CHAPTER V

DISCUSSION AND CONCLUSIONS

The analytical method for catechin assay in the green tea extracts using HPLC was validated according to USP 26. The parameters were accuracy, precision, linearity and specificity as shown in Table 28 (Appendix B). The values for % recovery were between 98-102%, for %RSD were less than 2, for R^2 were more than 0.995 between the range that covered assay concentration and no peak overlapped. Therefore it concluded that the developed method is suitable for quantitative analysis of cathechins in green tea extracts.

Green tea extracts, used in this study, are better than dried leaves in terms of convenience to handle, keep stock and assay the active contents. Green tea polyphenols have been shown to be very stable in acidic solutions (Zhu et al., 1997). Citric acid was used in the extraction method because it is safe and widely contained in food.

Less EGCG , ECG and more EGC, EC content was obtained from extraction with distilled water than the other solvents. This might be because in some of the EGCG and ECG gallate side chains were lost, thus EGCG changed to EGC and ECG changed to EC.

The extraction method was focused on the amount of EGCG retrieved because the most active antioxidant in green tea is EGCG (Salah et al. 1995) ..The EGCG content of extraction with distilled water was significantly different ($p= 0.05$) from extraction with citrate buffer p H 3, 3.5, 4 and 4.5; while there was no significant difference of content in the group of extraction with citrate buffer. For high yields of EGCG, the demonstrated extraction method of choice, is with citrate buffer at p H 3.0-4.50. Green tea extract from citrate buffer pH 4.5 extraction was chosen for this study because the results (Table 5) with this extraction buffer gave high yields of EGCG and EGC and was close to skin pH.

Keratinocytes, melanocytes and, melanocyte/ keratinocyte co-cultures could be prepared from epidermis by using selective media. Melanocytes were rounded up and detached in trypsin solution before keratinocytes, thus with correct timing they could be separated from the co-culture. Fibroblasts could be prepared from dermis and subcultured to expand the population for more than 2 months without changing morphology. Melanocytes after 1 month, lose dendrites; the nucleus and cell membrane of keratinocytes were indistinct, these cells might lose activity. It was determined that the melanocyte/ keratinocyte co-cultures, and monolayer of keratinocytes or melanocytes should be used within 1 month.

Large amounts of melanocytes were found in the cultures prepared from dark skin. In this study, dark skins were selected to prepare melanocytes and keratinocyte / melanocyte coculture. To reduce the inherent amount of melanocytes white skins were used to prepare keratinocyte cultures.

Within the first few days the fibroblasts in the collagen rafts contracted resulting in a dense raft. Epidermal cells in keratinocyte growth medium when seeded onto the rafts attached well and proliferated to form a monolayer. After 10 days; DMEM containing FBS was added to the level of the raft surface, so that the keratinocytes were grown at the air - liquid interface; this condition allowed cell differentiation forming a differentiated multicell culture. H/E stained sections of the 3D skin, showed a small amount of living cells (dark nucleus) under a thick stratum corneum layer. This gives a 3D model with similar cell content and diversity as found in normal skin.

GTE and EGCG showed similar cytological effects: dose dependent inhibited proliferation of L929, NHFM, NHFF cells and stimulated proliferation of NHFK cells. Doses below 90 μg/ml of GTE and 60 μg/ml of EGCG showed no effect and are considered the safe threshold doses.

It has been shown that melanocyte growth is complex phenomenon involving regulation of keratinocytes (Luga *et al.* 1994). Melanocyte/keratinocyte co-culture has been used in the model system for testing the compounds that might affect melanocyte and/or keratinocyte function (Lei *et al.* 2002).

Inhibition of melanin synthesis study by GTE on melanocyte / keratinocyte co-cultures has been shown. From this study, qualitative analysis by Dopa reaction (Figure 32, Figure 33 and Figure 34) and quantitative analysis by measurement of OD (Figure 36) show that compared to controls GTE inhibits melanin synthesis on melanocyte / keratinocyte co-cultures but less than kojic acid.

The dose of UVB 400 mj was used to study 3D skin model and excised skin because GTE.(60 μg/ml), EGCG (30 μg/ml) and Ectoin (4 mM) could protect cell (keratinocyte) death while at 600 mj these samples could not protect.

A limitation of this study was the lack of newborn foreskin. This was overcome through the use of a skin model prepared from excised human breast skin. The results showed that quality of this skin was not as good as the skin prepared from newborn foreskin (Figure 30 and Figure 39) and sunburn cells could not be identified. However, different morphologies could be identified between these skin models (Figure 39, Figure 40, Figure 41 and Figure 42). UVB irradiated untreated skin model showed that both epidermis and raft were damaged (Figure 40). GTE could prevent the damage of raft (Figure 42) while Ectoin could prevent both epidermis and raft (Figure 41).

The study on fresh excised human breast skin less than 24 h after surgery gave effectively and good results. There is a marked increase in sunburn cells in the irradiated unprotected excised skin, this is reduced when GTE is present, and more markedly when Ectoin acid is present. Therefore fresh excised human breast skin might be a model of choice for UV protection study.

Green tea extracted with citrate buffer (pH 4.5) has been shown with *in vitro* technology to be an effective UVB protectant. An efficient extraction method has been devised to give good overall yields of the catechins of choice. Safety parameters of dose have been defined. The effects of GTE on UV protection have been shown on irradiated skin samples, both *in vitro* skin models and excised skin, to be similar to *in vivo* effects. GTE inhibited melanin production (Figure 37) in cell cultures and mimicked the growth stimulation of keratinocytes observed *in vivo* (Figure 42). The results from this work confirm the utility of using *in vitro* systems to assess the activity of GTE and specific catechins in their roles as UVB protectants.