6. Biological screening

Biological screening of the *Echinacea* extracts was performed in *Medical Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technology Application, Borg El-Arab, Alexandria, Egypt.*

6.1. *In vitro* cytotoxicity assay

In vitro cytotoxicity assay was performed to assess the viability of normal cells (Human peripheral blood mononuclear cells, PBMCs) after incubation with different extracts. Viability of cells was measured using neutral red uptake assay as described by *Borenfreund* and *Puerner*[97] to determine the non-cytotoxic concentration of each extract. This assay depends on the fact that neutral red dye can be incorporated into the lysosomes of living cells providing a quantitative assay to the cytotoxic effects[98].

Cytotoxicity assay involved three main steps. First, the isolation of PBMCs from freshly collected human blood sample, then incubating PBMCs with different concentrations of the extracts for 72 hours and finally, measuring cells' viability using neutral red uptake assay.

Method

Human PBMCs were isolated from heparinized healthy volunteer peripheral blood by density gradient centrifugation technique as described by *Boyum*[99]. Blood samples were freshly collected into heparinized sterile tubes. Blood was diluted using equal volume of RPMI-1640 medium supplemented with 25 mM HEPES buffer and 4mM L-glutamine. Diluted blood was layered over equal volume of Ficoll-Pague™ Plus (density 1.077 g/L) (lymphocyte Separation Medium LSM) and centrifuged at 2000 rpm for 30 minutes with no acceleration and break at room temperature. The buffy mononuclear cell layer was collected using sterile Pasteur pipette into 50 ml sterile Falcon tube and washed twice in phosphate buffered saline (PBS :1x pH 7.4 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 dissolved in 1 L Milli-Q water) using centrifugation at 1650 rpm for 5 min. The isolated PBMCs' viability was assessed by Trypan blue exclusion method. PBMCs were re-suspended at 1 x 10^6 cells/ml in RPMI-1640 medium containing 25 mM HEPES, 4mM L-Glutamine supplemented with 10% heat-inactivated FBS.

A stock solution of each extract (2 mg/ml) was dissolved in 7% ethanol followed by sterilization using a 0.2 µm syringe filter. The desired concentrations (100, 50, 25, 12.5 and 6.25 µg/ml) were prepared using serial dilution in a 96-well plate. Tested extracts' wells were prepared by adding 100 µl of suspended cells at 1 x 10^6 cells/ml (final cell number/well was equal to 1×10^5 PBMCs in 100 µl culture media). Parallel concentrations of the solvent were prepared to be used as controls. Control wells were prepared by adding 100 µl culture media to a suspension of 1×10^5 PBMCs in 100 µl culture media. Blank wells contained 200 µl culture media only (without cells or compound solution). Each set of samples was pipetted in duplicate. The plate was then gently shaken then incubated at 37 ºC, 5% CO_2 for 72 hours.

After incubation, the plate was centrifuged (2000 rpm for 10 min. Followed by discarding of 100 µl media and 100 µl neutral red working solution (80 µg /ml) stain was added to each well then the plate was gently shaken. Followed by incubation at 37 ºC in humidified 5% CO_2 for three hours, and then centrifuged at 2000 rpm for 10 min. Excessive dyes were discarded, and cells were washed using PBS and centrifugation at 2000 rpm for 10 min. The stained cells were fixed with 100 µl fixing solution (0.5% formalin with 1% calcium chloride) for 1 min. Cells were destained in 100 µl destaining solution (50% ethanol with 1% glacial acetic acid) for 5 min by shaking. The stain intensity was assayed using automated microplate reader spectrophotometer adjusted at 540 nm. Viable cell fraction was calculated according to the following equation of cell viability:

Cell viability (%) = (E / C) * 100

Cell inhibition (%) by blank =100 – [(B / C) *100]

Actual cell viability (%) = Cell viability (%) + cell inhibition (%) by blank

Where:

E: The mean absorbance of extract/compound treated wells.
B: The mean absorbance of blank wells.
C: The mean absorbance of control wells.

Results were interpreted to calculate both the effective concentration that kills 50% of cells (EC_{50}) and the maximum safe concentration that keep 100% (EC_{100}) cell viability for each compound using GraphPad InStat 3.0 software.[100] The results of the *in vitro* cytotoxicity assay are listed in **Table 3**. While the effect of different tested concentrations from each extract on % viability of lymphocytes was presented in **Figure 36 to Figure 42**.

6.2. Total Antioxidant activity

The total antioxidant status was determined by 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS-based colorimetric method as described by *Erel.*[101] with some modifications. The reduced ABTS molecule is oxidized to $ABTS^{+}$ using hydrogen peroxide alone in acidic medium (deep green color). Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related with the total antioxidant activity of the sample.

Method

In a 96 well plate, 200 µl of **reagent 1**(400 mM acetate buffer solution (weigh 6.804gm sodium acetate tri-hydrate in 100ml distilled water then adjust the pH to 5.8 using acetic acid) was added to 5µl of different extracts' concentrations, the plate was gently shaken and the absorbance was measured at 660 nm as a sample blank, then 20µl **reagent 2**(10 mM ABTS in 1mM H_2O_2 prepared in 30 mM acetate buffer solution pH 3.8 (weigh 0.137gm ABTS in 25ml buffered H_2O_2 which is prepared by adding 8.1µl H_2O_2 (30% v/v) in 25 ml 0.03M acetate buffer(weigh 0.102gm of sodium acetate tri-hydrate in 25ml distilled water then adjust the pH to 3.8) was added and the mixture was incubated for 5 min, then the absorbance was measured again at 660 nm. The ascorbic acid solutions (200- 0.048828µg/ml) were prepared in 7% ethanol and treated similarly as samples and standard curve in **Figure 43** was established. Antioxidant potential of various extracts based on IC_{50} was presented in **Figure 44** and **Table 5 (in appendix).**

The percentage inhibition of ABTS bleaching for the sample and standards were obtained from the following equation:

$$\textbf{\% inhibition} = \frac{A_B - A_S}{A_B} \times 100$$

Where:

A_S: The absorbance of the sample (or standard)

A_B: The absorbance of the blank

6.3. *In vitro* phagocytic activity assay

In vitro phagocytic assay was performed to quantitate the phagocytic and intracellular microbicidal activity of phagocytes. Phagocytes are large white blood cells that can engulf and digest foreign invaders. They include monocytes, which circulate in the blood, and macrophages, which are found in tissues throughout the body, as well as neutrophils, cells that circulate in the blood but move into tissues where they are needed.

A powerful *in vitro* assay to assess host-pathogen interactions is the co-culture of phagocytic cells with the tested microorganism described by *Athamna*[102] was used with some modifications, neutrophils and macrophages were used as representative cell types, methylene blue (MB) was used as the indicator dye and the test organism was *C. albicans*. The method is based upon MB dye accumulation in vital phagocytes and yeast cells both are stained blue with the same dye. Starting from this fact, MB could be used to detect Candida ingestion and to differentiate whether or not *C. albicans* cells in the phagocytic vacuoles of vital neutrophils and

macrophages were dead. The phagocytic activity and intracellular killing were measured spectrophotometrically[103].

Methods

a. Extracts preparation

A stock solution of each extract (2 mg/ml) was dissolved in 7% ethanol followed by sterilization using a 0.2 µm syringe filter. The desired safe concentrations (IC_{100}) for each extract was selected based on its cytotoxicity on PBMCs and was prepared to provide a total of five drug concentrations using serial dilution. Parallel gradient concentrations of the **solvent** were prepared to be used as controls.

b. Cell isolation and preparation

Sterile technique was used throughout the entire procedure.

1. Polymorphonuclear neutrophils (PMN)[104] Five milliliters of blood were layered over an equal volume of lymphocyte separation medium Ficoll-Pague™ Plus (density 1.077 g/L), centrifuged at 2000 rpm for 30 min and the upper layers were discarded. Fill tube to capacity with fresh cold lysing solution (1x RBCs lysis buffer is composed of 0.802 gm of ammonium chloride, NH_4Cl, 0.084 g sodium bicarbonate, $NaHCO_3$ and 0.037gm of EDTA disodium dissolved in 100 ml Milli-Q water) to resuspend the bottom layer containing erythrocytes and neutrophils, invert or rock for 3~5 minutes at room temperature until liquid is clear red then centrifuge at 2000 rpm for 3 min and decant supernatant, the PMN pellet was washed twice using 30 ml of RPMI medium at 1650 rpm for 5 min / wash. Finally the PMN pellet was resuspended in RPMI1640 medium. The isolated PMN cells' viability was assessed by Haemocytometer count using the trypan blue exclusion method. The PMN cells were resuspended and cell concentration was adjusted to be 1 x 10^6 cells/ml in RPMI-1640 medium containing 25 mM HEPES, 4mM L-Glutamine supplemented with 10% heat-inactivated FBS.

2. Peritoneal macrophages (PM) isolation [105] One milliliter of fetal bovine serum was injected intraperitoneally into mice as a stimulant to elicit peritoneal macrophages. Three days later, the peritoneal exudate was collected by peritoneal lavage with 5 ml serum-free RPMI 1640 medium to which 1% of 100 Units penicillin, 100 µg streptomycin and 0. 25 µg/ml amphotericin B (Sigma, Germany) were previously added. The exudate was centrifuged at 1650 rpm for 5 min, the pellet was washed twice using the same media-antibiotic recipe stated before to remove any residual redness in the pellet by discarding the supernatant and resuspending cell pellet by gently tapping bottom of tube. The isolated cells viability was assessed by Haemocytometer count using the trypan blue exclusion method. The cells were re-suspended and cell concentration was adjusted to 1 x 10^6 cells/ml in RPMI-1640 medium supplemented with 20% heat-inactivated FBS and 1% 100 Units penicillin and 100 µg streptomycin. Cells were plated into 96-well plates (20 µl/ well), and the cells were allowed to attach overnight. After

attachment, plates were tilted to remove the supernatants as the pipette can be put directly into the "corner" of the well to aspirate the supernatant without disturbing the cells then add 20µl RPMI-1640 medium supplemented with 10% heat-inactivated FBS, now cells are ready for use.

c. Quantitated assay for phagocytosis and intracellular killing of *Candida albicans*

For Peritoneal macrophages: In two 96-well plates, 20 µl viable *C. albicans* suspension (5x107 *C. albicans* cells/ml), 20 µl of FBS without deactivation (as source for complement proteins), 40 µl of RPMI 1640 and 20 µl of different extract concentrations were added to 20 µl of macrophage suspension.

For PMN neutrophils: In two 96-well plates, 50 µl 0.4 x 10^5 PMN cells were added to 120 µl of viable *C. albicans* suspension (2x10^6 *C. albicans* cells/ml), 20 µl of FBS without deactivation (as source for complement proteins) and 10 µl of different extract concentrations .

The two plates were incubated at 37ºC in 5% CO_2 incubator for 10 min (first plate for phagocytosis). (phagocytosis was almost complete after 10 minutes, virtually no further phagocytosis appeared to occur and killing was negligible) and the second plate (for yeast killing) was incubated for 60 min. (best suited time for assessing the capacity of phagocytes to kill Candida).

After incubation time, centrifuge plates at 2000 rpm for 10 min then invert to remove previous mixture and to rinse wells gently with 1x PBS. Cells were stained and fixed by adding 50 µl methylene blue solution (1.25% glutaraldehyde and 0.6% methylene blue in HBSS) to each well. After incubation at 37 °C for 60 min in a CO_2 incubator, centrifuge the two plates at 2000 rpm for 10 min, then removed methylene blue solution from the wells by aspirating the supernatant without disturbing the cells two plates. Rinse wells by PBS (add 200 µl of PBS to each well, shake plate and centrifuge at 2000 rpm for 10 min then discard supernatant, repeat 6 times). The excess PBS was allowed to drain and the plate was air-dried. Add 100 µl elution solution (50% ethanol + 49% PBS + 1% acetic acid) to each well. After 15 min on a plate rotator at room temperature, the plates' absorbances were measured at 630 nm.

Data were presented as Phagocytosis index (the ratio of number of Candida engulfed to the total number of PMN cells or macrophage) and killing ratio (ratio of number of killed Candida to the total number of ingested Candida). Neutrophil phagocytosis activity was presented in **Figure 45** and that of macrophage was presented in **Figure 47** and **Table 6 (in appendix)**, while neutrophil yeast killing activity was presented in **Figure 46** and macrophage yeast killing activity in **Figure 48** and **Table 7 (in appendix)**.

6.4. *In vitro* immunostimulatory activity demonstrated by human peripheral blood mononuclear cells proliferation

Immunostimulatory effect of the extract was tested on peripheral blood mononuclear cells (PBMCs) of an immunosuppressed patient. Phytohemagglutinin (PHA), an extract from red kidney bean, *Phaseolus vulgaris*, has been extensively employed to produce mitotic activity in cultures of human WBC. Measuring lymphocyte response to mitogens is a mainstay of screening for cellular immunodeficiency. PHA is a potent stimulator of T-cell activation and proliferation. Therefore, abnormal T-cell responses to mitogens are considered a specific test of aberrant T-cell function and to assess the overall immuno-competence. Decreased lymphocyte responses to PHA have been reported in several diseases associated with anergy. In our study we are evaluating the efficacy of extracts to restore the proliferative responses of lymphocytes to PHA. This test was carried out in reference to a lot of previous studies with modifications[106-108]

Method

All cultures were run in duplicate and in each plate PBMCs from two subjects (normal subject and an immunosuppressed patient with autoimmune disorder under immune suppressant drugs) were cultured. The PBMCs from each subject were cultured in both the presence and absence of PHA to compare the cell proliferation in non-treated and extract-treated cultures.

Human PBMCs were isolated as previously described above in the *in vitro* cytotoxicity assay. The cells were suspended at 1 x 10^6 cells/ml in RPMI culture medium (RPMI supplemented medium and 10% FBS) 100 µl of this cell suspension were placed in each well (final cell number/well was equal to 1×10^5 PBMCs in 100 µl culture media). PHA final well concentration was 54.4µg/ml. At the time of drug addition, the stock solution of each extract (2 mg/ml) dissolved in 7% ethanol was diluted to the desired test concentration (safe dose) with complete RPMI 1640 culture medium, additional 2 fold serial dilution was made to provide a total of five concentrations, 50 µl of different extracts' dilutions were added to the wells containing 100 µl cells and 50µl of PHA resulting in the required final drug concentrations, parallel concentrations of the solvent were prepared to be used as blank. PBMCs in the presence of *Echinacea* extracts and PHA were incubated for 6 days at 37 ºC in humidified 5% CO_2. Cell viability was assessed by using Neutral red-based colorimetric assay as described in the cytotoxicity assay. Stimulation index (SI) obtained with PHA alone (without addition of *Echinacea* extract), was used as a control. Stimulation index obtained with PHA plus *Echinacea* extract was calculated by dividing the absorbance of the experimental well (PHA plus *Echinacea*) by the absorbance of the control well (PHA alone) . The potential of extracts to restore lymphocytes proliferative activity was presented as IC_{50} in **Figure 49** and **Table 8 (in appendix)**.

6.5. *In vitro* anti-inflammatory assay

It was reported that monocytes increase proliferative activity in bone marrow in response to inflammatory stimuli, leading to monocytosis, a clinical condition reflecting an increased number of circulating monocytes. This assay depends on the fact that monocytes play an important role in inflammatory diseases through the release of inflammatory mediators including nitric oxide (NO). Lipopolysaccharides (LPS) which is the most abundant component within the cell wall of Gram-negative bacteria enhances cellular oxidative stress via the generation of reactive oxygen species (ROS).Production of these intermediates has been determined in many inflammatory tissues, following exposure to LPS.

The aim of this study was to develop a rapid *in vitro* screening assay to evaluate the capacity of *Echinacea* extracts to inhibit the production of nitric oxide (NO) produced in response to LPS stimulated human peripheral blood mononuclear cells (PBMC). In parallel monocytes proliferation was measured to assess the general immune function. Method was described by *Hendra*[109] with some modifications.

Method

In each experiment one positive control (cells treated only with LPS) and one negative control (cells without any treatment) were included. All tested samples and controls were examined in duplicates.

Human PBMCs were isolated as previously described in the *in vitro* cytotoxicity assay. The cells were suspended at 1 x 10^6 cells/ml in RPMI culture medium (RPMI supplemented medium and 10% FBS) 100 µl of this cell suspension were placed in each well (final cell number/well was equal to 1×10^5 PBMCs in 100 µl culture media). LPS final well concentration was 39.8µg/ml. At the time of drug addition, the stock solution of each extract (2mg/ml) dissolved in 7% ethanol was diluted to the desired test concentration (safe dose) with complete RPMI 1640 culture medium, additional 2 fold serial dilution was made to provide a total of five concentrations, 50 µl of different extracts' dilutions were added to the wells containing 100 µl of cells and 50µl of LPS resulting in the required final drug concentrations, parallel concentrations of the solvent were prepared to be used as blank. PBMCs in the presence of *Echinacea* extracts and LPS were incubated for 6 days at 37 ºC in humidified 5% CO_2. Cell viability was assessed by using Neutral red-based colorimetric assay as described in the cytotoxicity assay. Stimulation index (SI) obtained with LPS plus *Echinacea* extract was calculated by dividing the absorbance of the stimulated well (LPS plus *Echinacea*) by the absorbance of the control well (LPS alone).Results were presented in **Figure 51** and **Table 9 (in appendix)**.

Total nitric oxide assay was performed to determine the levels of NO produced by PBMCs after 6 days of incubation; as an indicator of inflammatory status. Since most of the NO is oxidized to nitrite and nitrate, the concentrations of these anions have been used as a

quantitative measure of NO production. The nitrates are converted into nitrites by the enzyme nitrate reductase, and the spectrophotometric measurement of the total nitrites is accomplished by utilizing the Griess reagent (mixture of 0.1% naphthylethylenediamine dihydrochloride in distilled water and 1% sulfanilamide in 5% H_3PO_4 (1:1)).

In this method, nitrite is first treated with a diazotizing reagent, sulfanilamide, in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with a coupling reagent, N-naphthyl-ethylenediamine, to form a stable azo compound that can be spectrophotometrically quantitated according to Ding[110]. Briefly, 100 µL of cell-free culture supernatants were incubated with Griess reagent, which is freshly generated by mixing equal volumes of sulphanilamide in orthophosphoric acid and N-(1-naphthyl) ethylenediamine dihydrochloride in water, at room temperature for 10 minutes to yield an azochromophore (pink colored), the absorbance of which was measured at 540 nm using a microplate reader and NO concentration was determined by using the standard concentrations of sodium nitrite (0.03125–100 ng/ml) as shown in **Figure 50.** The ability of different extracts to scavenge NO was illustrated in terms of IC_{50} in **Figure 52** and **Table 10 (in appendix).**

Results and discussion

- ***In vitro* cytotoxicity of *Echinacea* extracts**

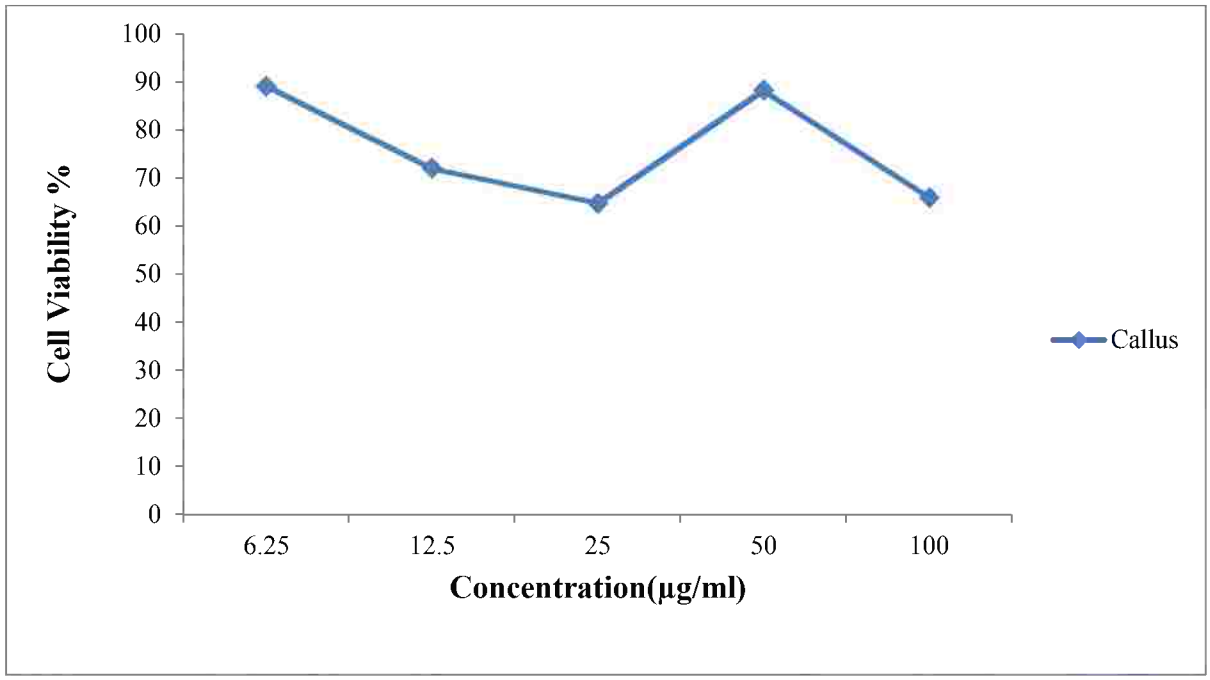

Figure 36: Effect of Untreated callus extracts on % viability of lymphocytes

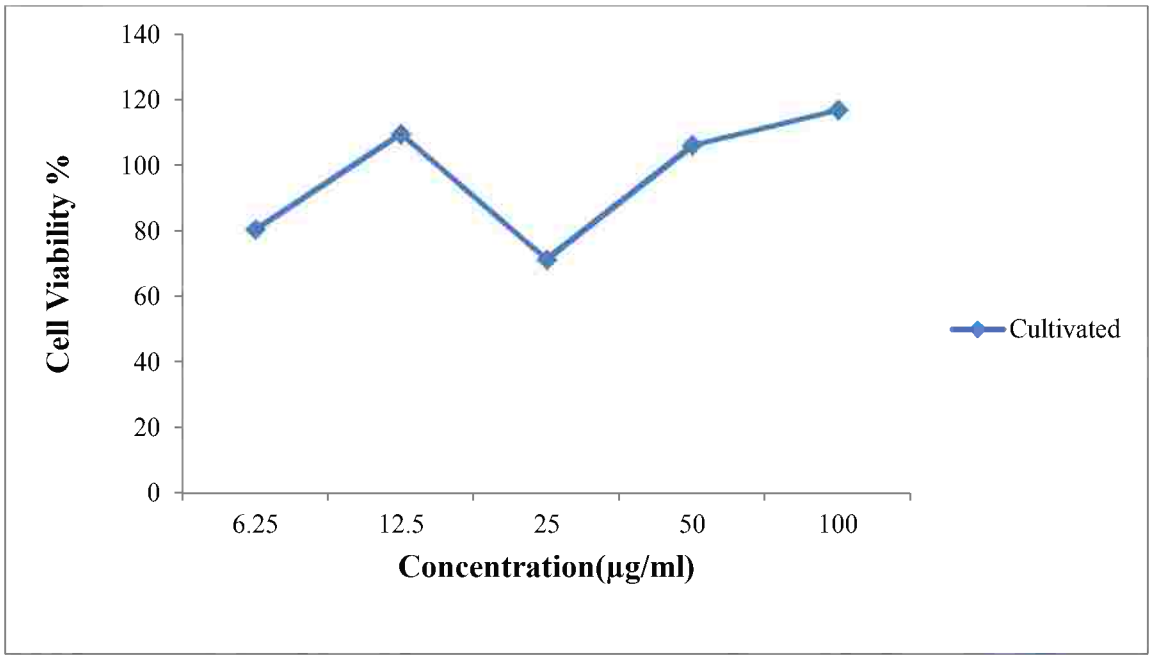

Figure 37: Effect of cultivated plant extracts on % viability of lymphocytes

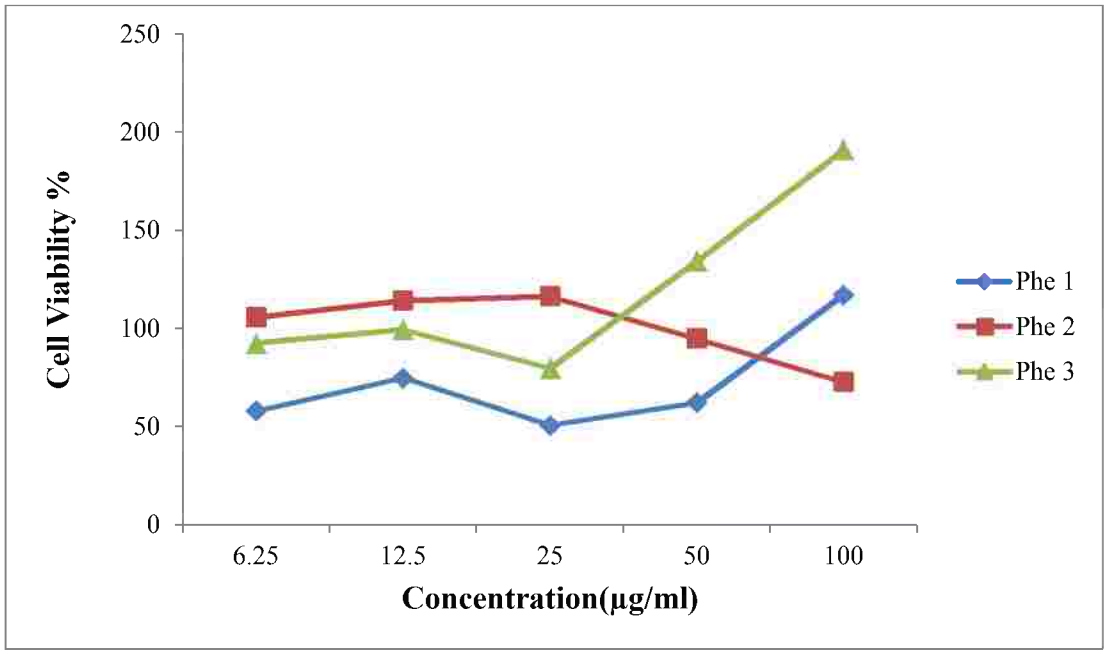

Figure 38: Effect of phenylalanine treated calli extracts on % viability of lymphocytes

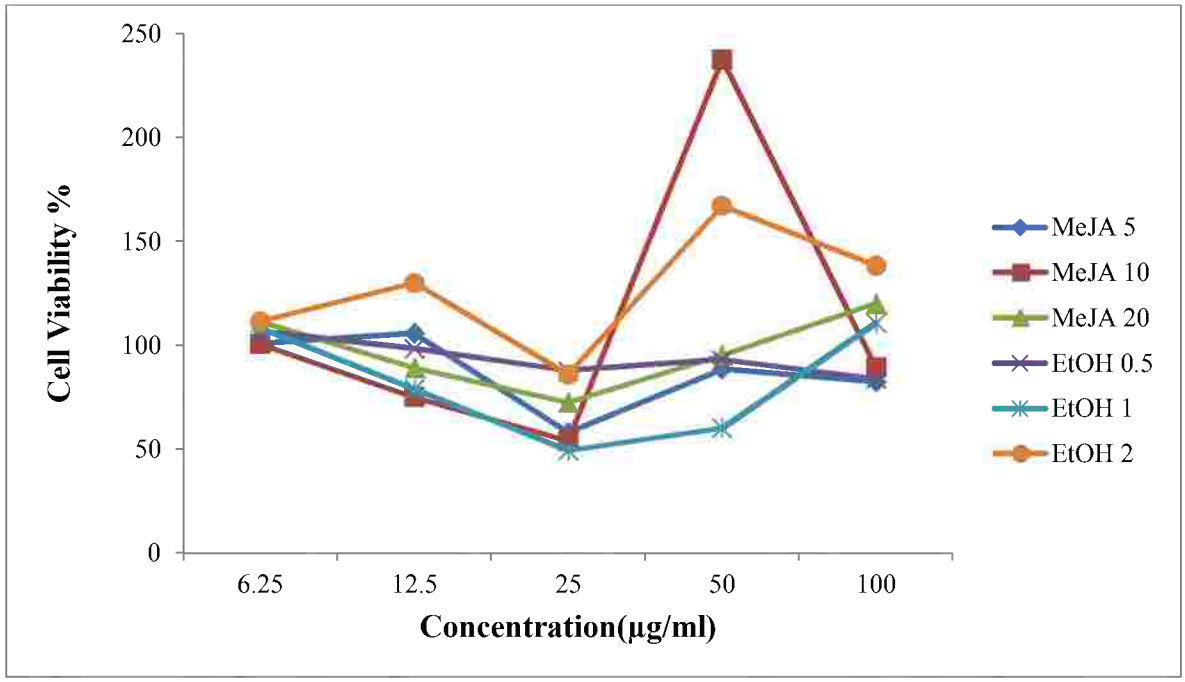

Figure 39: Effect of methyl jasmonate treated calli extracts on % viability of lymphocytes

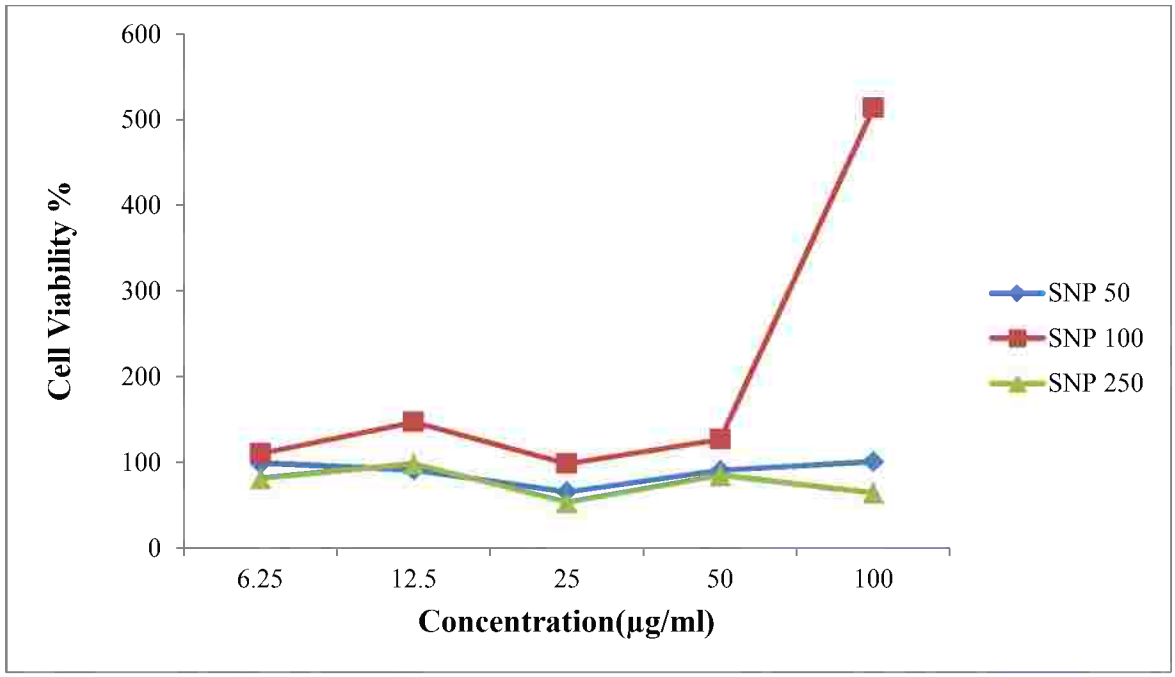

Figure 40: Effect of sodium nitroprussidete treated calli extracts on % viability of lymphocytes

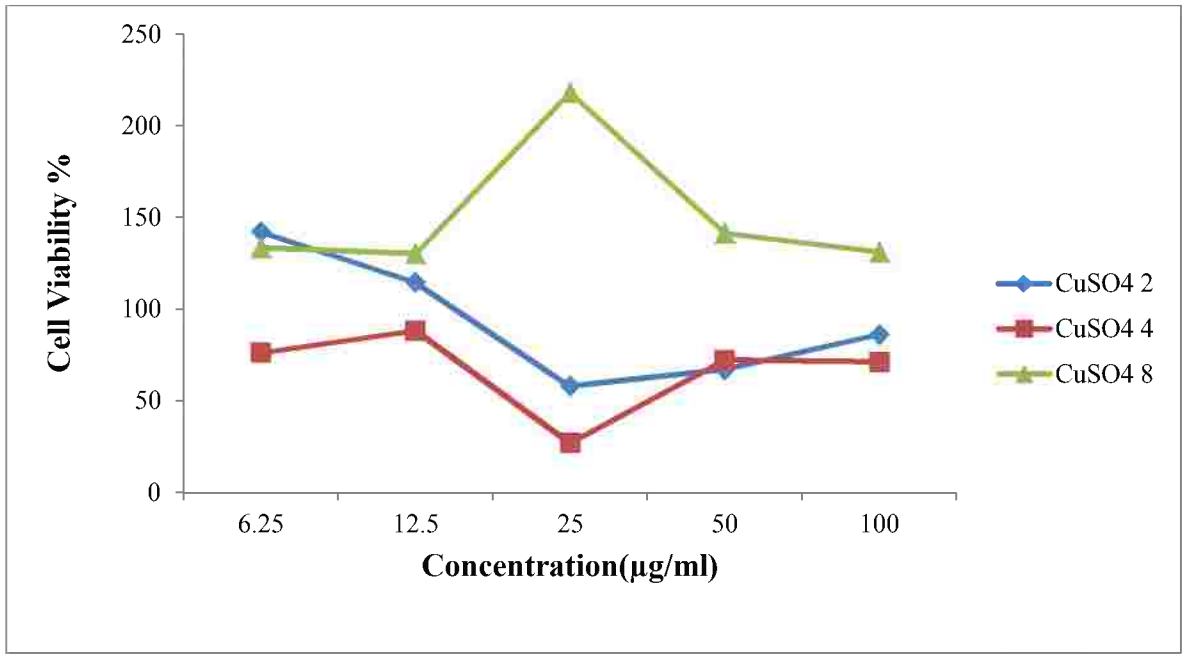

Figure 41: Effect of copper sulphate treated calli extracts on % viability of lymphocytes

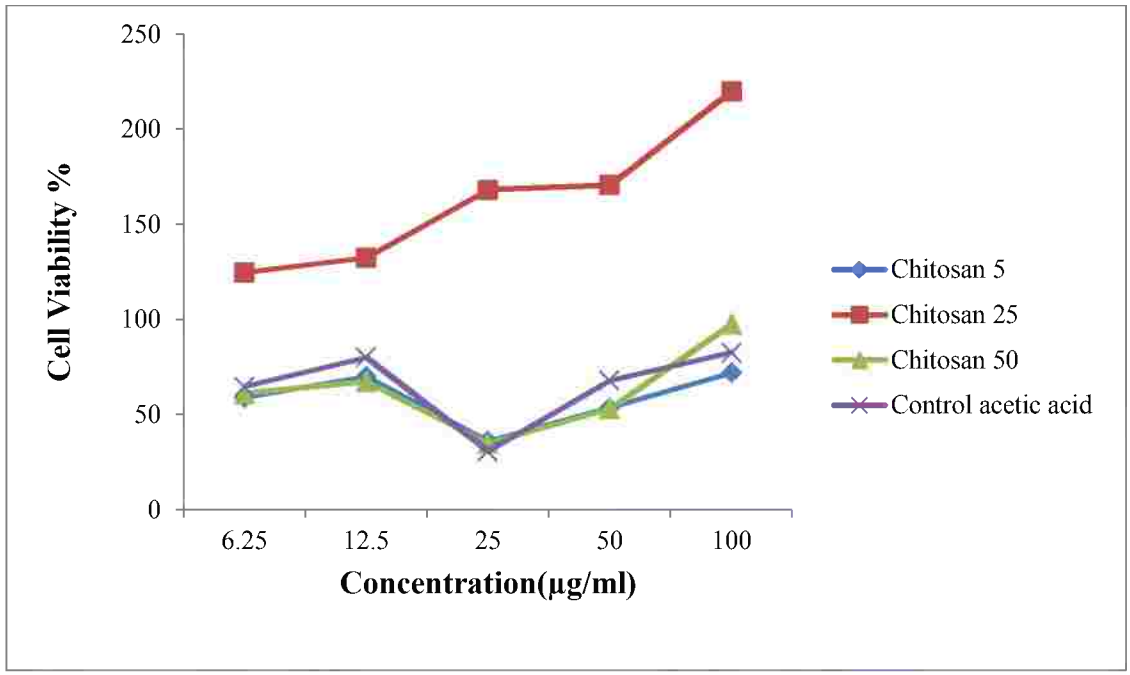

Figure 42: Effect of chitosan treated calli extracts on % viability of lymphocytes

Table 3: Results of *in vitro* cytotoxicity assay against PBMCs

Extract Identity	**EC_{100} in µg/ml**
Callus	176.979
Cultivated	48.309
Phe 2	40.319
MeJA 10	8.5
SNP 50	128.125
MeJA 5	53.207
EtOH 0.5	5.605
$CuSO_4$ 2	23.146
EtOH 1	129.176
Control acetic acid	207.577
$CuSO_4$ 4	22336.51
Chitosan 5	359.485
Chitosan 50	130.988
SNP 250	91.045
Phe 1	86.587
MeJA 20	47.825
Phe 3	21.437
SNP 100	14.762
Chitosan 25	27.402
EtOH 2	33.633
$CuSO_4$ 8	300.254

Our experimental data, provided by **Figures (36-42)**, demonstrate the lymphoproliferative activity level of the tested *Echinacea* extracts in relation to their applied concentrations (6.25, 12.5, 25, 50 and 100 µg/ml). To determine the concentrations of each extract that was not cytotoxic, the concentrations that retained 100% viability of the cells EC_{100} ranged from 5.6 to 91.04 µg/ml, it was noticeable that **50 µM SNP**, **EtOH 1**, **Control acetic acid**, **4 µM $CuSO_4$**, **8 µM $CuSO_4$**, **5 mg/L Chitosan** and **50 mg/L Chitosan** treated calli and the **untreated calli** extracts showed a very good safety profile. These doses were chosen to be used in all the *in vitro* biological assays. **(Table 3)**

- **Total antioxidant activity**

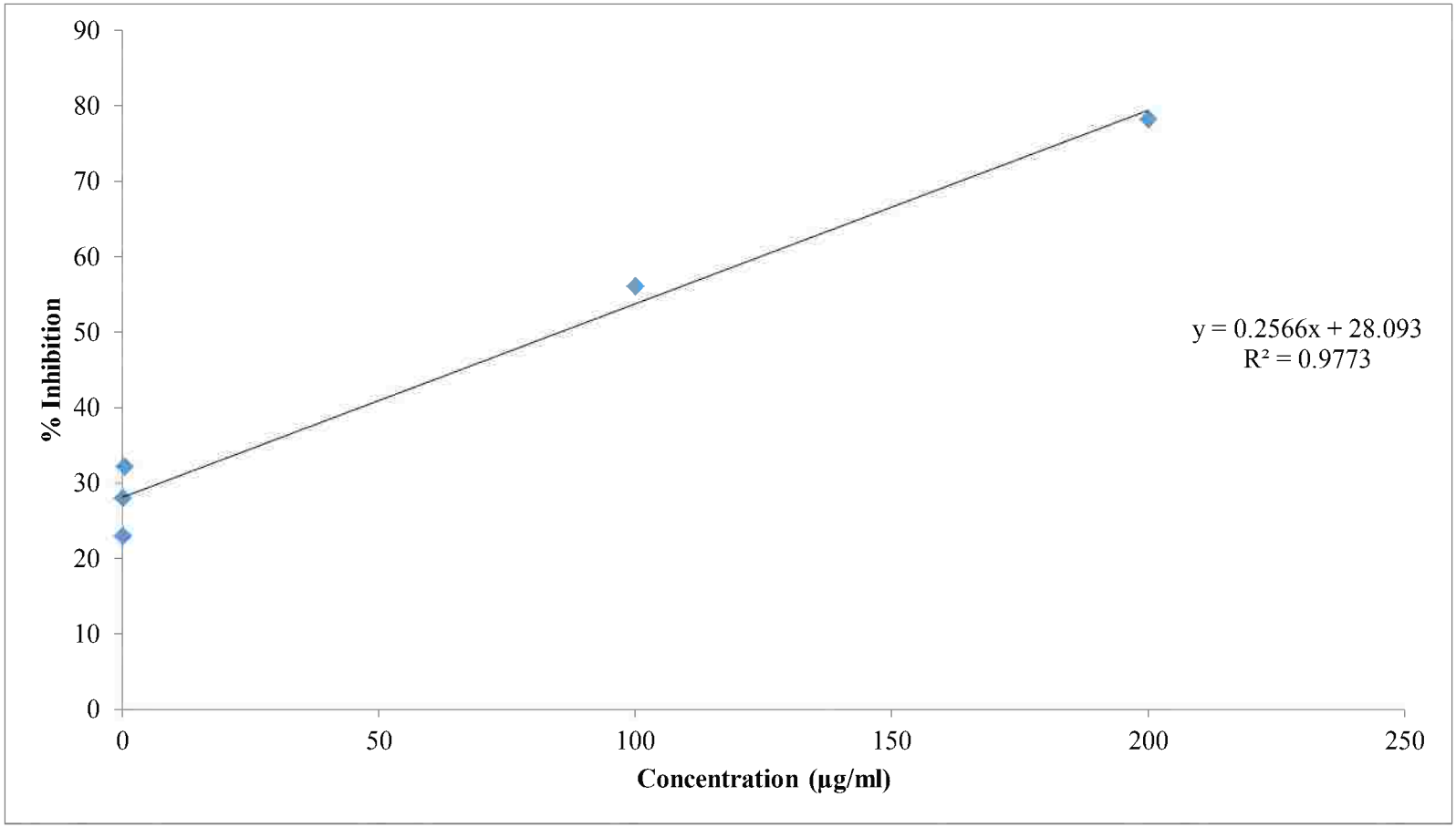

Figure 43: Standard curve (% inhibition versus concentration) of ascorbic acid

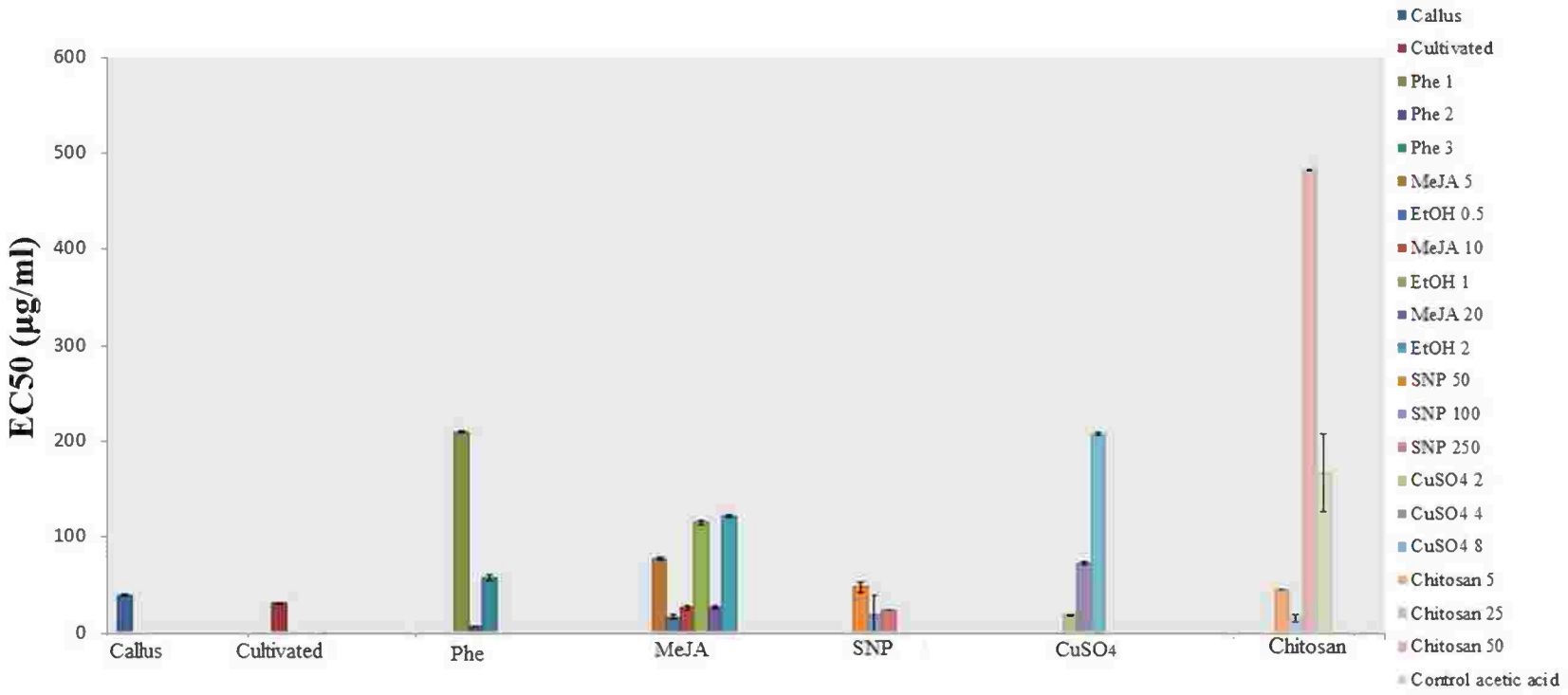

Figure 44: EC_{50} values (expressed in in µg/ml) of ABTS scavenging activity by treated *Echinacea* calli extracts compared with untreated callus and cultivated plant extracts**.**

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

Various phytochemical components, such as flavonoids, phenylpropanoids and phenolic acids, are known to be responsible for the antioxidant capacity of plants. Free radicals can be generated by metabolic pathways within body tissues; also they can be introduced by external sources, with food, drugs, can be caused by environmental pollution etc. Use of natural antioxidants, as food supplements for inactivating free radicals receives a lot of attention nowadays, not only for their scavenging properties, but also because they are natural, non-synthetic products, and their appreciation by consumers is favorable. Free radical scavenging is generally accepted to be the means by which antioxidant compounds inhibit lipid peroxidation. A screening method for antioxidant activity is the ABTS• radical cation decolourization assay. This assay is widely used to assess the antioxidant capacity. The reduction capability of ABTS• was determined by the decrease in its absorbance at 660 nm which is induced by antioxidants. In the present study, the EC_{50} value was used to measure the antioxidant activity of extracts: the lower the EC_{50}, the higher the value of the antioxidant activity. Generally, all the tested E. *purpurea* extracts showed a free radical scavenging activity. It was noticeable that **2 mM Phe, 25mg /L MeJA, EtOH 0.5, 2 µM $CuSO_4$, 100 µM SNP, 250 µM SNP, 10 mg /L MeJA** and **20 mg /L MeJA** treated calli extracts had the strongest capacity to quench ABTS• radicals and the lowest IC_{50} values (the concentration of the extract necessary to decrease ABTS• radical concentration by 50%) which was **7.1, 15.97, 16.72, 18.83, 19.44, 23.63, 26.61** and **27.46 µg/ml** respectively, but the IC_{50} values of these extracts were statistically insignificant to each other and in comparison with the cultivated plant which exhibit an IC_{50} value **30.8748 µg/ml**. Absence of statistical significance between the above listed IC_{50} values means that no one is preferable to the other. Concerning the least radical scavenging capacity, **50 mg/L chitosan** treated calli extracts was the weakest one, with an IC_{50} **483 µg/ml** which is significantly lower than all the other extracts. It is observed that **2 mM Phenylalanine, 10 mg/L MeJA, 2 µM $CuSO_4$, 250 µM SNP** treated calli extracts which bear the highest polyphenolic content **33.2, 40.02, 17.6 and 21.9 µg GAE/2 mg dry extract** possess high antioxidant activity which means that the radical scavenging activity of *Echinacea* extracts reflected their phenolic composition.

It is worth to mention that these findings are better than those previously published by Pellati.[111] who reported that the average EC_{50} values for *E. purpurea*, *E. pallida* and *E. angustifolia* extracts were 134, 167 and 231 µg/ml respectively and better than Stanisavljeviü[93] Findings, in which the IC_{50} value of *Echinacea* extracts was reported to 34.16 µg/ml. In this concern, Rininger[112] discussed free radical scavenging activity of *Echinacea* raw materials and finished products which are typically standardized to contain a minimum of (3–4% phenolics) was highly variable, with EC_{50} values ranging from 23 to 137 mg/ml[112].

- ***In vitro* phagocytic activity assay**

  - **<u>Using polymorphonuclear neutrophils (PMN)</u>**

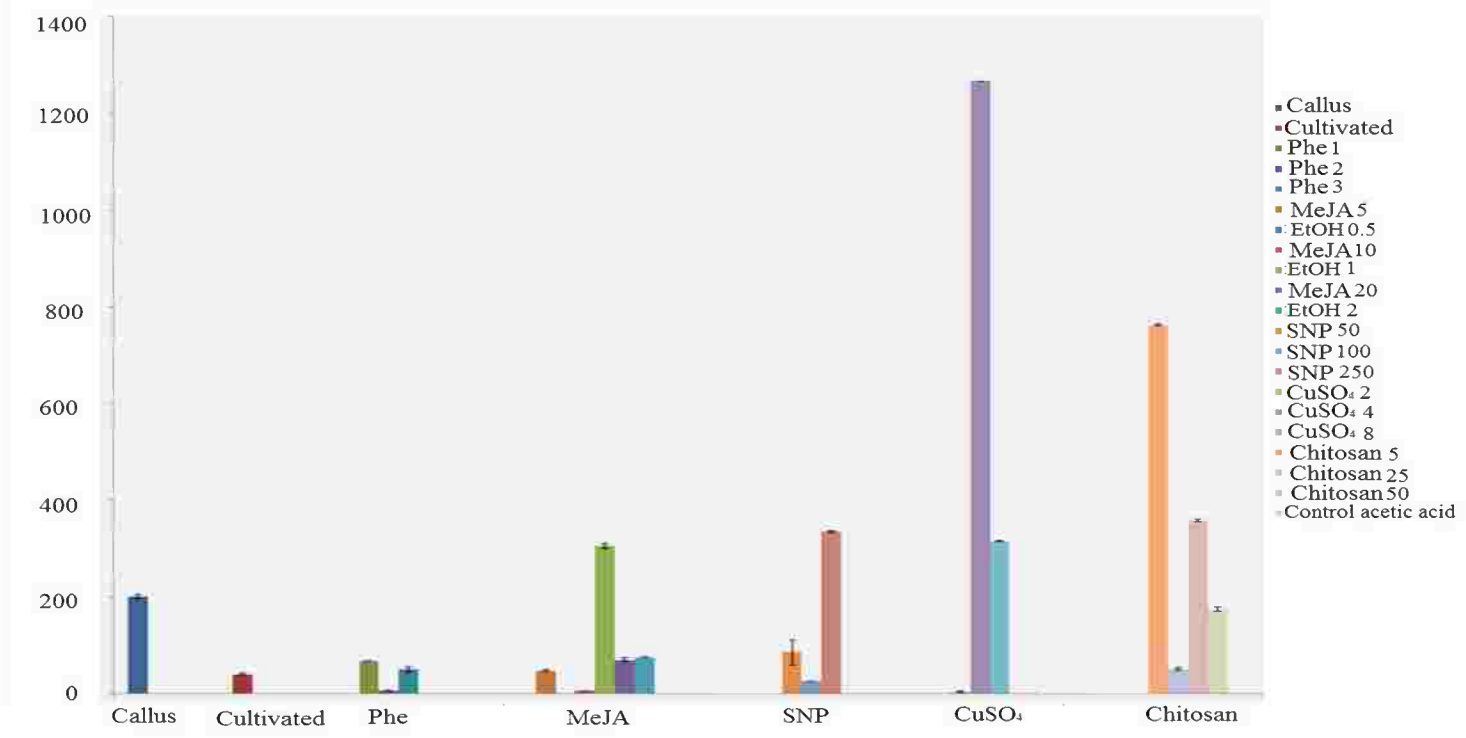

Figure 45: EC_{50} values (expressed in in µg/ml) of neutrophil phagocytosis activity by treated *Echinacea* calli extracts compared with untreated callus and cultivated plant extracts.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

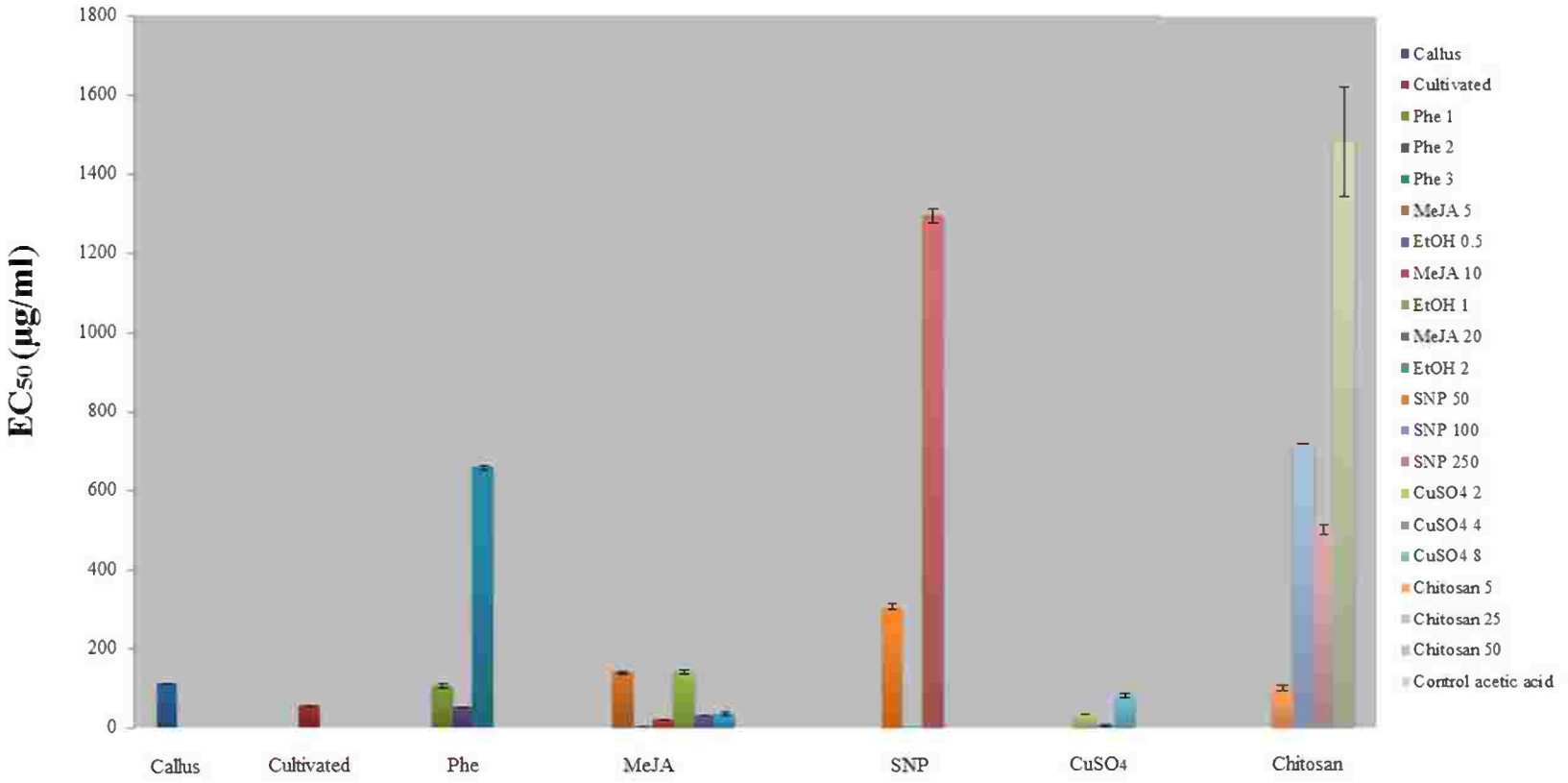

Figure 46: EC_{50} value (expressed in in µg/ml) of neutrophil yeast killing using by *Echinacea* calli extracts in comparison with cultivated plant and untreated calli extracts.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

- **Using peritoneal macrophages (PM)**

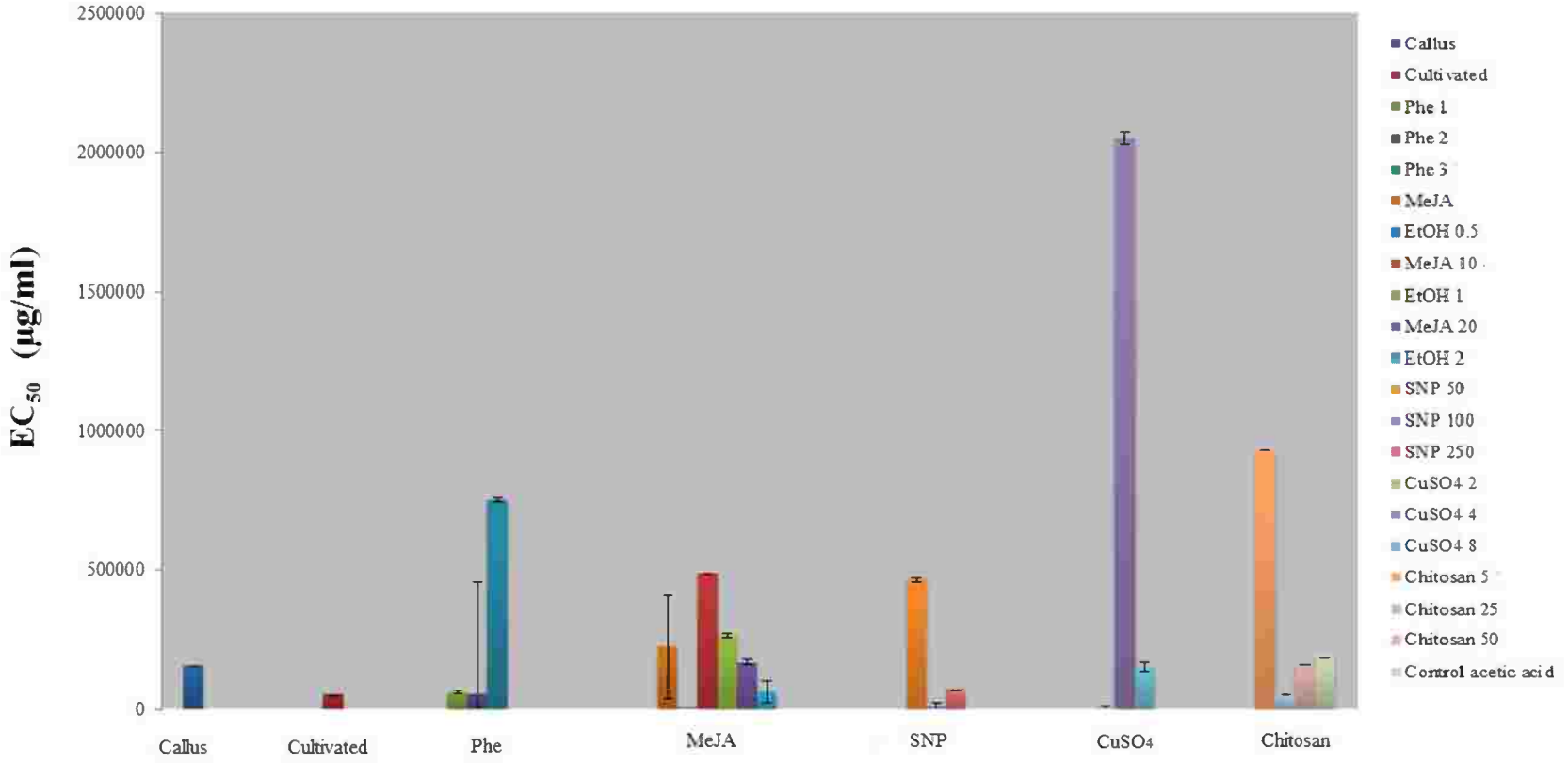

Figure 47: EC_{50} values (expressed in in µg/ml) of macrophage phagocytosis activity by treated *Echinacea purpurea* calli extracts compared with untreated callus and cultivated plant extracts.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

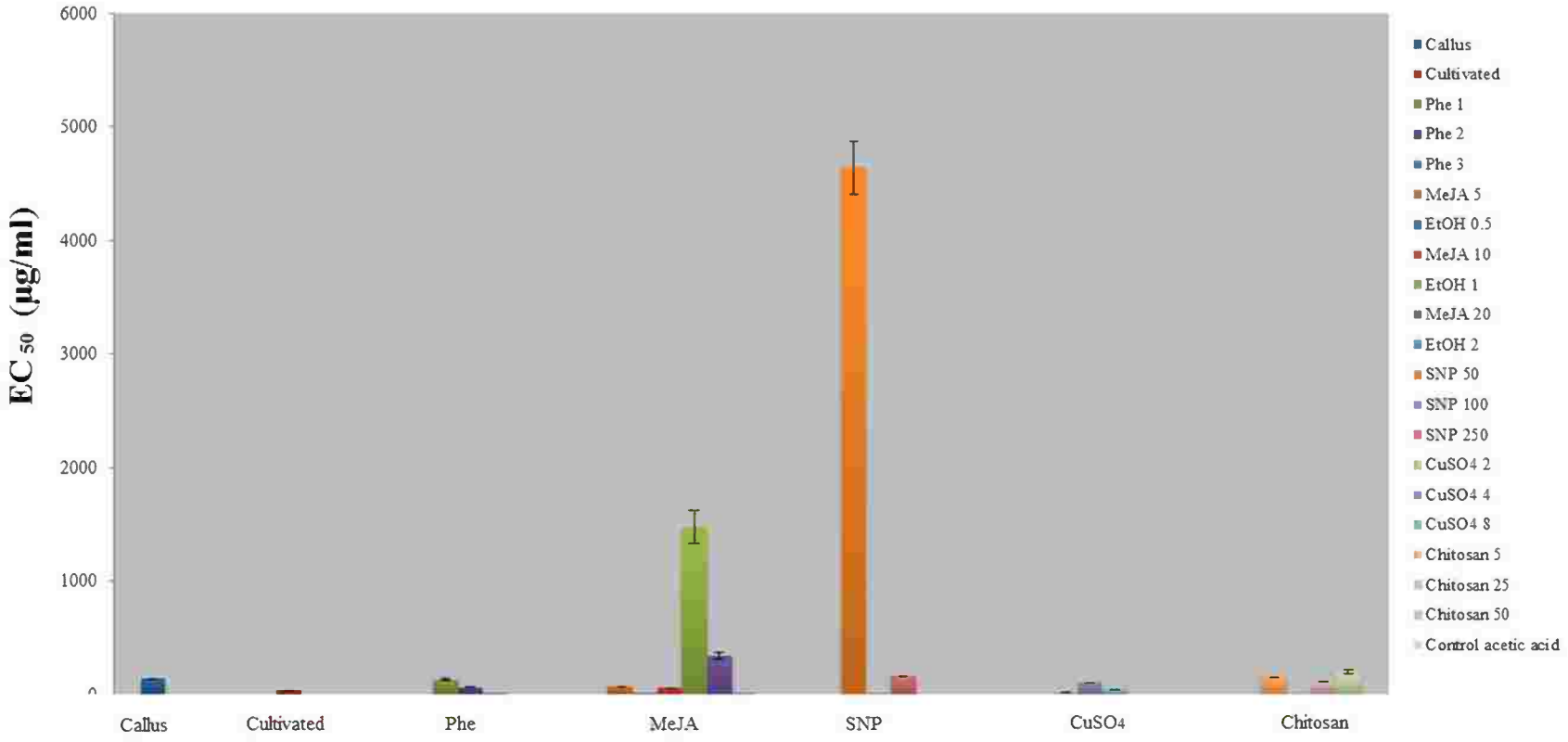

Figure 48: EC_{50} value (expressed in in µg/ml) of macrophage yeast killing using by *Echinacea* calli extracts in comparison with cultivated plant and untreated calli extracts.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

In this study, the phagocytic activity of different *Echinacea* extract was tested for phagocytosis activity and yeast killing activity of macrophages and neutrophils. In **Figure 45**, all tested extracts stimulated phagocytic activity **EtOH 0.5, 2 µM CuSO4, 10 mg/L MeJA** and **2mM Phe** treated calli significantly induced the phagocytic activity of neutrophils with an EC_{50} **1.09, 4.93, 6.2** and **7.7215 µg/ml** respectively compared with the cultivated plant which induced phagocytosis with an EC_{50} 40.98 **µg/ml.** In **Figure 46** all tested extracts stimulated yeast intracellular killing activity by neutrophils, the highest stimulation was achieved by extracts of **2 mM Phe, 10 mg/L MeJA, EtOH 0.5, 2 µM $CuSO_4$, 4 µM $CuSO_4$, 20 mg/L MeJA, 100 µM SNP** and **EtOH 2** treated calli induced killing with an EC_{50} **50.2625**, **22.8230**, **3.27**, **33.76**, **6.92**, **32.47**, **1.85** and **35.8 µg/ml** respectively, while the cultivated plant induced killing with an EC_{50} **56.2915** µg/ml, the previous extracts were statistically insignificant to each other and to the cultivated plant, in this concern these results were better than previously reported in Bauer *et al.*[42] study, as they reported that ethanolic extract obtained from the roots of *E. purpurea* (1:10) in a concentration of 10^{-3}% stimulated phagocytosis of yeast particles by human PMN in vitro by 33%.

In **Figure 47** all tested extracts stimulated phagocytocytic activity by macrophages, extracts from **10 mg/L MeJA, 50 µM SNP, 4 µM $CuSO_4$, 50 mg/L chitosan and 3 mM Phe** treated calli were significantly the lowest with an EC_{50} **487.36, 467.72, 2052.3, 932.97, and 752.54 mg/ml** respectively. In **Figure 48,** all tested extracts stimulated yeast intracellular killing activity by macrophages, extracts from **50 µM SNP, EtOH 1** and **20 mg/L MeJA** treated calli were significantly the lowest in activity with an EC_{50} **4600, 1400** and **339 µg/ml** respectively. Results and findings are in accordance with Zhai[113] who recorded that after incubation with *Echinacea* alcoholic extracts overnight, RAW 264.7 cells were assessed for phagocytosis and bacterial killing after the addition of Salmonella *E. angustifolia* at 100–200 µg/ml enhanced macrophage phagocytosis , but inhibited bacterial killing at 4 hours and 24 hours post-infection while *E. pallida* displayed no significant effects on bacterial phagocytosis, but increased bacterial killing over 24 h incubation at 200 µg/ml. *E. purpurea* had no effect on bacterial phagocytosis, but later decreased bacterial killing. *E. purpurea* at 100–200 µg/ml significantly inhibited bacterial killing 4 h post-infection. At 24 h post-infection, *E. purpurea* at 10 µg/ml also showed an inhibitory effect on bacterial killing [113].

- ***In vitro* immunostimulatory activity demonstrated by human peripheral blood mononuclear cells proliferation**

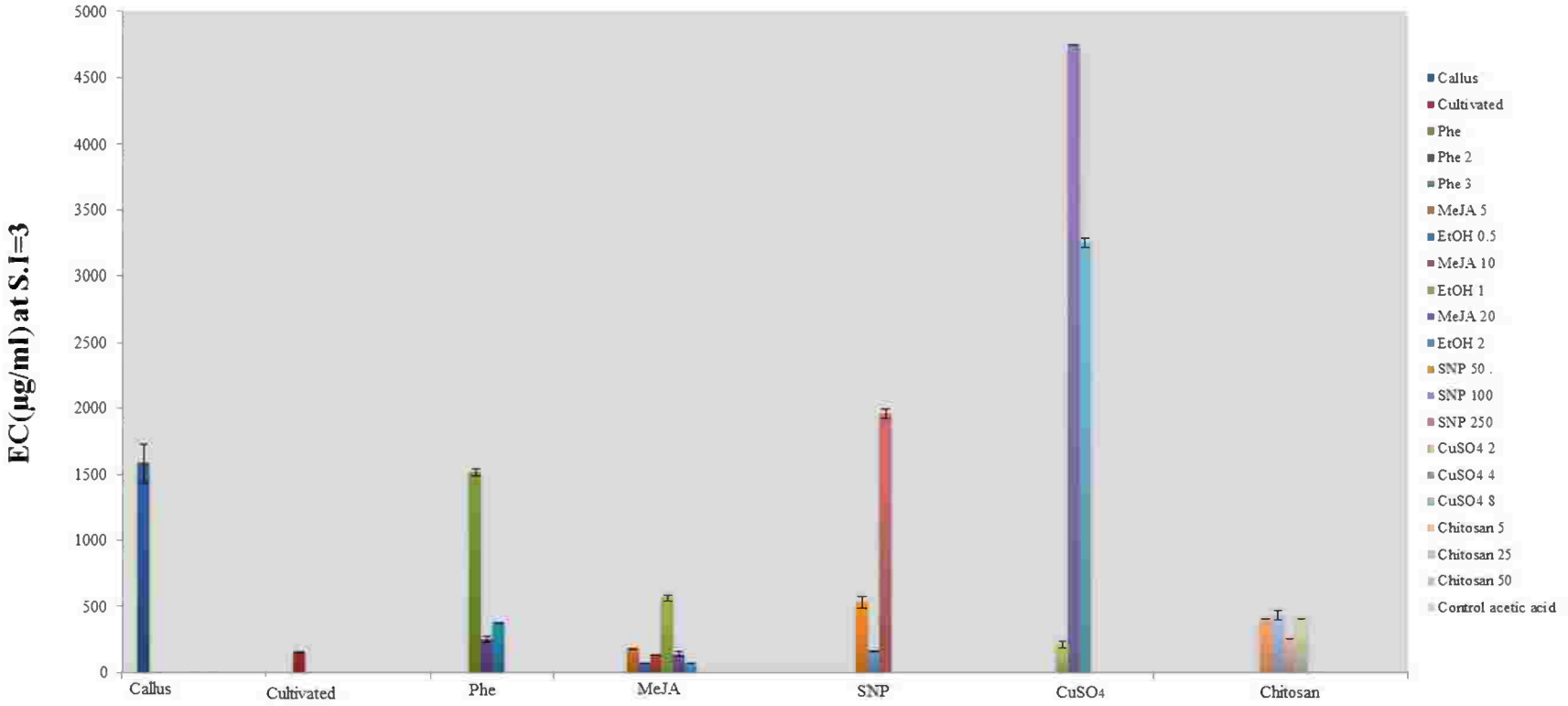

Figure 49: Effective concentration in µg/ml of different *Echinacea* extracts when lymphocyte proliferation was measured at S.I 3.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

Measuring lymphocyte in response to mitogens is a mainstay of screening for cellular immunodeficiency. The efficacy of *Echinacea* extracts to restore the proliferative activity of lymphocytes (300% stimulation) in response to PHA in patients' with attenuated response to mitogen was evaluated. In **Figure 49** all the tested extracts showed immunostimmulant activity and effective concentrations (EC_{50}) were recorded for each extract, all the extracts were significantly lower than the cultivated, even those extracts of **10mg/L MeJA**, **EtOH 0.5**, **20mg/L MeJA** and **EtOH 2** treated calli that exhibit a low EC_{50} **133, 72, 137, 74 µg/ml** are statistically insignificant with the cultivated plant. Chaves[114] reported immunostimmulant activity of **aqueous** extracts of *Echinacea*, the extracts stimulated lymphocyte proliferation 282% when PHA was used at 5 µg/ml with 4 µg/ml *Echinacea* extract and 215% stimulation was obtained when 0.5 µg/ml of PHA and 66 µg/ml of *Echinacea* extract was used[114].

- **In-vitro anti-inflammatory assay**

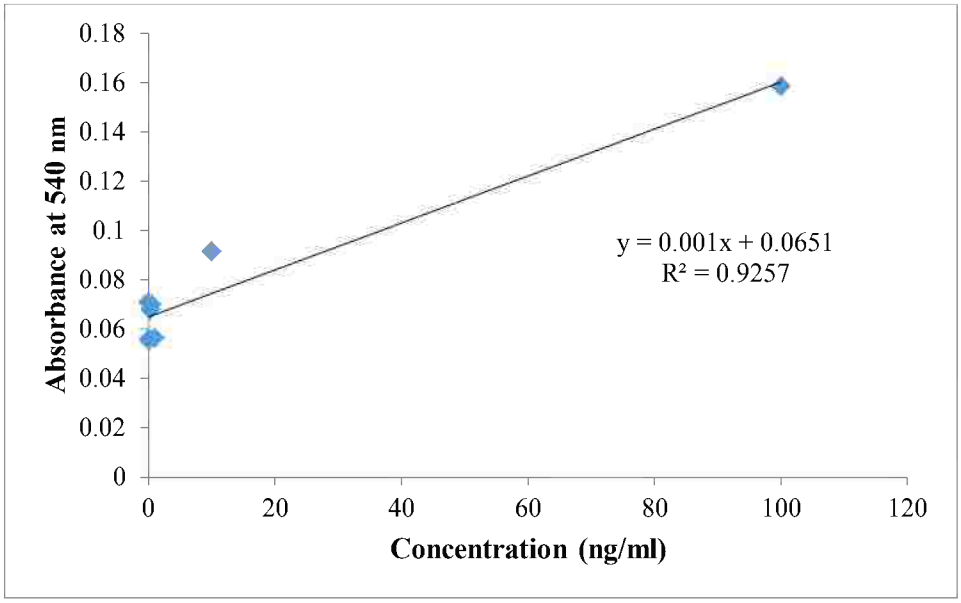

Figure 50: Standard curve (absorbance versus concentration) of sodium nitrite

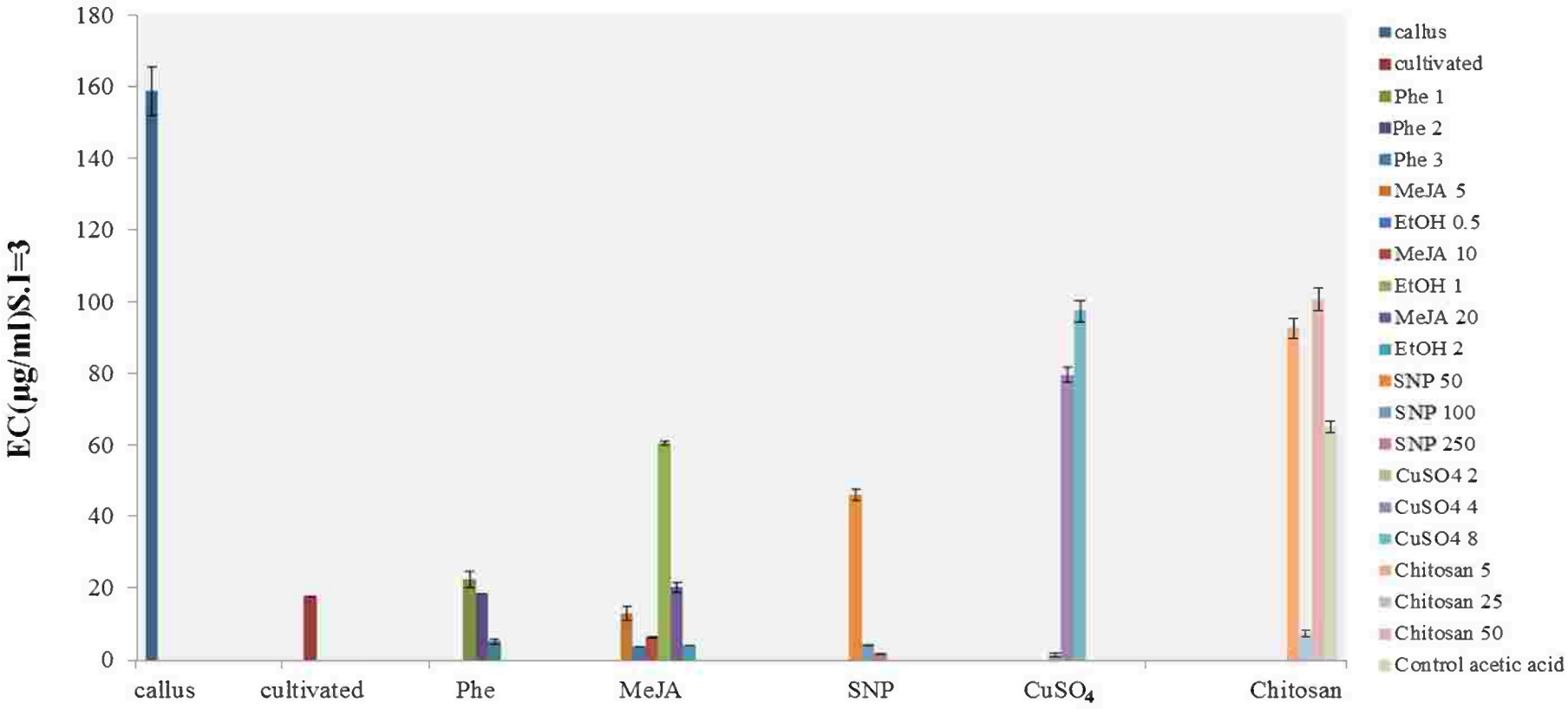

Figure 51: Effective concentration in µg/ml of different *Echinacea* extracts when lymphocyte proliferation was measured at S.I 3.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

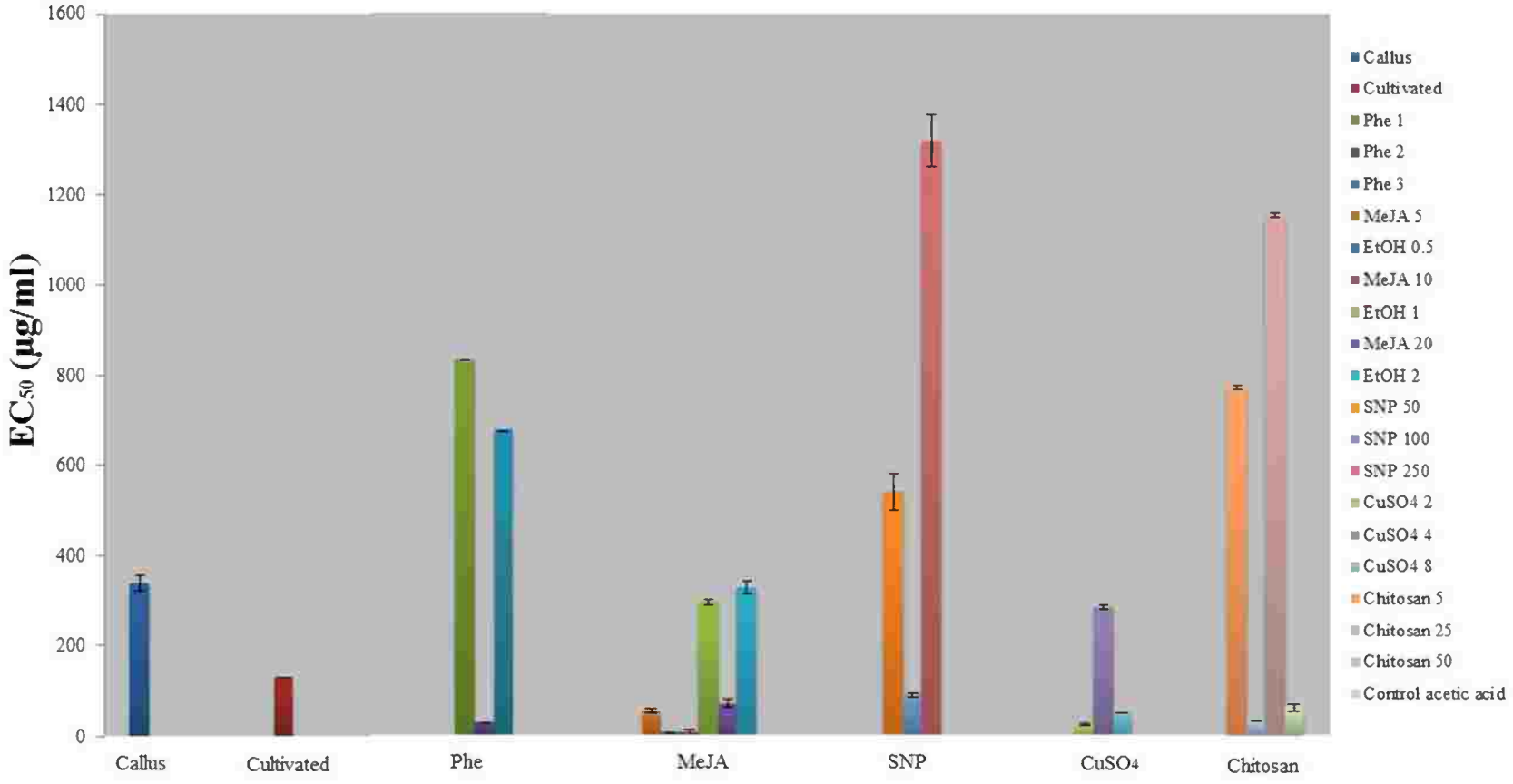

Figure 52: EC_{50} values of NO scavenging activity by treated *Echinacea* calli extracts compared with untreated callus and cultivated plant extracts.

Results were presented as Mean ± SEM of three parallel measurements. Statistical evaluation was carried out by one-way analysis of variance (ANOVA). Statistical significance is expressed as $p < 0.05$

Free radicals and reactive oxygen metabolites trigger and/or amplify inflammation via the up-regulation of expression of a number of genes, including NF-κ_β leading to tissue damage. Taking the above information into consideration, it is conceivable that scavenging of free radicals by appropriate antioxidants might be a useful approach to combating endotoxin-mediated inflammation. Although synthetic antioxidants are widely used, their safety and toxicity issues are a major concern therefore; much attention has been focused on the use of natural antioxidants. A number of studies have shown that many plants and herbal extracts and their products, such as polyphenolic substances, exert potent antioxidant actions. The capacity of *Echinacea* extracts to inhibit the production of nitric oxide (NO) produced in response to LPS stimulated human peripheral blood mononuclear cells (PBMC). Extracts of **10 mg/L MeJA, EtOH 0.5, 2µM $CuSO_4$, 250µM SNP, 3mM Phe, 100µM SNP, 25mg/L Chitosan and EtOH 2** treated calli with EC_{50} **6.3, 3.86, 1.35, 2.02, 5.17, 4.47, 7.33** and **4.13 µg/ml** respectively**,** those extracts were the most powerful inhibitor of lymphproliferative activity induced by LPS as they significantly decreased lipopolysaccharide (LPS) induced inflammation in comparison with the cultivated plant extract that relived inflammation with an EC_{50} 21.3 µg/ml. While **EtOH 0.5, 10mg/L MeJA, 2µM $CuSO_4$, 2mM Phe, 25mg/L Chitosan** and **8µM $CuSO_4$** treated calli possessed the strongest NO scavenging activity with EC_{50} **5.21, 6.37, 22.4, 28.9, 32.7** and **49.98 µg/ml** respectively, results were statistically significant in comparison to the cultivated plant extracts.

These findings were much better than those reported by Zhai[113] who assessed the effect of *Echinacea* alcoholic extracts on NO production, RAW 264.7 cells were exposed to LPS plus alcohol extracts for 24 h. The alcoholic extracts reduced NO production in a dose-dependent manner. In comparison to the vehicle control, *Echinacea pallida* at100 µg/ml significantly decreased the production of NO. When the herbal concentration was raised to200 µg/ml, NO production was significantly inhibited by *Echinacea purpurea*, *Echinacea pallida* and *Echinacea angustifolia* alcoholic extracts. *Echinacea* alcoholic extracts have a potential anti-inflammatory activity, but this effect was mainly observed *in vitro*, especially at relatively high concentrations of the extracts. Increased knowledge of the biological properties and the mode of action of the physiologically relevant concentrations of *Echinacea* alcohol extracts are necessary[113].