

# Effects of *Salvia virgata* Jacq. on Jurkat Clone-E6

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## ABSTRACT

**Objectives:** The genus *Salvia* is one of the most widespread members of the Lamiaceae family and *S. virgata* Jacq. decoction is traditionally used for leukemia in Turkey. In this study, water extract and methanol extract of *S. virgata* were investigated for their antiproliferative effects on Jurkat (Clone E6-1) leukemia cell lines and also GSH-Px, SOD and MDA levels were evaluated as oxidative stress parameters. **Methods:** The cell proliferation was studied by using Alamar Blue assay and the cell viability determined using trypan blue assay. The phenolic composition of the extracts was also identified using HPLC and pro/antioxidative enzymes were measured to identify if their mechanism of action is related to these phenolic compounds. GSH-Px, SOD and MDA levels were measured using commercial kits. Rosmarinic acid was the main compound in all the extracts. **Results and Conclusion:** According to the viability results, rosmarinic acid reduced the cell viability around 45%. Methanol extract in both doses reduced the cell viability around 10% methanol extract and rosmarinic acid reduced the GSH-Px and SOD activity as parallel with the cell viability results. All these findings indicate that the water extract at a dose of 0.50 mg/mL and methanol extract, reducing GSH-Px increasing TBARS might be pro-oxidant on this cell line and probably shows its anti-cancerogen effect over rosmarinic acid. These results agreed with the traditional usage.

**Key words:** *Salvia virgata* Jacq, Jurkat cell line, Rosmarinic acid, SOD, GSH-Px, TBARS.

## INTRODUCTION

The genus *Salvia* with about 900 species, is one of the most widespread members of the *Lamiaceae* family.<sup>1,2</sup> *Salvia* have been used since ancient times in folk medicine and have been subjected to extensive investigations and it features prominently in the pharmacopoeias of many countries throughout the world<sup>3,4</sup> where the flora of Turkey includes 95 species of the genus *Salvia*.<sup>5,6</sup> Several of the almost 1000 *Salvia* species have been used in many aspects including their anti-cancerogen effects. For instance, *Salvia officinalis* L. Extract is shown to induce apoptosis in human leukemia and lenfoma cells.<sup>7</sup> Tanshinone IIA and Cryptotanshinone isolated from *Salvia* species are shown to be the most effective compounds in leukemia.<sup>8-10</sup> Furthermore, these compounds were also tested in several leukemia cell lines such as K562, KBM-5 cells and HL-60.<sup>11-14</sup> In addition, *Salvia multiorbiza* Bunge extracts and

Tanshinone IIA isolated from this *Salvia* species also have been shown to be effective on HeLa cells and P388 leukemia cells.<sup>15,16</sup>

In Turkey, *S. virgata* Jacq. is known as 'yılançık' and it's leaves used for externally wound healing. The decoction of *S. virgata* is also used for leukemia.<sup>5</sup> The objective of this work was to study *S. virgata*, hitherto not investigated for its antiproliferative activity. Therefore, water extract and methanol extract of *S. virgata* were investigated for antiproliferative effects on Jurkat (Clone E6-1) leukemia cell lines. The phenolic composition of the extracts was also identified by HPLC-PDA analysis. Furthermore, the malondialdehyde (MDA), glutathione peroxide (GSH-Px) and superoxide dismutase (SOD) levels were measured in these cell lines to identify if their mechanism of action is related to these phenolic compounds and their pro/antioxidative effects.

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## MATERIALS AND METHODS

### Cell lines, chemicals and biochemicals

Jurkat (Clone E6-1) leukemia cell lines were purchased from ATCC (TIB-152). Rosmarinic acid and gallic acid were purchased from Sigma Aldrich. ELISA kits for TBARS levels, GSH-Px and SOD activities were purchased from Cayman. The assays were performed according to manufacturer's instructions. Chromatographic standards were purchased from Sigma Chemical Company (St. Louis, MO, USA). Ultra-pure water was used throughout and was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., Billerica, MA, USA). All remaining reagents were of the highest purity available and obtained from the Sigma Chemical Company (St. Louis, MO, USA).

### Plant material and preparation of the extracts

Air-dried aerial plant material (*S. virgata* Jacq., Lamiaceae) was collected from University of Uludağ /Bursa on 22/06/2009. Air-dried *S. virgata* was cut into small pieces and sequentially extracted with sufficient amount of 70% methanol and water for 24 h at 40°C in a water bath with shaking and was filtered. This procedure was repeated three times using the same batch of starting material and the resultant filtrates were combined and the solvent was removed in vacuo (40°C). All extracts stored at -20°C until the analysis time. Prior to the analysis, an aliquot of each extract was dissolved and filtered through 0.45 µm membrane (Whatman, Maidstone, UK) and used in all subsequent experiments.

### Qualitative–quantitative chromatographic analysis

All extracts and standards were analyzed with using HPLC systems (Agilent Tech. 1200, Mississauga, ON, Canada). Separations were performed on a 250×4.6 mm i.d., 5 µm particle size, reverse-phase Discovery-C<sub>18</sub> analytical column (Supelco, Bellefonte, PA, USA) operating at room temperature (22°C) at a flow rate of 1 mL min<sup>-1</sup>. Detection was carried out between the wavelengths of 200 and 550 nm. Elution was carried out using a ternary non-linear gradient of the solvent mixture MeOH/H<sub>2</sub>O/CH<sub>3</sub>COOH (10:88:2, v/v/v) (solvent A), MeOH/H<sub>2</sub>O/CH<sub>3</sub>COOH (90:8:2, v/v/v) (solvent B) and MeOH (solvent C). The composition of B was increased from 15% to 30% in 15 min, increased to 40% in 3 min and held for 12 min, increased to 100% in 5 min then the composition of C was increased to 15% in 2 min, increased to 30% in 11 min and then returned to the initial conditions in 2 min. Components were identified by comparison of their retention times

to those of authentic standards under identical analysis conditions and UV spectra using our inhouse PDA-library. A 10 min equilibrium time was allowed between injections. All the standard and sample solutions were injected triplicate.<sup>17,18</sup>

### Cell viability

The cell proliferation was studied by using Alamar Blue assay and the cell viability determined using trypan blue assay. % Inhibition of the cell proliferation compared to control group was calculated.

### TBARS, GSH-Px and SOD activities

TBARS levels, GSH-Px (Cayman) and SOD activities (Cayman) were measured using commercial kits according to the kit procedure.

### Statistical analysis

All statistical analyses were performed using the SPSS software, version 12.0. Data are presented as mean values ±95% confidence interval. Analysis of variance was performed using ANOVA procedures. Significant differences between means were determined by Tukey's pairwise comparison test at a level of p<0.05.

## RESULTS

### Qualitative–quantitative chromatographic analysis

The qualitative–quantitative analyses of the extracts, carried out using an HPLC apparatus coupled to a PDA detector, are presented in Table 1. Phenolic compounds were identified and quantified at 280, 320, and 360 nm. Gallic, *p*-OH-benzoic, caffeic, *o*-coumaric, rosmarinic acids, luteolin-7-*O*-glycoside, and luteolin were identified by comparison with the retention times and UV spectra of authentic standards, while quantitative data were calculated from their calibration curves. For *S. virgata*, the aqueous methanol extract (SME) was found to be the richest in phenolics as measured by HPLC. Rosmarinic acid was the main compound in all the extracts. The rosmarinic acid concentration of methanolic extract was found 66.94 ± 0.47 mg/g and in water extract (SSE) it was found 26.81 ± 0.05 mg/g. *o*-Coumaric acid and caffeic acid was found in the *S. virgata* extracts as the second hydroxycinnamic acids. Also it was found that the extracts content flavonoid glycosides more than aglycones. The luteolin concentrations of the extracts were 0.97 ± 0.03 mg/g for methanolic extract and 0.22 ± 0.00 mg/g for water extract (Table 1). HPLC chromatograms were given in Figure 1.

**Table 1: HPLC Results of *S. virgata* extracts**

Compounds	Extracts*	
	SSE	SME
<i>p</i> -OH-benzoic acid	0.83 ± 0.013 <sup>a</sup>	0.69 ± 0.040
caffeic acid	0.77 ± 0.001	0.97 ± 0.004
<i>o</i> -coumaric acid	0.90 ± 0.002	3.25 ± 0.240
rosmarinic acid	26.81 ± 0.047	66.94 ± 0.471
luteolin	0.22 ± 0.003	0.97 ± 0.031

\* (SME), 70% methanol extracts; (SSE), water extracts; &mg/g, mean ± SD

### Cell viability

The effects of gallic acid, rosmarinic acid in addition to methanol and water extracts of *Salvia virgata* on cell viability have been investigated. The effects of the extracts on cell viability varied in a dose dependent manner according to the extract used. According to the viability results, ascorbic acid and gallic acid caused 25-45% inhibition in cell viability in both doses used and rosmarinic acid reduced the cell viability around 45%. Methanol extract in both doses reduced the cell viability around 10% and 0.50 mg/mL dose of water extract affected the cell viability approximately 20% (Figure 2). Lower doses of rosmarinic acid and water extract increased the cell viability revealing that they show protective effects in these doses. However, the higher doses of water extract and rosmarinic acid reduced the cell viability significantly.

### GSH-Px activity

GSH-Px activity reduced to the non detectable values in methanol extract in both doses and increased in water extract at a dose of 0.25 mg/mL and decreased at a dose of 0.50 mg/mL. Rosmarinic acid and gallic acid also reduced the GSH-Px activity ( $p < 0.05$ ). These results reveal that the lower is the GSH-Px activity, the lower is the cell viability.

The GSH-Px activity reduced by rosmarinic acid (Figure 3). Methanol extract of *Salvia virgata* which has more rosmarinic acid than the water extract reduced the GSH-Px to non detectable values. These results were parallel with the cell viability results revealing that the lower is the GSH-Px activity, the lower is the cell viability. Furthermore, water extract as in the cell viability results, in low doses did not affect the GSH-Px activity whereas it reduced the activity in high dose where rosmarinic acid can be found.

### SOD Activity

Both rosmarinic acid and gallic acid reduced the SOD activity significantly ( $p < 0.05$ ) whereas the water and methanol extracts of *Salvia virgata* did not affect the SOD activity significantly as compared to control (Figure 4).

### TBARS levels

Methanol extract for both doses decreased the TBARS levels especially with 0.25 mg/mL significantly ( $p < 0.05$ ). However, the water extract decreased the TBARS levels with low doses but increased these levels significantly ( $p < 0.05$ ) with high doses. The water extract reduced the TBARS but increased the TBARS at high dose (Figure 5).

### DISCUSSION

*Salvia* species are widely investigated for its antioxidant effects and especially for wound-healing properties.<sup>19,20</sup> In this study, antiproliferative effect of *Salvia virgata* which is especially found in Turkey has been investigated and the mechanism of action was tried to be identified over oxidant and antioxidant status of the Jurkat leukemia cells. This leukemia cell lines were chosen for the antiproliferative effects of *Salvia virgata* since some other *Salvia* species have anti-cancerogen effects in the pathology of leukemia. For instance, *Salvia officinalis* and *Salvia miltiorrhiza* Bunge. have been investigated for their anti-cancerogen effects in several leukemia cell lines and the main component of these species namely Tanshinone IIA was shown to be responsible from this effect.

In this study, depending on our hypothesis, the phenolic components which are shown to be responsible from the antioxidant effects of these species were investigated whether they were pro-or anti-oxidant in Jurkat cell line. The main phenolic components of the *Salvia virgata* have been identified using HPLC. Gallic, *p*-OH-benzoic, caffeic, *o*-coumaric, rosmarinic acids, luteolin-7-*O*-glycoside, and luteolin were identified by comparison with the retention times and UV spectra of authentic standards and rosmarinic acid was found to be the main phenolic compound in each methanol and water extracts. According to the HPLC results rosmarinic acid was much more than the water extract which might explain why the methanol extract reduced the cell viability in both doses where as the water extract could only reduce the viability at higher doses where more rosmarinic acid can be found.

The previous studies on the anti-cancerogen effects of *Salvia* species on different leukemia cell lines such as K562, KBM-5 cells, HeLa, P388 and HL-60 proposed that Tanshinone IIA was responsible from the anti-cancerogen effect of some other *Salvia* species such as *Salvia officinalis* and *Salvia miltiorrhiza* Bunge.<sup>7-16</sup> Our study is the known first study to show the antiproliferative effect of a *Salvia virgata* on Jurkat cell line. The findings can show that rosmarinic acid might be responsible from the antiproliferative effect of methanol extract of

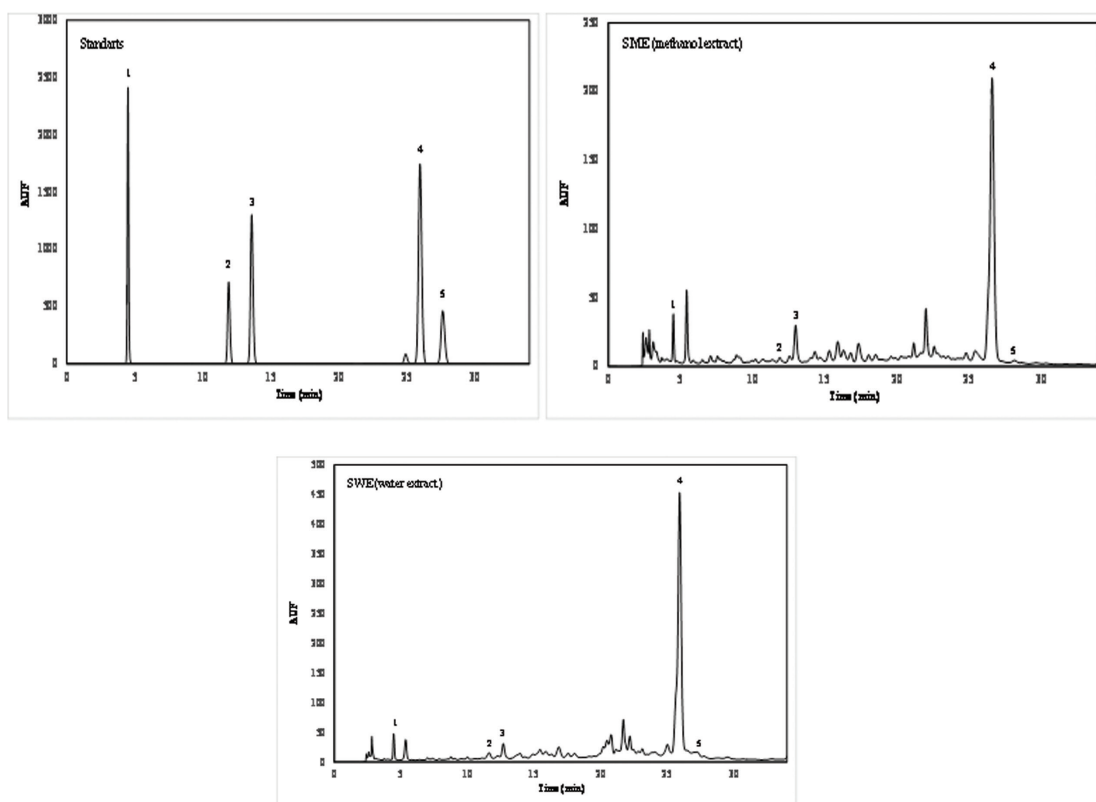


Figure 1: HPLC chromatogram of *Salvia virgata* extracts and standards. A; Standards, B; Methanol extract, C; water extract, 1; *p*-OH-benzoic acid, 2; caffeic acid, 3; *o*-coumaric acid, 4; rosmarinic acid, 5; luteolin

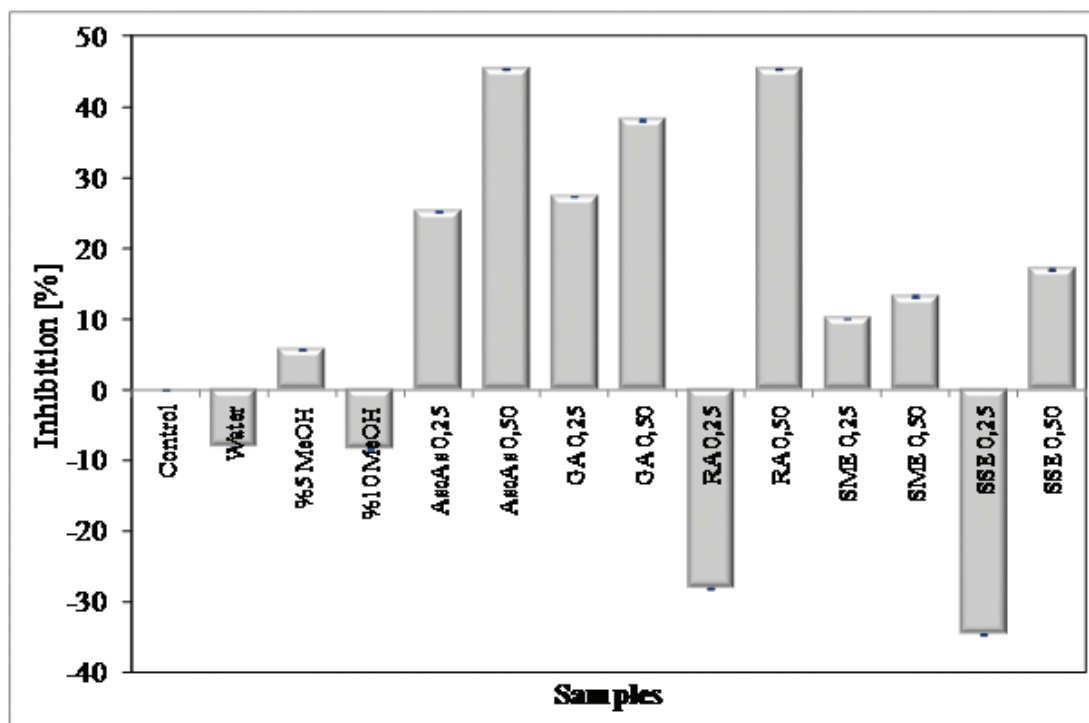


Figure 2: The cytotoxic effects of *Salvia virgata* extracts and some basic phenolic compounds on Jurkat Clone E6. MeOH, Methanol; AscAs, ascorbic acid; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanilin; GA, gallic acid; RA, rosmarinic acid; SME 70% methanol extract; SSE water extract

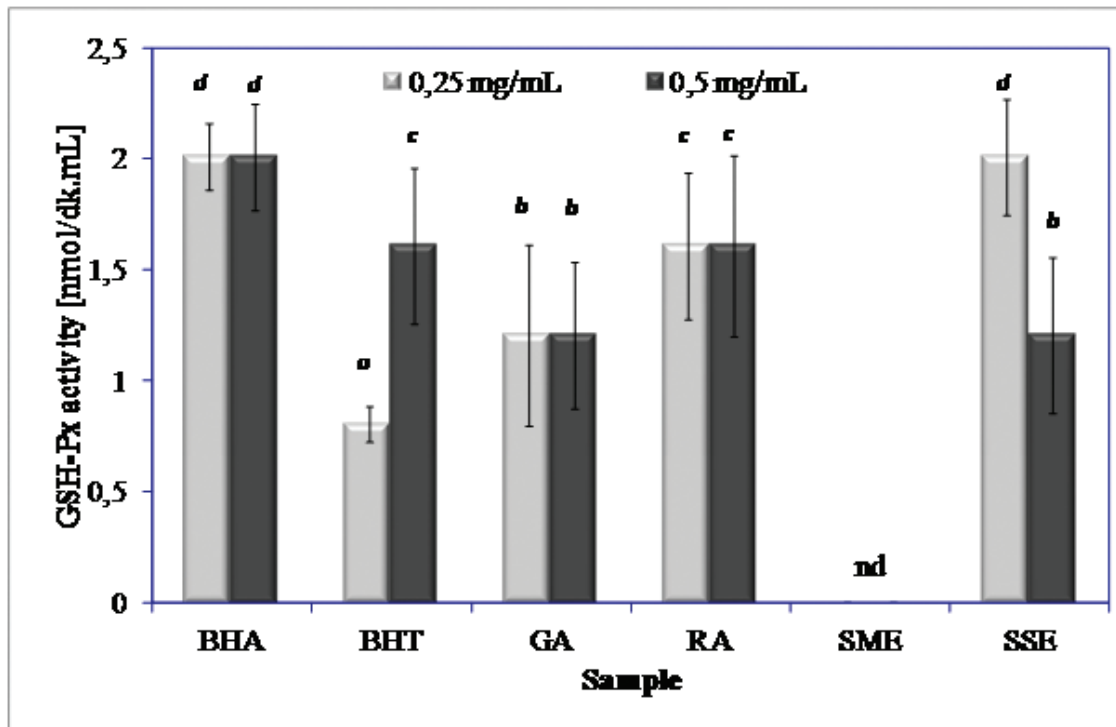


Figure 3: The GSH-Px activities in Jurkat Clone E6 with *Salvia virgata* extracts and some basic phenolic compounds. BHT, butylated hydroxytoluene; BHA, butylated hydroxyanilin; GA, gallic acid; RA, rosmarinic acid; SME 70% methanol extract; SSE water extract. Bars with the same letter (a-c), are not significantly ( $p < 0,05$ ) different

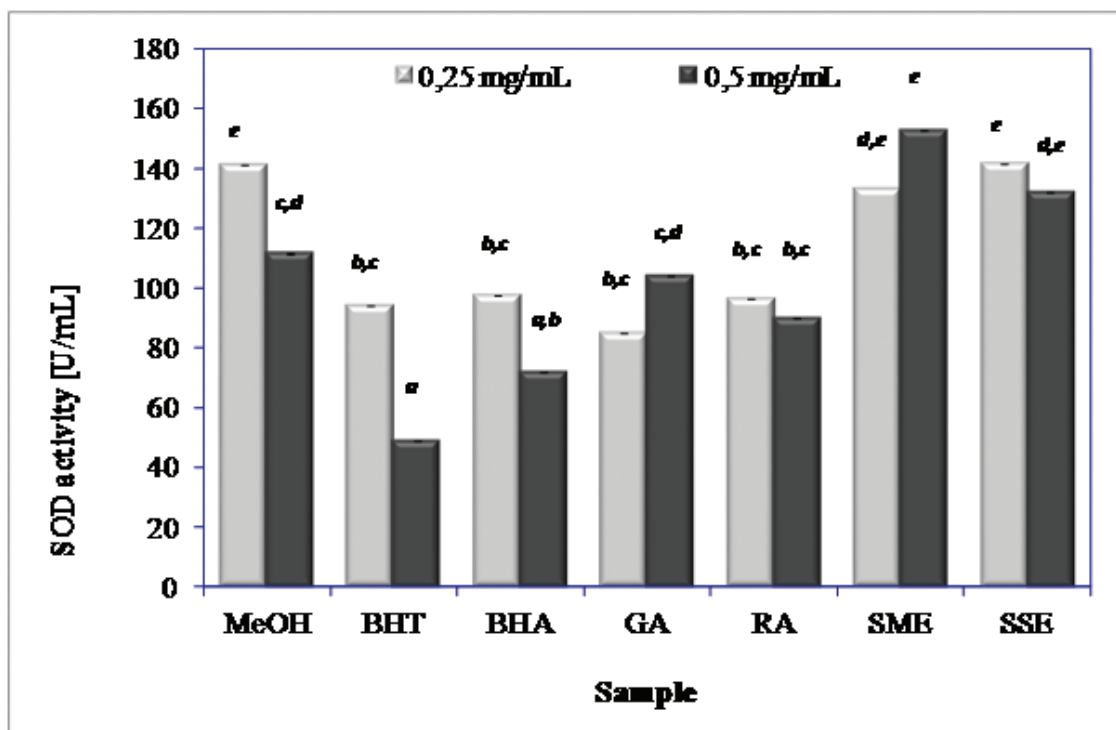
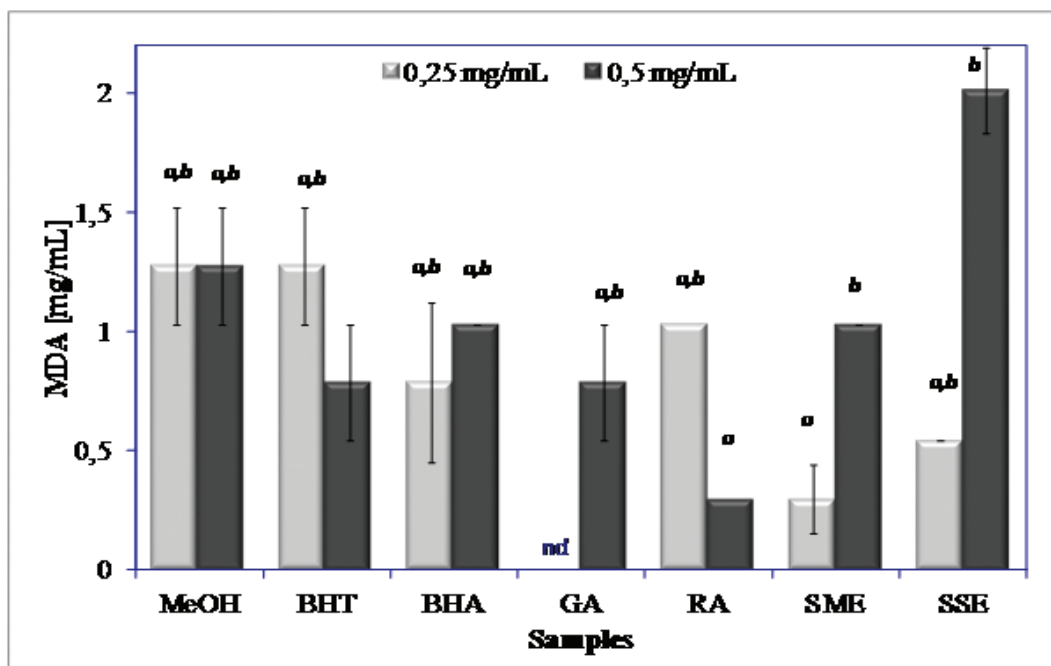


Figure 4: The SOD activities in Jurkat Clone E6 with *Salvia virgata* extracts and some basic phenolic compounds. BHT, butylated hydroxytoluene; BHA, butylated hydroxyanilin; GA, gallic acid; RA, rosmarinic acid; SME 70% methanol extract; SSE water extract. Bars with the same letter (a-e), are not significantly ( $p < 0,05$ ) different



**Figure 5: The TBARS activities in Jurkat Clone E6 with *Salvia virgata* extracts and some basic phenolic compounds. BHT, butylated hydroxytoluene; BHA, butylated hydroxyanilin; GA, gallic acid; RA, rosmarinic acid; SME 70% methanol extract; SSE water extract. Bars with the same letter (a-b), are not significantly ( $p < 0,05$ ) different**

*Salvia virgata*. The water extract reduced the TBARS but increased the TBARS at high dose. All these findings indicate that the water extract at a dose of 0.50 mg/mL, reducing GSH-Px and increasing TBARS might be pro-oxidant on this cell line at this dose and probably shows its antiproliferative effect over rosmarinic acid.

## CONCLUSION

In conclusion, more studies should be carried out to clarify the underlying mechanisms of anti-cancerogen effects of rosmarinic acid and water extract of *Salvia virgata* on leukemia cell lines. Therefore, this study shown the activity of *S. virgata* on leukemia as traditionally use. In further study, these extracts can be tested in *in vivo*.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

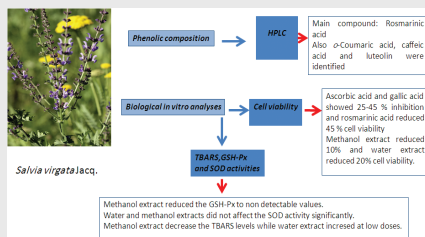
There is no conflict of interest.

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## PICTORIAL ABSTRACT



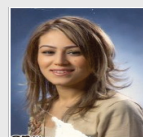
## ABBREVIATIONS USED

**GSH-Px:** Glutathione peroxidase; **SOD:** Superoxide dismutase; **MDA:** Malondialdehyde; **TBARS:** Thiobarbituric acid reactive substances; **K562:** Human immortalised myelogenous leukemia line; **KBM-5:** Chronic Myelogenous Leukemia Cells; **HL-60:** Human promyelocytic leukemia cells; **HeLa:** Homo sapiens cervix adenocarcinoma cells; **P388:** Mus musculus monocyte lymphoma; **HPLC:** high-pressure liquid chromatography.

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## SUMMARY

- Rosmarinic acid was the main compound in all the extracts.
- Rosmarinic acid reduced the cell viability around 45% and methanol extract in both doses reduced the cell viability around 10%.
- Rosmarinic acid reduced the GSH-Px and SOD activity as parallel with the cell viability results.
- The water extract at a dose of 0.50 mg/mL and methanol extract, reducing GSH-Px increasing TBARS might be pro-oxidant on this cell line e samples showed no significant cytotoxic effect.