemicals. Ann NY Acad Sci 1030:434–441

26. Islam SS, Abousselha A (2019) Sequential combination of cisplatin with eugenol targets ovarian cancer stem cells through the Notch-Hes1 signalling pathway. J Exp Clin Cancer Res 38(1):382

27. Sandamali JAN, Hewawasam RP, Jayatilaka KA, Mudduwa LKB (2018) Protective effect of Cinnamomum zeylanicum bark extract against doxorubicin induced cardiotoxicity: a preliminary study. Int J Med Health Sci 12(7):2018

28. Larasati YA, Meiyanto E (2018) Revealing the potency of Cinnamomum zeylanicum as an anti-cancer and chemopreventive agent. Indonesian J Cancer Chemoprev 9(1):47–62

29. Handschuh L, Łuczak M, Milewski MC, Góralski M, Łuczak M, Wojtaszewska M, Uszczyńska-Ratajczak B, Lewandowski K, Komarnicki M, Figlerowicz M (2018) Gene expression profiling of acute myeloid leukemia samples from adult patients with AML-M1 and M2 through boutique microarrays, real-time PCR and droplet digital PCR. Int J Oncol 52(3):656–678

30. Kazemi F, Najafabadi TA, Araabi BN (2016) Automatic recognition of acute myelogenous leukaemia in blood microscopic images using k-means clustering and support vector machine. J Med Signals Sens 6(3):183

31. Shahab F, Raziq F (2014) Clinical presentations of acute leukemia. J Coll Physicians Surg Pak 24(7):472–476

32. Dorri M, Hashemitabar S, Hosseinzadeh H (2018) Cinnamon (Cinnamomum zeylanicum) as an antidote or a protective agent against natural or chemical toxicities: a review. Drug Chem Toxicol 41(3):338–351

33. Khaifaji SS (2018) Study the effect of ceylon cinnamon (Cinnamomum zeylanicum) powder on some physiological parameters in broiler chicks. J Glob Pharma Technol 10(7):236–242

34. Morgan AM, El-Ballal SS, El-Bialy BE, El-Borai NB (2014) Studies on the potential protective effect of cinnamon against bisphenol A-and octylphenol-induced oxidative stress in male albino rats. Toxicol Rep 1:92–101

35. Ghonim A, Abdeen A, El-Shawarby R, Abdel-Aleem N, El-Shewy E, Abdo M, Abdelhiee E (2017) Protective effect of cinnamon against cadmium-induced hepatorenal oxidative damage in rats. Int J Pharmacol Toxicol 5(1):17–22

36. Shen Y, Shen H, Shi C, Ong C (1996) Benzene metabolites enhance reactive oxygen species generation in HL60 human leukemia cells. Hum Exp Toxicol 15(5):422–427

37. Dere E, Ari F (2009) Effect of benzone on liver functions in rats (Rattus norvegicus). Environ Monit Assess 154(1–4):23–27

38. Sandamali JAN, Hewawasam RP, Jayatilaka KA, Mudduwa LKB (2021) Cinnamomum zeylanicum Blume (Cinnamon cinnamon) bark extract attenuates doxorubicin induced cardiotoxicity in Wistar rats. Saudi Pharma J 29(8):820–832

39. Guo C, Liu S, Sun MZ (2013) Novel insight into the role of GAPDH playing in tumor. Clin Transl Oncol 15(3):167–172

40. Jones W, Bianchi K (2015) Aerobic glycolysis: beyond proliferation. Front Immunol 6:227

41. Boley SE, Anderson EE, French JE, Nonehower LA, Walker DB, Recio L (2000) Loss of p53 in benzene-induced thymic lymphomas in p53+/− mice: evidence of chromosomal recombination. Cancer Res 60(11):2831–2835

42. Greenblatt M (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 54:4855–4878

43. Tsang W, Chau SP, Kong S, Fung K, Kwok T (2003) Reactive oxygen species mediate doxorubicin induced p53-independent apoptosis. Life Sci 73(16):2047–2058

44. Yang F, Chen H, Liu Y, Yin K, Wang Y, Li X, Wang G, Wang S, Tan X, Xu C (2013) Doxorubicin caused apoptosis of mesenchymal stem cells via p38, JNK and p53 pathway. Cell Physiol Biochem 32(4):1072–1082

45. Schoene NW, Kelly MA, Polansky MM, Anderson RA (2005) Water-soluble polymeric polyphenols from cinnamon inhibit proliferation and alter cell cycle distribution patterns of hematologic tumor cell lines. Cancer Lett 230(1):134–140

46. Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, Kalyanaraman B (2004) Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms intermediacy of H2O2- and p53-dependent pathways. J Biol Chem 279(24):25535–25543

47. Kelly MM, Hoel BD, Voelkel-Johnson C (2002) Doxorubicin pretreatment sensitizes prostate cancer cell lines to TRAIL-induced apoptosis which correlates with the loss of c-FLIP expression. Cancer Biol Ther 1(5):520–527

48. Kang J, Bu J, Hao Y, Chen F (2005) Subtoxic concentration of doxorubicin enhances TRAIL-induced apoptosis in human prostate cancer cell line LNCaP. Prostate Cancer Prostatic Dis 8(3):274–279

49. Anees M, Horak P, El-Gazzar A, Susami M, Heinze G, Perco P, Loda M, Lis R, Kainer M, Oh WK (2011) Recurrence-free survival in prostate cancer is related to increased stromal TRAIL expression. Cancer 117(6):1172–1182

50. Huang TC, Fu HY, Ho CT, Tan D, Huang YT, Pan MH (2007) Induction of apoptosis by cinnamaldehyde from indigenous cinnamon Cinnamomum osmophloeum Kaneh through reactive oxygen species production, glutathione depletion, and caspase activation in human leukemia K562 cells. Food Chem 103(2):434–443

51. Wu SJ, Ng LT, Lin CC (2005) Cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins and MAPK pathway. Life Sci 77(8):938–951

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.