7. GENERAL DISCUSSION AND RECOMMENDATIONS

Plant cell, tissue, and organ culture has an inherent capacity to manufacture valuable chemical compounds as the parent plant does in nature. *In vitro* plant materials are one of the good sources for the production of secondary metabolite and elicitation can be used as one of the important tool in order to improve the synthesis of these compounds. The use of elicitor is also important in order to meet the market demands, for reducing production costs and for in-depth investigation of biochemical and metabolic pathways. This information helps us in manipulation of biosynthetic pathways which can be used as a powerful tool to make natural product-like compounds[115]. Recently it has become a popular need to increase production rates of known compounds or to find new natural products for biotechnological application and drug discovery[116]. In our laboratory *Echinacea purpurea* cell cultures have been established for the production of potential bioactive compounds using elicitation techniques.

In our investigation we assumed studying the effect of elicitors (MeJA, Chitosan, $CuSO_4$ and SNP) and one precursor (Phe) on the following:

-Total yield of polyphenols
-Yields of 4 phenolic acids (CADs): Caffeic, Caftaric, Chlorogenic, Cichoric acids

In addition we evaluated the biological activities of different *Echinacea* extracts (cytotoxicity, anti-oxidant, influence on phagocytic activity of neutrophils and macrophage, immunostimmulant and anti-inflammatory activities) and the results of our experimental work revealed that:

- Calli extracts from **10mg/L MeJA** (elicitor) and **2 mM Phe** (precursor) treatments influenced the polyphenolic content in *Echinacea* by **1.86-fold** and **1.5-fold** respectively in comparison with the cultivated plant.

- As far as CADs are concerned in this study no significant effect on the yields of phenolic acids was observed except for extracts from untreated calli tissues which yielded caffeic and chlorogenic acids with 27.6 and 52.16 fold higher than the cultivated plant.

- Obviously in ABTS radical scavenging assay, **2 mM Phe, 25mg /L Chitosan, EtOH 0.5, 2 µM CuSO4, 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, which was **7.1, 15.97, 16.72, 18.83, 19.44, 23.63, 26.61 and 27.46 µg/ml** respectively, but the IC50 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.

- The influence of *Echinacea* on phagocytosis activity and yeast killing activity of macrophages and neutrophils was studied and the following results were obtained:

- Extracts from **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**.
- The highest stimulation of yeast intracellular killing activity by neutrophils was achieved by extracts of **2 mM Phe, 10 mg/L MeJA, EtOH 0.5, 2 µM CuSO4, 4 µM CuSO4, 20 mg/L MeJA, 100 µM SNP and EtOH 2 treated calli** that 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.
- All *Echinacea* extracts stimulated phagocytocytic activity by macrophages, extracts from **10 mg/L MeJA, 50 µM SNP, 4 µM CuSO4, 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.
- All *Echinacea* extracts stimulated yeast intracellular killing activity by macrophages, although the extracts from **EtOH 0.5, 2 µM CuSO4, 3 mM Phe, 100 µM SNP, 25mg/L Chitosan, EtOH 2** and **8 µM CuSO4** treated calli exhibited lower EC_{50} it was statistically insignificant with the EC_{50} of the cultivated plant.

- 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. All EC_{50} of 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 low EC_{50}, are statistically insignificant with the cultivated plant.

- 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 CuSO4, 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 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 CuSO4, 2mM Phe, 25mg/L Chitosan and 8µM CuSO4** 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.

Based on our findings and observations we recommend the following:

- A broader quantitative analysis to be performed to measure the yield of alkamides and polysaccharides present in the treated calli extracts to correlate the levels of the three main constituents (CADs, alkamides and polysaccharides) with *Echinacea* biological

activity, analysis would also involve measurement of the yield of the major three constituents in tissue culture media.

- Further LC/MS/MS analysis of the composition of **10 mg/L MeJA** treated calli extracts is needed to illustrate and explore the detailed differences between the control and treated calli extracts to test for the presence of newly formed compounds and their accumulation, as these compounds may prove to be more potent contributors to the efficacy *Echinacea* extracts.
- Also, a pilot study was carried out to evaluate the biological efficacy of the extracts just to ensure the methods and its feasibility, so launching a larger study can be performed in which more volunteers can be involved which will open the door for more extensive research to discover the exact effect of different components of each extract on immune parameters like signal transduction, cytokines production and cell phenotypes.
- It was clear that upon *in vitro* biological evaluation of differently treated *Echinacea* calli the efficiency and potency of the extracts was not the same, and some (not all) extracts showed more potent activity in comparison with the cultivated plant, so further investigations are needed to involve the effect of several parameters such as elicitor concentration, duration of elicitor exposure, age of culture and nutrient composition in elicited *Echinacea* cell cultures also the effect of more elicitors other than MeJA, CuSO4, chitosan and SNP could be involved in further studies.
- This study focused mainly on the final product without any investigation of the influence of treatments on biomass production, oxidative stress levels and activities of the enzyme involved in plant stress response and overproduction of bioactive compounds, so this would be recommended for future studies.
- Precursor feeding, usually combined with elicitation, is widely and successfully used in *in vitro* cultures for the production of secondary metabolites so this trend would be helpful to explore the combination impact on *Echinacea* active components.

In summary, there is still a need for more research in *Echinacea*, especially on the active components and the mode of action. *Echinacea* will be accepted as a rational drug only on the basis of more valid pharmacological and clinical data, obtained from standardized consistent preparations.