Chapter 5

Discussion

Skin cancer has become an international epidemic with an alarming incidence rate which has steadily increased over the past few decades (Cancer Research UK, 2011). The rate of melanoma had doubled over many years (Chin et al., 1998) and it is the 5\textsuperscript{th} most common cancer among men and the 6\textsuperscript{th} most common cancer among women in United States (National Institute Cancer Facts, 2008). To date, an effective therapy is yet to emerge from the medical field for treatment of skin cancers. The drugs that have been approved by FDA to treat metastatic melanoma have showed very low response rates and have been largely unsuccessful (Middleton et al., 2000).

According to Cragg and Newman (2005), plant-derived compounds have played an important role in the development of several clinically useful anticancer agents. In recent years, plant polyphenols have gained a lot of importance because of their potential use as prophylactic and therapeutic agents in many diseases including cancers (Norman et al., 2003). Various plant secondary metabolites such as flavonoids, flavonols, anthraquinones, sesquiterpene lactone, triterpene, triterpenoid, etc., have also showed immense potentials in anticancer research (Zahin et al., 2010). Vincristine, irinotecan, etoposide and paclitaxel are classic examples of plant-derived compounds used to treat cancer. Taxanes, anthracyclines, vinca alkaloids, camptothecins, epothilones and their derivatives have been developed and exhibited promising effects in various \textit{in vitro} and \textit{in vivo} studies (Boulikas and Tsogas, 2008).

However, the search from natural sources still continued to find an attractive chemopreventive agent in skin cancer treatment. Hence the present study investigated the properties of four plant-derived compounds including parthenolide (PN), a sesquiterpene lactone; betulinic acid (BA), a pentacyclic triterpene; luteolin (LUT), a flavonoid; oleanic acid (OA), a triterpenoid and vitamin D\textsubscript{3} (positive control) to elucidate their mechanism of action in skin cancer management. Though preliminary studies showed the potentials of these selected compounds in various cancer model systems, their efficacy in skin cancer still remains unclear.
ROS had been widely implicated in development and progression of skin cancers (Waris and Ahsan, 2006). Increased levels of ROS could result in stress which in turn might induce various biological responses in skin (Davies, 1999). In biological systems especially in epidermis, during the cellular respiration, ROS like hydroxyl radical (OH), superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are generated, as a natural consequence of oxidation reactions (Tarnawski et al., 2005). These higher levels of ROS can damage the living cells causing lipid, protein and DNA oxidation (Shukla et al., 2010). Antioxidant compounds scavenge these radicals and there by prevent cell and tissue damages (Raha and Robinson, 2000). Large number of plant-derived compounds has been studied as new sources of natural antioxidants which showed the potentials to mitigate oxidative stress induced by ROS in the cells (Muanda et al., 2009).

Several methods have been developed to quantify the antioxidant activity of these compounds individually. Based on the involvement of chemical reaction they are divided into two basic categories, (i) hydrogen atom transfer reaction (HAT) and (ii) single electron transfer (ET) reaction based system. These techniques are different in terms of mechanism of reaction, effectiveness and sensitivity (Khal and Hilderbrant, 1986; Frankel, 1993; Koleva et al., 2002). Consequently, single method/assay is not sufficient to completely evaluate the antioxidant capacity of plant pure compounds (Ozgen et al., 2006). Hence, in the present study, DPPH and FRAP assays which are considered as direct, rapid and simple method to estimate the antioxidant activity of the plant compounds have been employed.

The compounds selected in this study showed minimal to moderate DPPH$^*$ scavenging activities with comparison to ascorbic acid (standard), except for LUT with a lower IC$_{50}$ value of 84.8 µM. However, FRAP assay showed a non-significant response for LUT with IC$_{50} > 200$ µM. This might be because of the differential scavenging activities of LUT against DPPH$^*$ and Fe$^{+3}$ radicals, where such differences in mechanisms of the radical-antioxidant reactions have been reported previously (George et al., 2012). The relatively lesser polarity of LUT in comparison with its sugar derivatives, which showed more antioxidant potentials, may also be the reason for its observed non-significant FRAP activity (Romanova et al., 2001; Xu et al., 2009; Ozgen et al., 2011). Moreover, flavonoids are well known group of plant
compounds which were reported earlier for their ability to scavenge DPPH free radicals when compare to other class of compounds (Hirano et al., 2001; Khanduja and Bhardwaj, 2003).

However, OA, BA and PN had failed to demonstrate significant antioxidant potential when compared to the standard. According to Wei et al. (2010) and Watson, (2013), most of the anticancer drugs act either by scavenging the free radicals or inducing cell death mechanisms. Secon et al. (2010) and Olayinka et al. (2012) have also stated that anticancer compounds accomplished their effects either by scavenging excess free radicals with minimum cytotoxicity or with significant apoptotic potentials and minimum antioxidant capacities. This suggested that these two parameters might be inversely correlated and may not appear in parallel. BA, OA and PN were previously reported for their significant cytotoxic potentials in various cell lines and proved to possess cell death inducing potentials (Parada-Turska et al., 2007; Feng et al., 2009; Faujan et al., 2010), which might be the reason for the observed non-significant levels of their radical scavenging potentials in the present study.

In vitro cell culture techniques has become one of the major tools used in cell and molecular biology for drug screening and development. Hence for the current study two cell line models, HaCaT (human immortalized keratinocytes) and A375 (human melanoma) were employed to evaluate the potentials of the selected plant compounds. HaCaT cell system has been commonly used for studying skin alterations and related diseases (Lippens et al., 2004; Lloyd et al., 2009) and also been utilized as a model system by many investigators to study the normal skin maturation process by inducing expression of caspase-14 using agents like EGCG and Vitamin D$_3$ (Lippens et al., 2004; Hsu et al., 2007a). Similarly, the A375 melanoma cells are widely used by many researchers for studying melanoma skin cancer for decades (Okazawa et al., 1998; Fei et al., 2002; Kumar and Ansari, 2012). Chung et al. (2011) further reported a close and important functional association between keratinocytes and melanocytes. Moreover, caspase-14 expression was observed only in terminally differentiated cells that all were a part of epidermal layer of the skin. Hence, the selection of the above mentioned cell systems for the current study is justified.
The most important characteristic feature of a chemotherapeutic drug is the ability of it to exterminate cancer cells (Mooney, 2005; Kumar et al., 2011). Hence, it was imperative to assess the cytotoxicity of these compounds prior to testing their proficiency as drugs against skin cancer. Cytotoxicity of these compounds was tested using XTT assay which is considered as a simple, reliable and efficient method to determine cell viability and/or cytotoxic properties of crude biological materials as well as purified chemical substances (Williams et al., 2003).

In the current study, LUT exhibited significant cytotoxicity with IC$_{50}$ values of 37.17 µM and 115.1 µM in HaCaT and A375 cells respectively. Conversely, it is also evident that LUT had the ability to inhibit cell proliferation as reported by other investigators in many other cancer cell lines (Kang et al., 2010; Wang et al., 2010). BA showed significant cytotoxic levels with IC$_{50}$ values of 23.02 µM and 97.1 µM in HaCaT and A375 cells respectively. Galgon et al. (2005) also demonstrated the cytotoxic effects of BA in HaCaT cells. Keratinocytes (HaCaT cells) was found to be much more sensitive than melanoma (A375 cells) when exposed to LUT and BA. This might be either by the differential mechanism of LUT and BA towards the metastatic melanoma (Krishnamurthy and Maly, 2010), or by the phosphorylation of signal transducer and activator of transcription (STAT) -3 and -5 in melanoma cells (Miimohammadsadesh et al., 2006).

PN was found to be the most cytotoxic among the other compounds tested with very low IC$_{50}$ values of 1.45 µM and 2.9 µM in HaCaT and A375 cells respectively. Several studies had already demonstrated the high cytotoxic nature of this sesquiterpene lactone in various cell lines with IC$_{50}$ values of 0.5 µg/ml in A549 cells, 4.3 µg/ml in TE671 and 2.8 µg/ml in HUVEC cells (Jolanta et al., 2007). All these reports were consistent with the observed effects of PN in the present study. Interestingly OA did not show significant cytotoxicity in both the cells. Its IC$_{50}$ values were found to be non-significant with 1424 µM and 277.5 µM in HaCaT and A375 cells respectively. Further, similar results were observed by Liu, (2005) using the same cell line. However, OA has been proved to be cytotoxic in other cell lines such as A-549, HCT-8 and MCF-7 (Ko et al., 1999; Jie et al., 2002; Fu et al., 2005; Fecker et al., 2005).
Cytotoxicity of a chemotherapeutic drug often induces alterations in cellular morphologies which may result in cell death by activation of various upstream/downstream elements (proteins) in apoptotic pathways (Ricci and Zong, 2006). Researchers used various fluorescence staining methods to detect the morphological changes occurred during this event in the cells. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) can be scored in the dying cells by DAPI staining (Radha and Padma, 2011). The presence of nuclear apoptotic bodies and chromatin margination can also be observed after DAPI staining and was thus employed in this study.

The degree of damage to the nucleus caused by the compounds was assessed and found to be high in PN exposed cells (HaCaT and A375). Similar type of observation for the effects of PN was also reported by Liu et al. (2009) and Chun et al. (2013). Other compounds (LUT, OA and BA) also exhibited significant levels of damage to the nuclear components in both HaCaT and A375 cells. Nuclear damage showed by DAPI staining suggested the possibility of cell death through apoptotic induction. Hence these results further extended to investigate the apoptotic induction potentials of the compounds in both HaCaT and A375 cells.

Recent literature reports have indicated that many anticancer drugs/chemopreventive agents act through the induction of apoptosis to prevent tumor promotion/progression (Kumar et al., 2012). Apoptotic induction has been a new target for innovative mechanisms-based drug discovery (Senderowicz et al., 2004; Rajkumar et al., 2011; Huang et al., 2012). It was also considered as a protective mechanism against development and progression of cancer (Wang et al., 2012). Naturally occurring plant compounds were already reported to induce apoptosis and target cancer cells towards death (Fulda, 2008; Ali et al., 2011; Deng et al., 2011). Hence the potential of the compounds selected in this study, to induce apoptosis in both HaCaT and A375 cells were investigated.

All the compounds in the present study showed significant (p<0.05) DNA fragmentation potentials in a dose-dependent manner in HaCaT and A375 cells. The results obtained for LUT substantiate the apoptotic potentials of this flavonoid in HaCaT and A375 cells which has not been reported earlier and corroborates with the
findings of others in various cell lines (Chiand et al., 2007; Kim et al., 2012). Similarly OA, BA and PN showed higher apoptotic potentials in HaCaT cells but the effects were limited in A375 cells at identical dosages. Differential level of DNA fragments observed in both cells at similar concentrations of compounds could possibly be due to the over expression of extracellular signal-regulated kinase (ERK)1/2 in A375 cells (miimohammadsadesh et al., 2007). Interestingly, OA at lower concentrations (12.5, 24 and 50 µM) had exhibited moderate DNA fragmentation and cytotoxicity when compared with the higher (almost two fold) cell death at elevated doses (100 and 200 µM). Similar results were previously reported in human skin fibroblast cells (Wojciak-Kosior et al., 2011). The observed apoptotic potentials of BA in HaCaT cells also correlate with that reported by Galgon et al. (2005). When taken together, these compounds possessed significant apoptotic abilities in these cells and hence subjected for further analysis.

The most desirable approach to treat cancer would be to identify the signalling molecules that induce growth arrest and trigger terminal differentiation of skin cancerous cells. Cell cycle arrest is one of the common effects shared by many anti-cancer drugs. Consternation of cell cycle progression could cause severe damage to cells and may trigger apoptosis (Voland et al., 2006). Intensive research have shown that cell cycle-mediated apoptosis can be considered as a novel way to address various problems like multi-drug resistance, decreased mutagenesis and reduced toxicity (Katsman et al., 2009; Kogel et al., 2010). To date, many chemotherapeutic agents have been shown to impart anti-proliferative effects via arrest of cell division at certain check points in the cell cycle (Adhami et al., 2004).

Consequently, the present study scrutinized the compounds for their potential to arrest/block cell cycle progression in both HaCaT and A375 cells. The results revealed that LUT was able to arrest the cell cycle in HaCaT and A375 cells by accumulating the cell populations in G2/M & G0/G1 phase respectively and subsequent reduction of cells in other phases. Incidentally, similar results of LUT have been reported earlier (Chang et al., 2005), in immortalized human hepatoma cell lines. In another study by Lim et al. (2007), LUT was able to arrest cell cycle in G2/M phase by the down regulation of cyclin B1 expression and inducing apoptosis in HT-29 human colon cancer cells at concentrations from 20 to 60 µM. Various other
studies have also demonstrated the potential of this flavonoid to inhibit cell growth by arresting the cells at various phases of cell cycle (Plaumann et al., 1996; Leung et al., 2005; Fang et al., 2007). Similarly, BA also demonstrated its potential to arrest cell cycle at G2/M phase in A375 cells. This result correlates with the observations made by Jianwen et al. (2009) and Santos et al. (2011). However, BA in HaCaT cells did not show any cell cycle arrest. In another study, Yang et al. (2010) showed the potential of BA in arresting human gastric adenocarcinoma (AGS) cells at G2/M phase by down regulating cyclin B1. PN also exhibited significant cell cycle arresting potentials at S & G0/G1 phase respectively in HaCaT and A375 cells respectively. Similarly, Guang and Xie. (2011) demonstrated its potential to arrest cell cycle at G1 phase in human 5637 bladder cancer cells by modulating cyclin D1 and phosphorylated cyclin-dependent kinase 2. OA was not studied for its potential to arrest cell cycle since it showed lesser cytotoxic levels in both the cells.

Caspases are known to be the downstream effector molecules irrespective of the upstream and signal transduction players involved. Till date, many plant compounds were involved in induction of apoptosis through activation of several caspases (Khan et al., 2006; Yusuf et al., 2007; Zhang et al., 2009; Chilampalli et al., 2010).

The apoptotic cell death pathway in skin cells was found to be different than other organs in the body. Therefore, the molecular elements involved in skin cancer remains distinctive and termed as specialized apoptosis (Kokileva, 1994). Keratinocytes can die by two different processes: apoptotic cell death induced by damaging agents such as UV-B, chemicals and cytotoxic cytokines or by a continuous process of differentiation known as terminal differentiation, leading to the formation of corneocytes (Denecker et al., 2008). But the complete cellular mechanism involved in skin cancer still remains unclear (Yang et al., 2013).

A recently discovered caspase (caspase-14), is found to be essential for normal skin development which is expressed suprabasally in normal human epidermal keratinocytes (Eckhart, 2000). Unlike other caspases, caspase-14 is also a part of the family of cysteine-aspartic acid proteases which is non-apoptotic in nature but involved in terminal differentiation, a process essential for the skin cells to enter apoptotic pathway (Hu et al., 1998; Rendl et al., 2002).
Hence, this study further investigated the potentials of the selected compounds in the context of caspase-14 expression in HaCaT and A375 cells. The quantitative assessment (ELISA) suggested that only LUT was found to have significant (P<0.05) potential to induce caspase-14 in HaCaT cells. All other compounds failed to demonstrate its ability to induce caspase-14 in HaCaT cells and have not been studied further. However, LUT was not able to induce the expression of caspase-14 in A375 cells. This was because A375 cells lack the caspase-14 gene in its genome and the result was also supported by many investigators who suggested the mode of apoptotic cell death in A375 cells through caspase-3 mediated pathway (Hsiao et al., 2012; Wang et al., 2012).

Vitamin D$_3$ was used as a positive control for inducing caspase-14 which when treated, have decreased the psoriatic phenotype and increased the levels of caspase-14 in skin lesions (Lippens et al., 2004). Pistritto et al. (2002) have also showed that when the keratinocytes are treated with vitamin D$_3$ in vitro, it forced these cells to differentiate by the induction of caspase-14 expressions. The effect of LUT was thus compared with vitamin D$_3$ in order to find out its effectiveness in induction of caspase-14. LUT was able to induce 3.19 ng/ml of caspase-14 protein in HaCaT cells when compared to the positive control vitamin D$_3$ which had only showed 1.29 ng/ml of caspase-14 protein levels at identical dosages of 80 µM. This data thus revealed the efficacy of LUT for the first time, in inducing caspase-14 expressions in human keratinocytes (HaCaT) with almost three folds higher potentials than reported activity for vitamin D$_3$.

Further, the study investigated the potency of LUT in HaCaT cells in relation to its caspase-14 induction abilities through RT-PCR (reverse transcriptase) methods, a confirmatory proof-of-principal approach. This method was successfully employed by many investigators for studying caspase-14 expression in skin lesions as well as in skin cells (Eckhart et al., 2000; Pistritto et al., 2002; Hsu et al., 2004).

PCR screening of cDNA derived from HaCaT cells revealed that caspase-14 mRNA was expressed at significant levels in LUT treated cells but not in control (untreated) cells. The less intensive bands observed for vitamin D$_3$ treated cells probably suggested a lower abundance of caspase-14 expression in HaCaT cells when
compare to LUT treated cells. This result corroborate with the results of quantitative
assessment of caspase-14 expression. Similar results were also reported by Eckhart et
al. (2000). Thus this study further confirmed the potential of LUT in inducing
caspase-14 expression in HaCaT cells.

So far, many investigators studied the expression of caspase-14 and its
association with skin cancer. Hsu et al. (2007) showed that green tea polyphenols,
particularly epigallocatechin-3-gallate (EGCG) provoked the expression of caspase-
14 in A431 cells that reduced tumorigenicity of skin cancer cells. EGCG was also
reported to induce caspase-14 expression in NHEK (normal human epidermal
also demonstrated the effects of green tea polyphenols in epidermal keratinocyte cells
by inducing differentiation with coordinated expression of p57/KIP2 and caspase-14
in NHEK. However, the exact role of p57/KIP2 in association with caspase-14 left
undiscovered. However, till date the molecular mechanism involving upstream and
downstream molecules that regulates the expression of caspase-14 in skin remains
unknown.

Conversely, a series of elegant experiments proved that induction of caspase-
14 at the transcriptional level improves the formation of stratum corneum, whereas its
diminished level was found to be associated with poor cell differentiation (Eckhart et
al., 2000a; Rendl et al., 2002). From these studies it was clear that caspase-14 had
specifically modulated epidermal differentiation, possibly by inducing terminal
differentiation and cornification of the epidermis (Mikolajczyk et al., 2004).

Induction of terminal differentiation may lead to the eventual elimination of
tumorigenic cells and rebalance of normal cellular homeostasis. Therefore, the present
study also investigated the potential of LUT towards inducing terminal differentiation by
the expression of caspase-14 in HaCaT cells by studying the human involucrin, a
potential marker exclusively seen in terminally differentiated cells, employing RT-PCR
method (Woodcock-Mitchell et al., 1982; Candi et al., 2002; Balasubramanian et al.,
2005). The results revealed the high expression of human involucrin gene when compare
to its diminished expression in untreated cells (control). This clearly indicated that LUT
has the ability to trigger terminal differentiation process in HaCaT cells.
The findings of the above study clearly demonstrated the promising potentials of LUT for the first time, to induce terminal differentiation through caspase-14 activation and consequent apoptosis induction in immortalized human keratinocytes (HaCaT cells) and thereby could possibly play an important role in reducing skin tumors. Thus LUT had provided a substantial reason to develop it as an efficient and safer chemotherapeutic drug against human skin cancers.