CHAPTER VII

HAEMOLYTIC ACTIVITY OF DERIVED COMPOUNDS FROM GLYPTOPETALUM CALOCARPUM
ABSTRACT

Background: Toxic effect of any drug on the host cells, and in animal model may result in abandoning the study. Toxicity of the active molecule is a key factor during drug designing, and haemolytic activity represents a useful starting point in this regard, as it provides the primary information on the interaction between molecules and biological entities at cellular level. In this study, an attempt was made to determine the cytotoxicity of crude extract and bioactive compounds isolated from *Glyptopetalum calocarpum*, using haemolytic assay method.

Materials and Methods: *In vitro* haemolytic activity was performed by spectrophotometer method. A volume of 0.5 ml of cell suspension was mixed with 0.5 ml of plant crude extracts, and the compounds were derived. 5% SDS and PBS served as positive and negative control respectively, which were incubated for one hour at 37°C. Cell suspensions were centrifuged, supernatants were transferred to a 96-well plate, and the absorbance was read at 492nm.

Results and Discussion: Compounds Lupenone, Stigmasterol, Lupeol, β- amyrin and β-amyrin acetate had negligible or no haemolytic activity, exhibiting IC$_{50}$ values of greater than 5mg/ml. The compound α–Lupene showed very high haemolytic activity, by exhibiting IC$_{50}$ value of 1.04 ± 4.77mg/ml. The IC$_{50}$ value of the crude extract of *Glyptopetalum calocarpum* was 19.26 ± 2.44mg/ml.

Conclusions: High haemolytic activity was observed for the compound, α–Lupene. Further in vivo studies are needed to investigate the pharmacological and toxicological properties of *Glyptopetalum calocarpum* before it can be considered as a new anti-leptospiral agent.

INTRODUCTION

Medicinal plants are the rich source of medicinally important compounds. Since ancient time, plants and plant derived products are used as medicine in traditional and folk medicinal system (Halberstein, 2005). Initially, the herbal drugs were used in the form of dried powder, gums, extracts or formulations from more than one plant product (Bhattarai et al., 2010). Advanced scientific techniques brought a revaluation in herbal medicine industry, and all focus was concentrated on the active principles present in plants (bioactive molecule). However, a cumbersome processing procedure is required, in order to develop a drug from the natural sources.
Cytotoxicity is the cell-killing property of a chemical compound (such as food, cosmetics or pharmaceuticals), or a mediator cell (such as a cytotoxic T cell), independent from the mechanisms of death. Toxicity studies play a pivotal role in herbal medicine. Toxic effects of any drug on the host cells and in the animal model may result in abandoning the study. The objective of toxicological studies is to establish a dose-reaction relationship. Instead of searching for the mechanism of toxicity, it is important to determine the drug dosage effective to the target organism, but harmless to mammalian cells or body. Toxicity can be studied by different physiological, morphological and biochemical examination. There are various methods used for the determination of cytotoxicity in vitro, such as brine shrimp, lactate dehydrogenase (LDH) assay and colorimetric assays like haemolytic assay (Ali et al., 2013; Decker and Lohmann-matthes, 1988; Nachlas et al., 1960; Riaz et al. 2012).

Toxicity of the active molecule is a key factor during drug designing, and haemolytic activity represents a useful starting point in this regard, as it provides the primary information on the interaction between molecules and biological entities at cellular level. Haemolytic activity of any compound is an indicator of general cytotoxicity towards normal healthy cells (Silva et al., 2004). Usually, saponins (a group of phytochemical) present in the plants show haemolytic activity by creating changes in the erythrocyte membrane. In vitro haemolytic assay by spectroscopy method provides an easy and effective method for the quantitative measurement of haemolysis. This method provides, an evaluation of the effect of different concentrations of biomolecules on human erythrocytes at different concentrations.

In this study, an attempt was made to determine the cytotoxicity of crude extract and the bioactive compounds, which were isolated from *Glyptopetalum calocarpum* (Kurz.) Prain, using haemolytic assay. Although this plant is a well-known as a potent medicinal plant in the Nicobarese community, their effects on Human Red Blood Corpuscles (HRBCs) was evaluated.

**MATERIALS AND METHODS**

The haemolytic activity of the methanol crude extract and six derived compounds were assayed with heparinized Human Red Blood Corpuscles.
**Preparation of erythrocyte suspension**

Five millilitres of blood was collected from a healthy individual (blood group O +ve) in a tube containing heparin. The blood was centrifuged at 1500 rpm for three minutes. Plasma (supernatant) was discarded, and the pellet was washed three times with sterile phosphate buffer saline solution (pH 7.2±0.2) by centrifugation at 1500 rpm for 5 min. The cells were resuspended in normal saline to obtain a final concentration of 0.5%.

**Haemolytic activity**

*In vitro* haemolytic activity was performed by spectrophotometer method (Yang et al., 2005). A volume of 0.5 ml of the cell suspension was mixed with 0.5 ml of the plant crude extracts (156.25, 312.5, 625, 1250, 2500 and 5000μg/ml concentrations in phosphate buffer saline) and the derived compounds (31.25, 62.5, 125, 250, 500 and 1000μg/ml concentrations in phosphate buffer saline). 5% SDS and PBS served as positive and negative control respectively. After 1hr of incubation at 37°C in an incubator, cell suspensions were centrifuged for 10 minutes at 1500 x g, and the supernatants were transferred to a flat bottom 96-well plate. The absorbance (A) was read at 492nm by ELISA reader (Tecon). Since the crude extract of the plant was coloured, separate blank was prepared for each concentration of the extract. For preparation of blanks, plant extracts were taken in separate tubes without blood, treated in the same manner as that of the tests. Each experiment was performed in triplicate for each concentration. The percentage haemolysis was calculated using the formula,

\[
\text{Haemolysis (\% of control) = } \frac{(A_{TS} - A_{SC})}{(A_{WC} - A_{SC})} \times 100
\]

Here: \(A_{TS}\) is the absorbance of test sample.

\(A_{SC}\) is absorbance of the control (saline control)

\(A_{WC}\) is the absorbance of the control (water control)

**Statistical Analysis**

All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). Results were analysed statistically using Microsoft Excel 2013. Graphs were plotted using Prism 5.
RESULTS AND DISCUSSION

The IC$_{50}$ values of methanol crude extract and six pure compounds isolated from *Glyptopetalum calocarpum* are shown in graphs (Figure 7.1 – 7.7). The crude extract and isolated compounds were evaluated *in vitro* for their haemolytic activity against the human red blood corpuscles. The highest concentration tested for the crude extract was 5000μg/ml, and for the derived compounds it was 1000μg/ml. The results estimated from the spectrometer readings indicated that the crude extract and the compounds, *viz.*, Lupenone, Stigmasterol, Lupeol, β- amyrin and β- amyrin acetate have negligible or no haemolytic activity, exhibiting IC$_{50}$ values of greater than 5mg/ml. The compound α–Lupene showed the highest haemolytic activity, by exhibiting IC$_{50}$ value of 1.04 ± 4.77mg/ml. The IC$_{50}$ value of the crude extract of *Glyptopetalum calocarpum* was 19.26 ± 2.44mg/ml. Among the isolated compounds, β- amyrin acetate seemed to show the least haemolytic activity.

**Figure 7.1:** Haemolytic activity of methanol crude extract of *Glyptopetalum calocarpum*

IC$_{50}$ value (mg/ml ± SD) of 19.26 ± 2.44 mg/ml

Where (mg/ml ± SD) = values are means ± standard deviation.
**Figure 7.2:** Haemolytic activity of derived compound Lupenone

IC$_{50}$ value (mg/ml ± SD) of 13.51 ± 0.36 mg/ml

![Graph showing haemolytic activity of Lupenone](image)

**Figure 7.3:** Haemolytic activity of derived compound Stigmasterol

IC$_{50}$ value (mg/ml ± SD) of 9.78 ± 0.56 mg/ml

![Graph showing haemolytic activity of Stigmasterol](image)
**Figure 7.4:** Haemolytic activity of derived compound α–Lupene

IC$_{50}$ value (mg/ml ± SD) of 1.04 ± 4.77 mg/ml

![Graph showing haemolytic activity of α–Lupene](image)

**Figure 7.5:** Haemolytic activity of derived compound Lupeol

IC$_{50}$ value (mg/ml ± SD) of 9.65 ± 0.44 mg/ml

![Graph showing haemolytic activity of Lupeol](image)
**Figure 7.6:** Haemolytic activity of derived compound β- amyrin

IC$_{50}$ value (mg/ml ± SD) of 7.55 ± 0.47 mg/ml

**Figure 7.7:** Haemolytic activity of derived compound β- amyrin acetate

IC$_{50}$ value (mg/ml ± SD) of 28.57 ± 0.54 mg/ml
CONCLUSIONS

Further studies, including in vivo experiments and toxicity tests are necessary to gain a full understanding of the effectiveness and possible toxic nature of these compounds.