CHAPTER 4

4.1. Motivations for this study

Cancer is the second leading cause of death in many nations after cardiovascular diseases in the world. Chemoprevention or chemotherapy via natural based agents could be one approach for decreasing the incidence of different type of cancers. Despite the discovery of numerous natural and synthetic anticancer agents, attempts for sighting of an effective anticancer agent with low toxicity effects on healthy cells, high efficacy against multiple cancers, and low cost have been absorbed many attentions.

For centuries people have used plants as medicine or food additives with varying success to cure and prevent diseases. Written records about medicinal plants date back at least 5,000 years to the Sumerians. According to World Health Organization (WHO) around 80% of the population in developing countries is dependent on herbal medicine for basic healthcare needs. Even at the dawn of the twenty-first century, 11% of the 252 drugs considered as basic and essential by WHO were exclusively of flowering plant origin.

The selected plants for present investigation were indigenous to South Africa. Only a few studies were found on the biological activities of *Hyaenanche globosa*. Genus *Maytenus* has been studied intensively in different countries while astonishingly; literature review demonstrated a gap of biological index about *M. procumbens* in between. Thus, we were encouraged to evaluate the new possible biological activities from these species with a particular emphasis on their antiproliferative properties.

4.2. BIOLOGICAL ACTIVITIES OF *HYAENANCHE GLOBOSA*

Ethnobotany and traditional usage of *Hyaenache globosa* Lamb. (Euphorbeaceae) led to the selection of this plant to explore its possible cytotoxicity effects. This genus contains several toxic sesquiterpene lactones, such as, tutin, mellitoxin, urushiol III, and isodihydrohyaenanchine (Van Wyk *et al.,* 1997).

Phytochemical studies of the ethanol extract of the fruits of *H. globosa* (F.E) resulted in isolation of two known pure sesquiterpene lactones; 'tutin **1**' and 'hyenanchin **2**'. The crude extract (F.E) and its isolated constituents were tested on four cancerous and a normal cell lines. F.E exhibited the highest antiproliferative activity on HeLa cells which followed by Caco-2 cells. None of the isolated compounds (**1** and **2**) were found to be toxic to the cells tested in this experiment.

Antioxidant/pro-oxidant activities of F.E, 'tutin **1**' and 'hyenanchin **2**' were determined extracellular (DPPH radical scavenging method) and intracellulary (in cultured HeLa cells) via three different methods; FRAP, TBARS and ROS assays. F.E demonstrated potent inhibition of DPPH radical activity similar to vitamin C (positive control). 'Tutin **1**' and 'hyenanchin **2**' were found with marginal antioxidant activity of which 'compound **1**' presented more potent than 'compound **2**'. As data characterized, F.E, 'tutin **1**' and 'hyenanchin **2**' enhanced FRAP values in HeLa cells at higher concentrations tested compared to non-treated cells. None of samples were capable to enhance lipid peroxidation in HeLa cells, significantly. Nevertheless, the amounts of ROS radicals formed by pure compounds (**1** and **2**) were not significantly higher than those of controls. However, F.E elevated the fluorescence intensity of DCF at the highest concentration tested (400 µg/ml).

The crude extract of *H. globosa* showed the highest antibacterial activity of 1 mg/ml against Gram-positive bacteria (*B. subtilis* and *S. aureus*) and the lowest of 8 mg/ml against Gram-negative bacteria (*P. aeruginosa*). Only 'tutin **1**' showed inhibitory activity exhibiting MICs of 400 and 800 µg/ml for *S. aureus* and *P. aeruginosa,* respectively.

Compound **2** did not show any significant growth inhibition of microorganisms tested. None of pure compounds inhibited the growth of fungi tested in this study.

In summary, in spite of our great expectation about the toxicity of pure compounds isolated from the ethanolic extract of the fruits of *H*. *globosa* ('tutin **1**' and 'hyenanchin **2**'), they did not show any significant cytotoxic effects on the examined cancer cell lines, while the crude extract was well known for its poisonous effects. The poisonous effect of this plant could be due to the activity of the compounds that were not isolated yet. Moreover, it has been proven the toxicity effects of sesquiterpene lactones are dose dependent. Although the data are still inconclusive and further scientific attempts are needed to confirm the traditional information or to investigate the novel medicinal aspects of this plant. A further study aims to determine the anticancer properties of other major constituents of *H*. *globosa*, as well as identify the unknown compounds is required to fully understand its bioactivity.

4.3. BIOLOGICAL ACTIVITIES OF *MAYTENUS PROCUMBENS*

Biochemical studies of the acetonic/ethanolic extract of the leaves of *M. procumbens* (L.M.P) resulted in isolation of two new triterpenes namely; '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'. In addition, a known terpene was isolated which identified as 'asiatic acid **4**'. Due to the unavailability of sufficient amount of 'asiatic acid **4**', this compound was not tested.

M. procumbens (L.M.P) exhibited the highest inhibition of cells growth on HeLa cells among tested cell lines. The reduction of proliferation was followed in Caco-2, T47D and HT29, though L.M.P showed cytotoxicity against normal NIH3T3 cells. Both pure compounds (**3** and **5)** showed cytotoxicity of all experimental cancer cell lines. They also appeared toxic to the normal NIH3T3 cells. The cytotoxicity of triterpenes have been proven frequently thus confirm our findings well.

Following the MTT assays, the induction of apoptosis by pure compounds (at the concentration of their IC_{50}) were investigated in HeLa cells. The affinity of the isolated compounds for Annexin V and PI were determined through microscopic and flow cytometric analysis. These compounds induced apoptosis in HeLa cells at their IC_{50} concentrations. Significant elevation of DNA damage in concept of tail moment was detected in cultured human HeLa cells by both compounds. 'Compound **3**' presented more apoptotic and genotoxic than 'compound **5**' *in vitro*.

To see whether induction of apoptosis by compounds **3** and **5** in HeLa cells depends on their prooxidant/antioxidant properties; RSC, FRAP, TBARS and ROS assays were utilized. L.M.P and its isolated constituents exhibited marginal antioxidant properties as compared to vitamin C. There were significant elevations of ferrous content by L.M.P and pure compounds in HeLa cells. The experimental samples were unable to increase HeLa cells TBARS significantly at any concentration tested as compared to those of controls. The ROS intensity of HeLa cells was elevated significantly by L.M.P and the isolated compounds compared to control cells ($P< 0.05$).

The moderate to weak antioxidant potential of L.M.P and the isolated compounds might be a logical explanation for enhancement of ROS levels *in vitro*. Therefore, ROS generation might be a part of the mechanisms by which these compounds induce apoptosis in HeLa cells. However, L.M.P, compounds **3** and **5** induced ROS generation dose and time-dependently in the HeLa cellular environment. Thus, the active components in L.M.P might serve as a mediator of the reactive oxygen scavenging system and have the potential to act as a prooxidant and an antioxidant, depending on the biological environment of the cells. Such a dual-property role for antioxidants has also been reported previously (Turley *et al.,* 1997; Yang *et al.,* 2006; Zou *et al.,* 2001).
This study proved compounds **3** and **5** were capable to induce apoptosis at their IC_{50} concentrations in HeLa cells as evidenced by DNA staining (PI), and plasma membrane permeability (Annexin V binding assay). In addition to genetical changes (as proved by comet assay) and the participation of ROS in mediating apoptosis induced by

compounds **3** and **5**, other pathways may also be involved. The elucidation of these mechanisms by which these samples induce apoptosis in different cancer cells will be helpful for better understanding new apoptotic signaling pathways and will benefit the clinical application of them in the prevention and treatment of cancer.

The present study revealed a new biological index of the acetonic/ethanolic extract of the leaves of *Maytenus procumbens*. This project resulted in isolation of two new triterpenes from L.M.P for the first time. There is no report until date on phytochemical index, anticancer, antioxidant and antibacterial properties of the acetonic/ethanolic extract of the leaves of *Maytenus procumbens* (L.M.P), '30-hydroxy-11α-hydroxyl-18β-olean-12-en-3-one **3**' and '30-hydroxy-11α-methoxy-18β-olean-12-en-3-one **5**'.

4.4. FUTURE PERSPECTIVES

Regarding the novelty of the isolated compounds, there are limited information about their medicinal properties except from those reported in this study. Wide spectrum of biological activities of triterpenes have been recognized such as bactericidal, fungicidal, antiviral, cytotoxic, analgetic, anticancer, spermicidal, cardiovascular, antiallergic, and so on. In the next step, the other biological aspects of this species should be explored.

Ovarian, epidermoid carcinoma, melanoma, and leukemic cell lines could be considered as next candidates for future cytotoxic assays. Several triterpenoids, including ursolic acid, oleanolic acid, betulinic acid, celastrol, pristimerin, lupeol, and avicins possess antitumor property and are evaluated for their cytotoxicity in mammalian cancer models *in vivo.* Therefore, the elucidation of the mechanisms by which the crude extract and pure compounds induce apoptosis in different cancer cells will be helpful for better understanding new apoptotic signaling pathways and will benefit the clinical application of them in the prevention, and treatment of cancer.

A proposed mechanism to explain the anticancer actions of these compounds might be mitochondrial swelling, which together with changes in the mitochondrial potential and

release of proapoptogenic proteins leads to the death of transformed cells. Further studies are required to understand the effect of different functional group substitutions and the mode of inhibition of cell proliferation by purified compounds. These compounds might be worth considering as new anticancer agents alone or in combination with other antiproliferative drugs.

Despite the large number of molecules exhibiting anti-cancer properties *in vitro*, only some of them are able to induce an effective antiproliferation effect measurable in clinical trials. This gap between *in vitro* and *in vivo* studies suggests that new strategies are needed for discovering new anticancer drugs and validating their efficacy and safety. The combination of two or more agents acting on different mechanisms to produce a synergistic anticancer effect should be considered.

4.5. REFERENCES

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