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PHENOLIC COMPOSITION, ANTIOXIDANT CAPACITY OF *SALVIA VERTICCILATA* AND EFFECT ON MULTIDRUG RESISTANT BACTERIA BY FLOW-CYTOMETRY

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Abstract

Background: Antioxidants are of great importance for preventing oxidative stress that may cause several degenerative diseases. Studies have indicated phytochemicals have high free-radical scavenging activity, which helps to reduce the risk of chronic diseases. The aim of the present study is the determination of antioxidant properties, polyphenolic content and multidrug resistant bacteria of Salvia verticcilata L.
Materials and Methods: Methanol was used as the extraction solvent. The total phenolic content was calculated using Folin-Ciocalteau method and phenolic composition was determined by HPLC. The radical scavenging activity of plant was evaluated *in vitro* based on the reduction of the stable DPPH free radical. The reducing capacity was identified by using the FRAP method. The ability of *Salvia verticcilata* L. to increase the permeability of multidrug resistant bacterial cells was conducted by flow cytometric assay on *Listeria innocua* and *E-coli.*
Results: The amount of total phenolics was found to be 347.5 mg GA/g extract. The IC_{50} value and FRAP assay are 0.61, and 0.944 respectively, Free radical scavenging effect and FRAP values are less than synthetic antioxidant compounds (BHA and BHT). Eight phenolic compounds were found in *Salvia verticcilata* L. Intense concentration of *S. verticcilata* L. has destroyed 97 % of living cells for *Listeria innocua* and 94.86% for *E-coli*
Conclusion: This studyshows that methanolic extracts of *Salvia verticcilata* L. is a potential source of natural antioxidants and antimicrobial agent and can form the basis for pharmacological studies.

Key words: *Salvia verticcilata*, medicinal plant, antioxidant, phenolic composition, multidrug resistant

Introduction

The genus *Salvia* (*Lamiaceae*) has 900 species spread throughout the world. In Turkey, Hedge (1982) recognized 86 species with two subspecies, two varieties, and one doubtful species (Kahraman et al., 2012), (Hedge 1982). In Turkey, the rate of endemism in genus *Salvia* is 50%. Most of *Salvia* are known as medicinal herbs and are used in folk medicine for treatments of many diseases, including the common cold, coughs, chest problems, etc. Also, this genus is used as herbal tea and for food flavouring, as well as in cosmetics and by the pharmaceutical industries (Saeidnia et al., 2011), (Tosun et al., 2009). Human beings are aerobic organisms and oxygen can be converted into molecules called superoxide free radicals such as superoxide anion, hydroxyl radical. These molecules ar highly reactive and contain unpaired electrons. Environmental stresses cause generations of free radicals faster than their degradation in the cell which in turn causes oxidative stress. Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues, resulting in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins. It can even lead to cell death induced by DNA fragmentation and lipid peroxidation.

The consequences of oxidative stress construct the molecular basis in the development of cancer, neurodegenerative disorders, cardiovascular diseases, diabetes and autoimmune disorders (Ratnam et al., 2006), (Asadi et al., 2010). Synthetic antioxidants such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole) and TBHQ (tertiary butyl hydroquinone) have high antioxidant capacity and commonly have been used to prevent oxidative deterioration of fats and oily foods (Topçu et al., 2008). But, these antioxidants are responsible for liver damage and carcinogenesis. For this reasons, use of synthetic antioxidants is limited in some countries. Therefore, the interest in natural antioxidants has been increasing in view of their protection of humans from oxidative stress damage (Tel et al., 2010). Furthermore, *salvia* as a natural antioxidant can be used as an alternative to synthetic antioxidants. Some of the secondary metabolites such as the phenolic compounds of plants belonging to this genus have also shown excellent antimicrobial activity, as well as scavenging activity (Tepe et al., 2005). Fluorescence-based flow cytometry is a technique for measuring characteristics of cells or biological particles and using flourescent dye as fluorogenic substrate provides. The datas obtained can be used to understand and monitor biological processes. Propidium iodide (PI) is an intercalating agent and a fluorescent molecule and it can be used to stain cells. The SYTO dyes can be used to stain nucleic acids in both live and dead cells, as well as in Gram-positive and Gram-negative bacteria.

In this study, PI and SYTO 13 are used as a fluorescent molecule. To stain cells, the combination of SYTO 13 and propidium iodide (PI), in other words a cell-permeant dye and a cell-impermeant dye can be used together to distinguish between live and dead cells in media to detect Multi Drug Resistant (MDR) (Riu et al., 2009), (Nguefack et al., 2004), (Johnson and Spence, 2011). *Listeria innocua* and *Escherichia Coli* were used as a Gram positive bacteria and Gram negative bacteria, respectively.

Material and Method

Plant Material

Salvia verticcilata L. was collected from Yayladagı road (3 km) Antakya in May, 2012 by Dr. Yelda Güzel. The voucher specimen (Y. Guzel 813 MKUF) stored in the Herbarium at the Science and Art Faculty of Mustafa Kemal University.

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Preparation of the methanolic extracts

The air-dried and finely ground samples (about 100 g) were extracted with methanol at 45°C for eight hours. Then the sample was lyophilized and kept in the dark at +4 °C until tested.

Antioxidant activity tests

Analysis of the total phenolic compounds in plant methanolic extracts

Sample extracts were prepared at 0.4 mg/ml. To proceed with the Folin-Ciocalteu method Atanassova et al., 2011), 0.25 mL of sample extract followed by the addition of 1.25 mL of Folin-Ciocalteu reagent and 3.75 mL sodium carbonate solution (7%) 0.1 M. The blank was prepared using the same chemical reagents excluding the extract. The flasks were mixed well and left in the dark, at room temperature (25°C) for 120 minutes, then the absorbance was read at λ = 765 nm. In order to establish the standard curve, gallic acid was used in the range of five different concentrations (0.4-0.125 mg / ml).

Radical scavenging activity test (DPPH assay)

The plant sample's radical scavenging activity was determined according to the method Brand Williams modified, (Williams et al., 1995) . The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured by bleaching the purple-colored methanol solution of DPPH. This spectrophotometer assay uses the stable radical, 1,1-diphenylpicrylhydrazyl (DPPH·), as a reagent. Sample and synthetic antioxidants were prepared at 0.4 mg / ml and diluted at 1:1 to obtain five different concentration. 1.25 ml sample and 3.75 ml of DPPH (6.10^{-5} M) solution were stirred. After a 30 minute incubation period at room temperature, the absorbance was read against a blank at 517 nm. The inhibition of free radical DPPH in percent (I %) was calculated in following way:

$$I\% = (A_{blank}\text{-}A_{sample}/A_{blank}) \text{ x}100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. An extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate. BHA and BHT were used as standart antioxidants.

Reducing power

The reducing power of each extract was determined according to the Oyaizu method which was modified (Oyaizu, 1986). Sample solutions (2.5 ml, five-fold dilution of prepared sample) in phosphate buffer (2.5 ml, 0.2 M, pH 6.6) were added to potassium ferricyanide (2.5 ml, 1.0%), and the mixture was incubated at 50°C for 20 minutes. Trichloroacetic acid (2.5 ml, 10%) was added and the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant (5.0 ml) was mixed with distilled water (5.0 ml) and ferric chloride (1.0 ml, 0.1%) was added, and then the solution's absorbance of the incident radiation was determined spectrophotometrically at 700 nm, with higher absorbance value indicating greater reducing power. BHA and BHT were used as standard antioxidants.

Determination phenolic content of *Salvia verticillata* subsp. *L. amasiaca* by HPLC

To determine the sample's phenolic content gallic acid, catechin, caffeic acid, epicatechin, coumaric acid, ferulic acid, vitexin, routine, hesperidin, rosmarinic acid, eriodictiol, and quercetin, morin and carvacrol were used as standards. The Young Lin HPLC 9100 model was used and its technical specifications are as follows:
Detector: MS 9160 PDA detector
Pump: Quaternary MS 9160
Column: tailor-phase ODS2 C18 (250 x 4.6 mm)
Particle Size: 5 µ

Sample Preparation and Injection: The sample was prepared with 0.5 mg / ml concentration, filtered with a diameter of 0.45 mmand, then 20 µL was injected into HPLC it has a "." 5% formic acid-water, and 5% formic acid-acetonitrile were used as mobile phases. The flow rate was 0.8 mL / minute and the column temperature was set to 30°C.

Flow cytometric analysis of the percentage of cell death of *Listeria innocua* and *E-coli*

This method was determined according to Kongcharoensuntorn (Kongcharoensuntorn et al., 2007). The plant extract was disolved in dimethylsulfoxide (DMSO) and filtered by 0.2 µm Nylon filtered paper. The ability of the plant extract to enhance the permeability of multidrug resistant bacterial cells was conducted by flow cytometric assay of uptaking PI (propidium iodide) and SYTO 13 by *Listeria innocua* and *E-coli* provided by the Department of Biotechnology in Applied Science Faculty, Flensburg University. Initially, the optimum conditions were determined. (Plant sample concentrations, amount of extract solution added into the bacterial culture and PI or SYTO 13 concentrations) Table 1.

Table 1: Extract concentrations and rate of dilution of PI and SYTO 13

for *Listeria innocua*	PI	10% (in FACS Solution)
	SYTO 13	10% (in FACS Solution)
for *Escherichia coli*	PI	10% (in FACS Solution)
	SYTO 13	Undiluted
Plant concentrations		6 mg/ml, 3 mg/ml and 1,5 mg/ml in 10% DMSO

PI and SYTO 13 can intervene in double-strand DNA and become highly flourescent, when damaged cells are present. So, they can be used as reporters to detect membrane integrity (Kongcharoensuntorn et al.,, 2007), (Vives-Rego et al.,, 2000). Bacterial cultures were grown at 37 °C in Mueller Hinton broth. Then, the cultures were diluted at a concentration of 10^7 CFU/ml in a phosphate buffer. Concentrations of PI and SYTO 13 were applied differently for *Listeria innocua* and *E-coli* (Table 1). For the positive control (multidrug resistant strains), 2,5 µL PI and 2,5 µL SYTO 13 were added in 500 µL of bacterial culture (*Listeria innocua* or *E-coli*) and incubated at 70 °C for one hour. For the negative control

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(non-multidrug resistant strains), distinct from the positive control, 200 µL DMSO (10%) was added and incubated at 25 °C for one hour. For all samples, the procedure was the same as with the negative control; however, instead of using the DMSO solution, *Salvia verticcilata* solutions (6 mg/ml, 3 mg/ml and 1,5 mg/ml in 10% DMSO) were added into test tubes. Next, test tubes were examined using a FACScan flow cytometer.

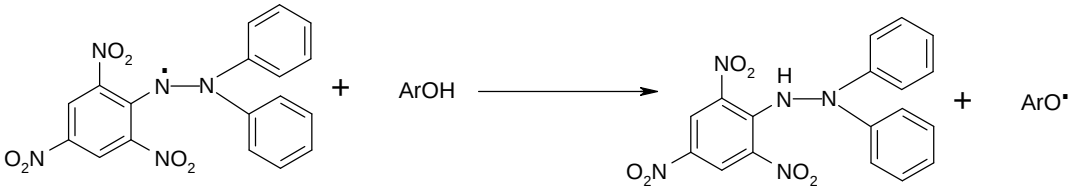
Statistical analysis

The experiments were replicated three times and all measurements were done in duplicates. Data for total phenols and antioxidant activity in the extract were subjected to analysis of variance (ANOVA), and the Fisher's LSD test was used to identify differences among the means at $p< 0.05$.

Result and Discussion

Polyphenolic compounds are secondary metabolites which are of considerable physiological and morphological importance in plants. Research showed secondary metabolites such as phenolic acids and flavonoids have effective chemical structure for free radical scavenging activity (Lo et al., 2005). In this study, the total phenolic compound assay was measured by using the Folin-Ciocalteu method and was expressed as mg/mL of gallic acid, equivalent to dry *Salvia verticcilata* extract solutions. The Folin-Ciocalteu reagent assay was used to determine the total phenolics content. The amount of phenolic compounds in the methanol extracts from the *Salvia verticcilata* was found to be 0.3475 mg gallic acid /mL equivalent (Table 2). DPPH is a stable-free radical and the extract of *Salvia verticcilata* contained antioxidant components, as mentioned above, that could react rapidly with DPPH radicals and reduce most DPPH radical molecules.

$$DPPH^{\bullet} + ArOH \rightarrow ArO^{\bullet} + DPPH\text{-}H$$



1,1-diphenyl-2-picryl hydrazyl Antioxidants

This method is currently popular and based upon the use of the stable-free radical compound 1,1 diphenylpicrylhydrazyl (DPPH·). In this method, a decrease in absorbance of the DPPH radical solution is due to its reduction by *Salvia verticcilata* and synthtetic antioxidant compounds. Absorbance decreases, as a result of a colour change from purple to yellow, the radical is scavenged by antioxidant through a donation of hydrogen to form the stable DPPH-H. Colour change is obsorbed in 517 nm by spectrophotometry. Primarily, the inhibition of free radical DPPH (I%) was calculated from absorbance values (Figure 1).

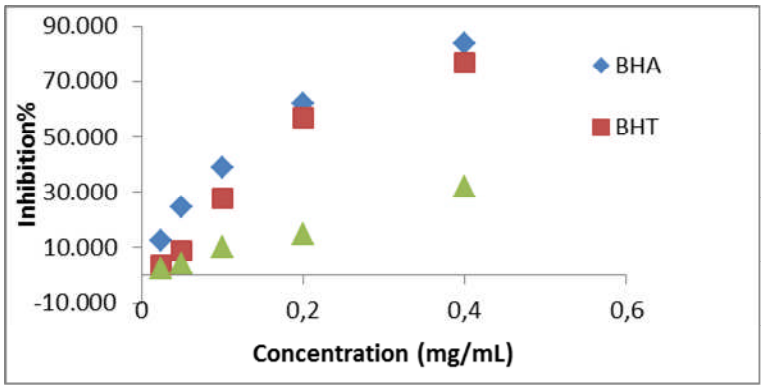


Figure 1: DPPH radical inhibition rate

The aim of this method is to examine the use of the parameter IC_{50} (IC_{50} = equivalent concentration to give 50% effect) (Hasan et al., 2009), (Jothy et al., 2011). As a result of the DPPH method, IC_{50} values of *Salvia verticcilata*, BHA and BHT are 0.61, 0.142 and 0.189 respectively (Table 2). A lower IC_{50} value indicates higher antioxidant activity. BHA and BHT are synthtetic antioxidants and phenolic structured compounds. Therefore, the lowest IC_{50} has the value of BHA, which is is perfectly normal. The IC_{50} value of *Salvia verticcilata* is 0.61, and furthermore, the *Salvia verticcilata* quenched DPPH radicals in medium just as synthetic antioxidant compounds BHA and BHT.

Many studies have demonstrated that the electron donation capacity, reflecting the reducing power of secondary metabolites, is related to their antioxidant activity, and that the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron. The presence of reducing agents, such as antioxidant effect compounds in the plant extracts, causes the reduction of the Fe^{3+} complex to Fe^{2+} form, and Fe^{2+} can be monitored by spectrophotometric methods at 700 nm. (Iris et al., 1996), (Beyhan et al., 2010), (Lim et al., 2010). In this method, the value of FRAP (ferric reducing antioxidant power) is calculated as follows:

$$\varepsilon = A / LxC ,$$

$$FRAP = \varepsilon_{sample} / \varepsilon_{trolox}$$

where ε is molar absorption coefficient, A is absorbance, C is concentration and L is the way of light. The same procedure was repeated with synthetic antioxidant BHT and BHA, as a positive control**.** Table 2 shows the antioxidant capacities of *Salvia verticillata L.* and synthtetic antioxidant compounds BHA and BHT. The synthtetic antioxidant compounds, in general, showed high antioxidant capacities. According to the results, for the FRAP assay, the lowest FRAP value belongs to *Salvia verticillata* L. Thus, the antioxidant capacity of *S. verticillata* L. is lower than the BHA and BHT. This result gives similar results for other methods.

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Table 2: Antioxidant value of *Salvia verticillata* L and synthetic antioxidant compounds

	Salvia verticillata L	BHA	BHT
Total phenolic compounds (mg GA/g extract)	347.5 ±0,24	-------	-------
IC_{50} (DPPH)	0,61	0,142	0,189
FRAP (TEAC, trolox equivalent antioxidant capacity)	0,944±0,017	1,066±0,011	1,027±0,011

The antioxidant activity of phenolic compounds depends on the structure, and the number and positions of the hydroxyl groups on the aromatic rings (Aberoumand and Deokule, 2008). The identification of phenolics of *Salvia verticcilata* by HPLC was achieved by comparing their retention times. The HPLC chromatogram of standard phenolic compounds is presented in Figure 2, and chromatograms of *Salvia verticcilata* are shown in Figure 3. Fourteen standard phenolics or phenolic acid compounds were used in the HPLC system of which eight phenolic compounds were found in *Salvia verticcilata* L. In the chromatograms, two different signals was detected from some fenolic compounds. These are p-coumaric acid, ferulic acid and quercetin. Ferulic acid, hesperidin and quercetin are the most characteristic phenolic compounds found in extract. As said before, pheolics are very strong antioxidant compounds and when table 1 and figures 2-3 are analyzed, it is evident that there are significant linear correlations between phenolics concentration and antioxidant capasities.

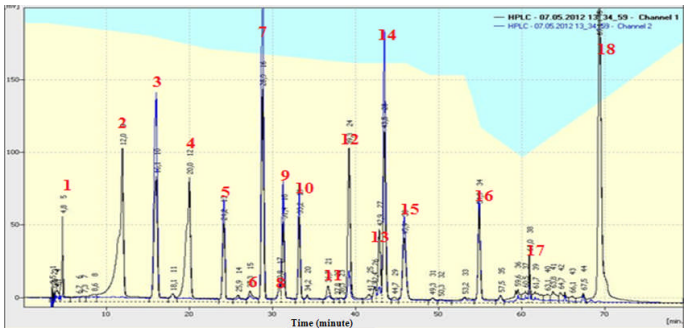

Figure 2. HPLC chromatogram of standart phenolic compounds. (1.Gallic acid, 2. catechin, 3. caffeic acid, 4.epicatechin, 5-6 p-coumaric acid II, 7-10 ferulic acid, 8. Vitexin, 9. routin, 11-14 hesperidin, 12. naringinin, 13.rosmarinic acid, 15. morin, 16-17 quercetin, 18. carvacrol)

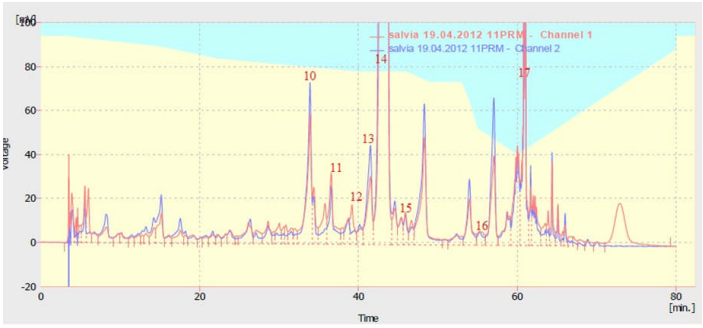

Figure 3. HPLC chromatogram of *Salvia verticillata* 10. Ferulic acid 11-14 Hesperidin, 12. Naringinin, 13. Rosmarinic acid, 15. Morin, 16-17. Quercetin.

In flow cytometry experiments on *Salvia verticcilata*, the cell of *Listeria innocua* and *E- coli* were more flourescent and more stained than cells treats with PI and SYTO 13 combined system for positive and negative control (Figure 4 and Figure 6). After exposure incubated at 25 °C for one hour, the herb extracts mediated staining of PI and SYTO 13 combined system were brighter and more uniform when the cell was treated at the increasing concentration. In figures 4-7, while the right side of figures shows living cells, the left side shows the dead cells. With reference to these figures, the lowest concentration of *Salvia verticcilata* L. revealed that the number of living cells more than high concentration (Figures 5 and 7). In the most intense concentration of *S. verticcilata* L., the percentage of dead cells was 97.77% for *Listeria innocua* and 94.86% for *E-coli* (Figures 5A and 7A). When the extract concentration in the bacterial culture reduced, the number of living cells increased. It can be said that the *Salvia verticcilata* extract may disrupt the membrane barrier and allow exogeneous solutes such as PI and SYTO 13 to permeabilize into the bacterial cells. Secondary metabolites in the *Salvia verticcilata* may have the ability to increase the permeability of bacterial membranes and allow antibiotics to access the bacterial targets. These results show that the flow cytometry method is more advantageous than traditional methods, such as disk diffusion and mikro dilution, for quickly generating a large amount of data. This technique can serve as a powerful tool in optimally combining different preservative factors in order to design an effective antimicrobial system for selected foods.

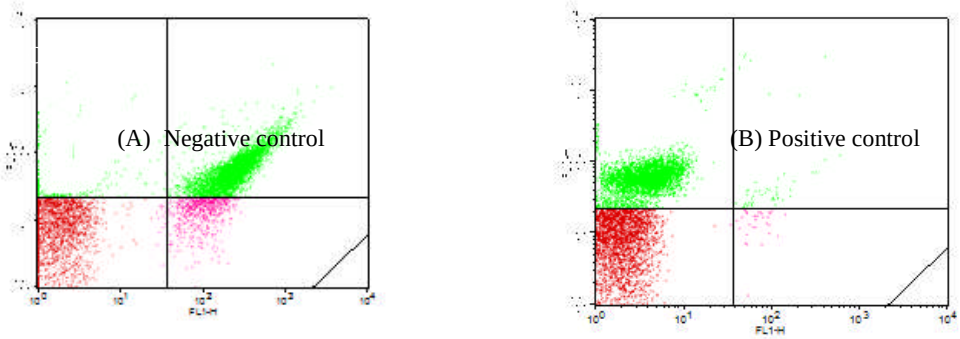

Figure 4..Positive and negative **control *of*** *Listeria innocua*

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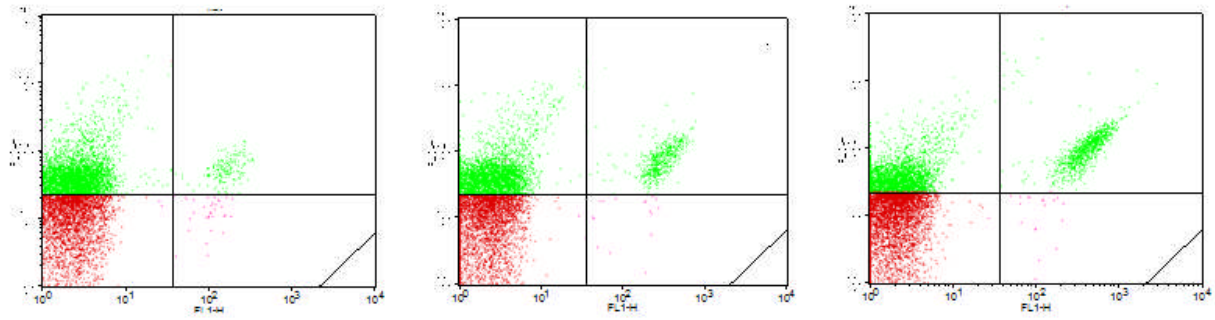

Figure 5. Percentage of dead cell in *Listeria innocua* bacterial culture.

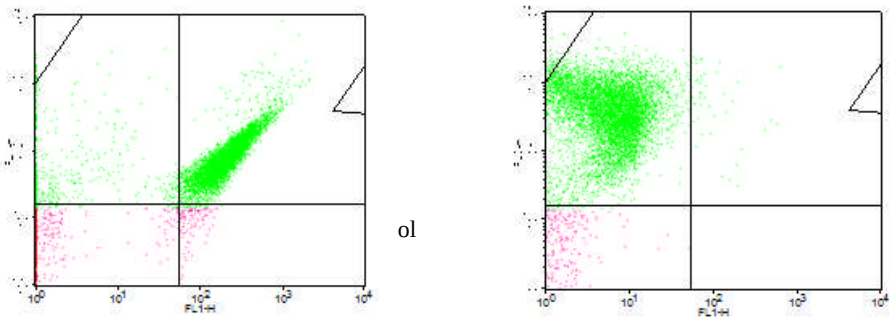

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Figure 6..Positive and negative control *of E- coli*

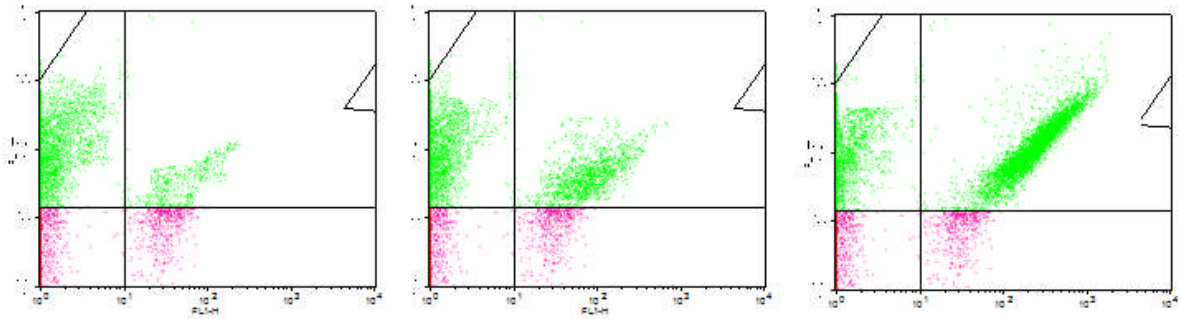

Figure 7. Percentage of dead cell in *E- coli* bacterial culture.

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