

## Research Article

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# *Eulophia Gracilis* Orchid Profers Mitigative Influence on Benzene-Initiated Onco-Hematologic Toxicities in Male Wistar Rats

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### Abstract

The steady use of benzene and benzene containing products in the industry and its relative environmental exposure necessitated the investigation and search for antidote that can abate its hematological toxicity preceding leukemogenesis. *Eulophia gracilis* (EG) is a medicinal orchids used traditionally as aphrodisiac and for the treatment of blood diseases. The effectiveness of aqueous-methanolic extract of *Eulophia gracilis* (AMEG) on benzene-initiated onco-hematological toxicity was investigated. Leukemogenesis was initiated in twenty-four rats ( $100 \pm 5$  g) out of total 36 rats by intravenous administration of 175 mg/kg benzene in 1/1 water:propan-2-ol solution 48-hourly for five weeks and were confirmed by anemia indices and blast appearance in peripheral blood. The leukemogenic-initiated rats (Leuk) and control baseline were grouped (n=6) as follows: A baseline receiving distilled-water (control), B: baseline receiving 200 mg/kg AMEG, C: Leuk received distilled-water (Leuk), D: Leuk + AMEG(200 mg/kg), E: Leuk + AMEG(200 mg/kg) + cyclophosphamide(2 mg/kg) and F: Leuk + cyclophosphamide (2 mg/kg). Plasma protein carbonyl, Advanced Oxidized Protein Products-(AOPPs) and total thiol in plasma were evaluated spectrophotometrically. The expression of gene products of p53, p38, inducible nitric oxide-(iNOS), interleukin-6 and CD79 in rat bone marrow were determined by immunohistochemistry. We observed that EG protected against BZ-induced hematological disturbances and blood morphology in leuk. AMEG significantly ( $p < 0.05$ ) reduced BZ-induced increase in plasma protein carbonyl and advanced oxidation protein products but increased total thiol relative to leuk group. AMEG down-regulated expression of increased pro-inflammatory mediators: p38, inducible nitric oxide-(iNOS), interleukin-6 and CD79 and abated reduced tumor suppressor protein p53 in the bone marrow of leuk rats. This work corroborated that oxido-inflammatory mediators are involved in benzene-mediated hematotoxicity and can be effectively intercepted by *Eulophia gracilis* through its antioxidant potential.

**Keywords:** Leukemogenesis; Benzene; Myeloprotective; Oxidative stress; *Eulophia gracilis*.

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## Introduction

The link between onco-hematologic diseases and oxidative stress has been reviewed where continued oxidative stress activated genes involved in growth factors, inflammatory cytokines, cell cycle regulatory molecules and anti-inflammatory molecules therefore leading to cancer [1]. Oxidative stress is a prominent feature of both acute and chronic diseases and cancer including leukemias [2]. Benzene is a widely utilized solvent but constitutes an occupational hazard as it caused adverse health effects such as acute myelogenous leukemia [3]. However, the mechanism employed in benzene's toxicity that led to acute myeloid leukemia and myelodysplastic syndromes remains largely unclarified [4].

Hematological toxicity by benzene may be linked to its active metabolite such as benzoquinone that induces DNA strand breaks [5]. The bioactivation of benzene resulted in increase in oxidative stress that plays key roles in its cellular toxicity. Benzene is metabolized into hydroquinone and catechol in liver which are further converted by myeloperoxidase to 1,4-benzoquinone and 1,2-benzoquinone, respectively in the bone marrow [6]. Many of metabolites of Benzene have been associated with increase myeloid cell growth in vitro by the formation of reactive oxygen species ROS [7].

The interest on medicinal herb is due to its secondary metabolites that possess medicinal properties and its essentiality in pharmaceutical development [8]. *Eulophia gracilis* is one of several species of orchids with medicinal utility for treatment of diabetes, cancer, blood diseases, sexual impotency and male sterility by traditional healers in Nigeria especially among indigenous people in Oyo town and Jigawa in Nigeria. The plant has been shown to be rich in phytochemicals like glycoside, alkaloids, tannins, phlobatanins and flavonoids which are of medicinal values [9]. Many of these phytochemicals have been extensively studied to possess antioxidant capacity that are of pharmacological importance in decreasing the harmful effects of "reactive species", such as reactive oxygen and nitrogen molecules on a cellular level in humans. It is therefore on this note, that the present study investigated the anti-leukemogenic and myeloprotective effect of *Eulophia gracilis* on Benzene-induced hematological perturbation.

## Materials and methods

### Chemicals and Reagents

Reduced glutathione, 5',5'-dithio-bis-2-nitrobenzoic acid, thio-barbituric acid, epinephrine, Potassium iodide, 1-chloro-2, 4-dinitrobenzene and hydrogen peroxide were purchased from Sigma® Chemical Company (London, UK).

## Plant collection, identification and preparation

Fresh *Eulophia gracilis* plants were collected from rocky area in Oyo, Nigeria. The plant was identified and authenticated by plant taxonomist at Herbarium section of Department of Botany, University of Ibadan, Nigeria with University of Ibadan Herbarium (UIH) number 22528. The tubers were thoroughly washed with tap water, sliced into pieces and shed dried. The dried tubers were pulverized into powder form using electric grinder. 200g of *Eulophia gracilis* fine powdered sample was extracted with 800 mL aqueous methanol (20:80) for 48 hours by cold maceration. The extract was evaporated under reduced pressure by using a rotary evaporator and further lyophilized using freeze-dryer machine into powdery marc tagged AMEG (aqueous methanolic extract of *Eulophia gracilis*).

### Experimental animals

Male wistar rats of weight range 100 g - 120 g were used for this study. The animals were got from McTemmy farm and acclimatized for two weeks in the animal house of the Department of Chemical Sciences, Ajayi Crowther University, Oyo. They were kept in a well ventilated research cages and fed with standard commercial rat feed (Ladokun feeds Nigeria Ltd.) and clean tap water supplied *ad libitum*. Anesthesia was not involved in this research and the protocol conformed to the guidelines of the National Institute of Health for laboratory animal care and use [10].

### Treatments and animal grouping

After adaptation period, the animals were randomly assigned into seven main experimental groups of 6 animals each. The rats were intravenously administered with 175 mg/kgbw of benzene (in water; 2-propanol, 1:1) every other day for 5 consecutive weeks and this successively initiated leukemogenesis in the rats. The doses of the extract was carefully chosen from pilot study and that of cyclophosphamide were chosen from literature which were administered once daily and orally as shown in the table 1.

### Collection of blood and liver samples

24 hours after the final treatment, the blood samples were collected from each animal through retro orbitals plexus into lithium heparinized tubes and thereafter sacrificed by cervical dislocation. The bone marrow from femurs was collected for histopathological examination.

### Measurement of haematological parameters

Haemoglobin concentration, % packed cell volume (PCV), red blood cell (RBC) count, haemoglobin concentration, white blood

**Table 1:** Experimental design.

Treatment groups	Treatment
A. Control	Distilled water
B. Leuk untreated (leukemia-initiated rats without extract treatment)	175 mg/kgbw benzene mixture every two days for 5 weeks
C. Leuk + AMEG Post-treated	Leukemia induction then followed by 2 weeks of 200 mg/kg AMEG treatment
D. Leuk + AMEG + CYP Post-treated	Leukemia induction then followed by 2 weeks of 200 mg/kg AMEG and 2 mg/kg cyclophosphamide treatment
E. Leuk + CYP Post-treated	Leukemia induction then followed by 2 weeks of 2 mg/kg cyclophosphamide treatment

cell (WBC) count, % Neutrophils, % Lymphocytes and platelets count were determined using the automated blood analyser, SYS-MEX KX21.

#### Assay for oxidative stress maker in the plasma and bone marrow gene expression

AOPP in plasma was estimated by the method described by Witko et al. [11] as modified by Zhang et al. [12]. Plasma total thiol were measured spectrophotometrically using DTNB (2, 2'-dinitro-5, 5'-dithiodibenzoic acid) [13]. Protein carbonyl content in liver was determined according to the procedure of Reznick and Packer [14] where 2,4-dinitrophenylhydrazine (DNPH) reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone. The amount of protein-hydrazone produced is quantified spectrophotometrically at 370 nm. The expression of gene products of p53, p38, inducible nitric oxide synthase, IL-6 and CD79 in rat bone marrow were determined by immunohistochemistry.

## Result

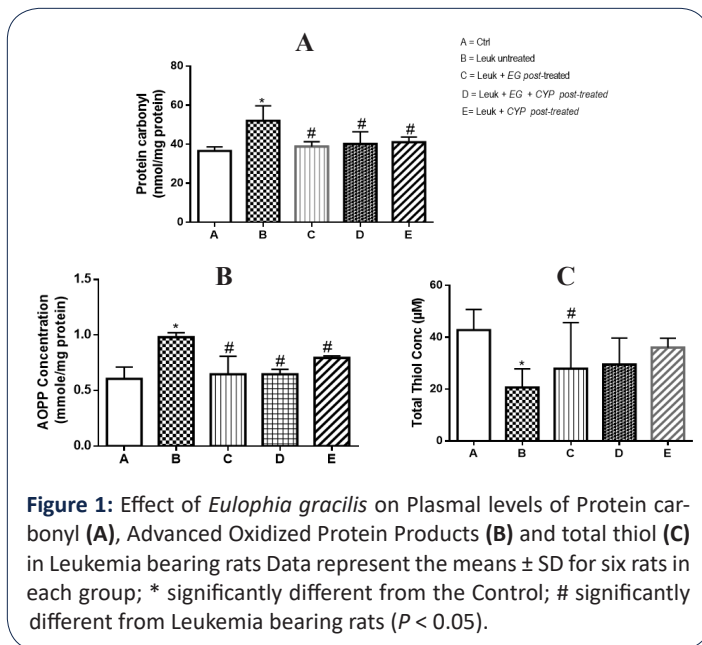
### Hematological parameters of control, post leukemia induction, and after treatment with extract of *Eulophia gracilis* (mean $\pm$ SD)

The protective effect of *Eulophia gracilis* on hematological perturbation by benzene was presented in table 2. PCV and platelets were drastically reduced in leukemic rats when compared with control group. However, white blood cells were greatly increased in rats treated with benzene when compared with control group. Moreover, anisocytosis, Poikilocytosis and formation of up to 4% blasts were observed in blood film of leukemic rats when compared with control group. However, post-treatment of leukemia-induced rats with aqueous methanolic extract of *Eulophia gracilis* protected against influence of benzene on these hematological parameters and caused a disappearance of blast in the blood film.

**Table 2:** Hematological parameters of control, post leukemia induction, and after treatment with extract of *Eulophia gracilis* (mean  $\pm$  SD).

GROUP	PCV	HGB	RBC	WBC $\times 10^3/\mu\text{L}$	platelet	Anisocytosis	Poikilocytosis	Percentage blast
Ctrl	50.5 $\pm$ 6.66	11.74 $\pm$ 0.83	6.65 $\pm$ 1.20	4500.00 $\pm$ 711.81	102666.7 $\pm$ 8326.664	Negative	Negative	0
Leuk untreated	40.75 $\pm$ 3.40	6.85 $\pm$ 0.57	4.09 $\pm$ 0.65	7250 $\pm$ 1138.71	248666.67 $\pm$ 55967.25	Positive	Positive	4
Leuk + EG Post-treated	42.33 $\pm$ 3.21	9.65 $\pm$ 0.28	6.25 $\pm$ 0.64	4933.33 $\pm$ 945.16	184666.7 $\pm$ 40451.62	Negative	Negative	0
Leuk + EG + CYP Post-treated	45.25 $\pm$ 4.11	10.60 $\pm$ 1.30	5.94 $\pm$ 0.66	6075 $\pm$ 411.3	159666.7 $\pm$ 16653.33	Negative	Negative	0
Leuk + CYP Post-treated	46.00 $\pm$ 2.16	11.60 $\pm$ 1.30	6.04 $\pm$ 0.66	5666.67 $\pm$ 1342.88	137333.3 $\pm$ 12096.83	Negative	Negative	0

**Abbreviations:** PCV: Packed Cell Volume; LYM: Lymphocytes; WBC: White Blood Cell

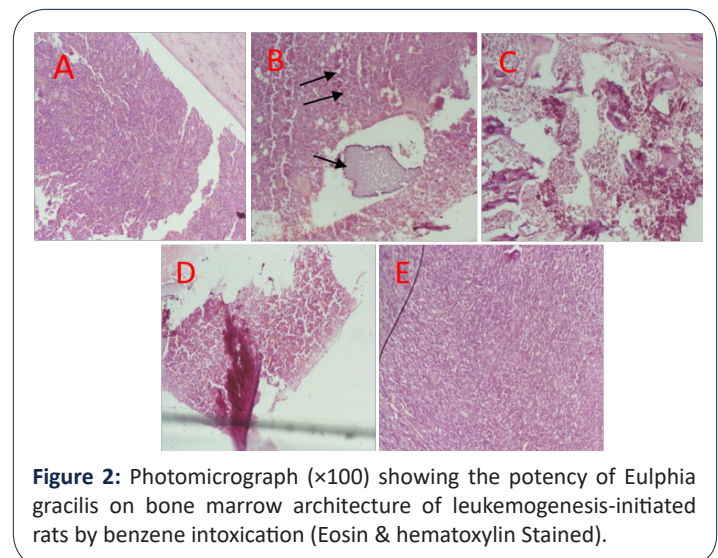


**Figure 1:** Effect of *Eulophia gracilis* on Plasmal levels of Protein carbonyl (A), Advanced Oxidized Protein Products (B) and total thiol (C) in Leukemia bearing rats Data represent the means  $\pm$  SD for six rats in each group; \* significantly different from the Control; # significantly different from Leukemia bearing rats ( $P < 0.05$ ).

#### Effect of *Eulophia gracilis* extract on Benzene – induced oxidative stress in the plasma of leukemia bearing rats

The protective effect of aqueous methanolic extract of *Eulophia gracilis* on Benzene – induced Oxidative Stress in the plasma of Leukemia bearing rats is shown in figure 1. The intravenous administration of Benzene to rats resulted into a significant elevation in the concentration of advanced oxidation protein products (AOPPs) and protein carbonyl content present in the plasma of

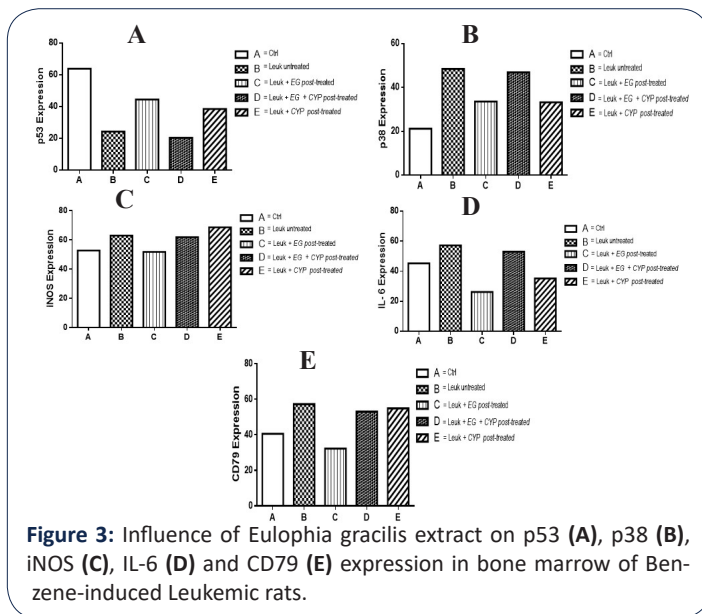
rats by 62.13% when compared with the control group (Figure 1A and B). There was also a concomitant significant reduction in total thiol content by 51.84% when compared to the control group (Figure 1C). However, post-treatment with aqueous methanolic extract of *Eulophia gracilis* significantly ( $p < 0.05$ ) attenuated the effect of Benzene toxicity by significantly reducing the generation of plasma AOPP and protein carbonyl and restore sulfhydryl level when compared to animal group exclusively treated with Benzene.



## Influence of *Eulophia gracilis* extract on p53, p38, iNOS, IL-6 and CD79 expression in bone marrow cell of benzene-induced leukemic rats

The results of Influence of *Eulophia gracilis* Extract on p53, p38, iNOS, IL-6 and CD79 expression in bone marrow cell of Benzene-induced Leukemic rats were presented in figure 3.

The expression of tumor suppressor protein p53 was low in leukemic rats relative to control animal group (figure 3A). However, treatment of rats with plant extract before and after leukemia induction upregulated the expression of p53 gene relative to untreated leukemic rats. There was an increase in expression of stress kinase p38, inducible nitric oxide (iNOS), proinflammatory cytokine IL-6 and CD79 gene proteins in leukemogenesis-initiated rats in figure 3B, C, D and E respectively when compared with control rats. However, rats co-treated and post-treated with AMEG showed lower expression of the stress kinase p38, iNOS, IL-6 and CD79 proteins relative to the leukemic rats. Also, there was upregulation of the p38 protein in animals post-treated with both extract and cyclophosphamide which might probably due to overload of the drug and the plant extract resulting in further stress to the animals.



**Figure 3:** Influence of *Eulophia gracilis* extract on p53 (A), p38 (B), iNOS (C), IL-6 (D) and CD79 (E) expression in bone marrow of Benzene-induced Leukemic rats.

## Discussion

The present work evaluated the potential of aqueous methanolic extract of *Eulophia gracilis* pseudobulbs against leukemogenic process initiated in Wistar. In this study, the significant increase in WBCs in leuk group relative to control group corroborated the leukocytosis that was earlier reported in leukemic rats and humans [15]. This was ameliorated in rats treated with aqueous methanolic extract of *Eulophia gracilis* before and after exposure to the leukemic-inducing agent. An anisocytosis has been listed as a morphological feature of the occurrence of acute panmyelosis with myelofibrosis. The result showed a presence of anisocytosis and poikilocytosis in leuk group due to exposure to benzene mixture. This result is in consonance with earlier finding of other works [16]. Moreover, the presence of blast and hypercellularity observed in the peripheral blood and bone marrow of rats in leuk group are notable laboratory picture of leukemia. However, these hematological alternances were buffered when the rats were

treated with AMEG extract.

Assessment of products of damaged protein and lipid contents can be used as indirect quantification of reactive oxygen species [17]. The sulfhydryl groups present on protein make up the major plasma antioxidants *in vivo* and are mostly present on albumin making it to be the reducing groups constituent in body fluids [18]. Proteins has the capacity through thiol groups present on them to scavenge 50% – 75% of reactive species generated [19]. Oxidation of critical sulfhydryl-containing proteins is also associated with depleted reduced glutathione which is a cellular nonprotein thiol defenses system [20]. The reduction in plasma levels of protein thiol correlated positively with the increased levels of lipid peroxides and advanced oxidation protein products (AOPPs) [21]. In this present work, the exposure of rats to benzene resulted in declined plasma total thiol with concomitant increase in AOPPs relative to control. The AOPPs are produced in plasma when the plasma albumin is subjected to oxidation by various oxidants [22] and its elevated levels has been associated with some pathological conditions such as atherosclerosis, diabetes, nephropathies and cancer [23]. However, administration of extract of *Eulophia gracilis* in this study significantly reduced the elevated generation of AOPP in leukemogenesis-initiated rats group and effectively restored the total thiol status when compared to untreated group. This shows that the extract may improve plasma antioxidant activity by preserving sulfhydryl protein pools.

Elevated level of protein carbonyl in leukemia bearing rats in this present work relative to control supports the hypothesis that high ROS generation occurs in neoplastic or cancer cells. Protein carbonyl is a product of irreversible non-enzymatic oxidation of protein and serves as biomarker of disease progression in oxidative stress-mediated pathophysiology [24]. Carbonylation of protein often leads to a loss of protein function and therefore marker of severe oxidatively damage and disease-derived protein dysfunction [25]. However, administration of *Eulophia gracilis* pseudobulb extract significantly reduced ( $p < 0.05$ ) the levels of protein carbonyl when compared to the untreated leukemia-bearing rats. This therefore suggested the antioxidant potential of this plant extract.

The tumor suppressor p53 plays an important role in regulating hematopoietic stem cells (HSC) quiescence and self-renewal during steady-state hematopoiesis [26] and its loss has been associated to decreased apoptosis and an increase in disease progression of leukemic cells and hematologic tumors [27]. In the present study after intravenous exposure of rats to benzene for 4 weeks consecutively, our results showed that the expression of p53 protein in benzene exposed animal group marrow cells declined markedly relative to control animal group. It has been demonstrated that decreased levels of p53 in bone marrow may lead to reduction in apoptosis and increase in bone marrow cellularity [28]. This may be implicated in hypercellularity observed in bone marrow histomicrogram observed in this study. However, the post-treatment of animal group exposed to benzene with methanolic extract of *Eulophia gracilis* offer protection by upregulating p53 protein expression in these animal bone marrow cells relative to untreated animal group and therefore lead to moderate cellularity observed in the bone marrow cells of animal group treated with the plant extract.



Benzene metabolites were shown to stimulate the production of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 by activated human peripheral blood mononuclear cells (PBMC) [29]. DNA damage induced by Benzene metabolites and Ionizing radiation have been shown to initiate the expression of various circulatory cytokines such as IL-6 and this response may be related to apoptosis. IL-6 is a pluripotent cytokine found to be involved in acute pro-inflammatory process associated with overexposure to ionizing radiation and leukemogenic benzene intermediates [30]. Our result showed a high expression of IL-6 in the bone marrow of rats exposed to benzene. This increase in IL-6 expression is in agreement with findings of other researchers on animal exposed to radiation which is also a leukemogenic factor and human peripheral blood mononuclear cells activated by benzene metabolites [29]. However, post-treatment of exposed animals with extract of *Eulophia gracilis* pseudobulb resulted into low positive expression of this cytokine. This result suggested that the protection of these rats from benzene-induced hematopoietic damage may also be mediated by cytokines and chemokines.

The contribution of aromatic hydrocarbons in mediating inflammatory signaling through stress kinase p38 MAP Kinase that influences production of various cytokines has been reported [31]. Protein phosphorylation and dephosphorylation mediated by protein kinase and phosphatase respectively is central to regulation of many cellular processes in biological system. Previous findings implicated benzene and its metabolites as activator of several key signaling pathways such as p38 and resultantly triggering apoptosis of cells of the marrow or malignant progression of human leukemia cells [32] and therefore suggesting the involvement of protein kinase in benzene-induced toxicity.

The present work showed the elevated expression of p38 protein in the rats exposed to benzene relative to control group. Benzene component of Tobacco smoke has been inferred to activate p38 in an oxidative stress dependent way [33]. Therefore, increased expression of p38 protein in marrow cells of animals exposed to benzene may be attributed to oxidative stress mediated by benzene or its metabolite. However, administration of *Eulophia gracilis* subsequent to leukemia induction down regulate the p38 protein expression. This show that the extract may offer hematoprotective via p38 down regulation in oxidative stress condition caused by environmental benzene pollutant.

Benzene has been shown to induces iNOS and generates nitric oxide and reactive oxygen species like hydroxyl radicals, superoxide anion (O<sub>2</sub> •-), hydrogen peroxide and singlet oxygen in the mice marrow cells [34]. The iNOS-deficient mice were reported to be partially protected from benzene induced bone marrow suppression and this supported a link between nitric oxide production and hemotoxicity [35]. The result of this work presented an elevated expression of iNOS in the bone marrow of animal exposed to benzene relative to control. However, administration of the extract to animal group that were induced with leukemia show relatively reduced expression of iNOS protein. More importantly, pre-treated or co-treated with extract show relatively more effect in lowering expression of iNOS while co-administration of extract and standard drug do not produce any noticeable effect. This means that the extract may offer preventive effect on iNOS induction by benzene intoxication.

CD79 is heterodimeric transmembrane protein that is associated with membrane immunoglobulin and expressed in B cell at early stages of its development until the last stage of maturation before differentiation to plasma cells [36]. It has also been found in biphenotypic leukemia cases of myeloid leukemia, where it was coexpressed with myeloid markers on bone marrow blast cells [37]. Previous work showed that CD79a plays an important functional role in maintaining the immature, immune suppressive phenotype of myeloid-derived suppressor cells and in inducing the secretion of protumorigenic cytokines. The result of this study revealed the higher positive expression of CD79 protein in bone marrow of animal group exposed to benzene relative to low positive expression in control group. This agree with the observation of other researchers that benzene is a risk factor for development of acute myeloid leukemia. However, in the animal group that were post-treated with extract of *Eulophia gracilis*, the expression of CD79 protein was reduced relative to the Leuk group. Therefore, it is suggested that the phytoactive principle in this extract may downregulate the expression of CD79 in myeloid-derived suppressor cells and prevent the induction of protumorigenic cytokine like interleukine-6 as observed in this study.

Overall, aqueous methanolic extract of *Eulophia gracilis* protected against the cellular and biomolecular oxidation in the blood system and modulated the expression of genes involved in biosignalling and regulation of myeloid proliferation. The effects of this plant may be due to antioxidant capacity of inherent active compounds.

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