V. SUMMARY

Chronic diseases like cancer require a large investment in therapy that often is only palliative. Prevention and attempts to stay healthy upto an old age should be thus the agenda of future scientific pursuits, considering that the life span of baby boomer generation has increased. Introduction of antioxidant therapies into mainstream medicine is one of the possible strategies which hold promise for such an approach. The medical significance of oxidative stress is being increasingly recognized such that it is now considered to be a component of virtually every disease process. As free radical/oxidative stress research enters the 21st century, we face the challenge of transferring our nascent knowledge of oxidative pathology from the laboratory into the clinic and the pharmacy. New therapeutic strategies, can, and will be developed which rationally incorporate antioxidants into the management of chronic diseases.

The agents tested in the present study were direct scavengers of the entire range of ROS (including superoxide anions, hydrogen peroxide, hydroxyl radicals, nitric oxide and lipid peroxides) against which their activities were evaluated. In general, catechin and epicatechin showed significantly higher activities except for nitric oxide scavenging (weak nitric oxide scavenging activity); closely followed by quercetin and rutin. Sesamol and NDGA though less active, showed a significantly higher activity than vitamin C and vitamin E respectively. However in case of hydrogen peroxide scavenging, NDGA showed a maximum effect with IC$_{50}$ = 32.68 nmoles and the nitric oxide scavenging achieved both by NDGA and sesamol was significantly better than catechin and epicatechin. Since, the antioxidant action also depends on the type free radical or oxidant being used in the assay, hence it may be said that a variation in the extent of scavenging achieved against different free radicals and the variation in sequence of activity of these agents in different test systems depends upon the type free radical being scavenged.

Since some flavonoids/polyphenolics are reported to autooxidize in chemical systems and accelerate hydroxyl radical formation at high concentrations (25-100 μM) indicative of a pro-oxidant effect (Sergediene et al., 1999). So we usually selected a lower concentration range with atleast 5-6 dilutions showing a linear response between concentration and
percentage scavenging to confirm the absence of any pro-oxidant effect in the dose range used. The higher activity observed by us for catechins could probably be because of a suitable dose selection in the lower range.

Another interesting feature of the study is a better lipid peroxidation activity in liver microsomes, shown by both vitamin E and C in comparison to the other molecules tested except for epicatechin and both epicatechin and catechin respectively. A better activity shown by vitamin E could probably be because of its high lipophilicity which may make it more interactive with the biomembranes. While the natural presence of ascorbic acid in biomembranes and the aqueous cytosol may help in the better uptake of ascorbic acid from the surrounding aqueous medium, it has a high solubility also. Green tea extract and ginseng extracts were also evaluated and their activities were compared with ascorbic acid. Invariably for all ROS scavenging, green tea extract showed a better or atleast an activity comparable to ascorbic acid while ginseng extract showed a several times lower activity.

Based on this observation, we feel that eventhough these polyphenolics are better antioxidants but due to certain limitations on their permeability and hence bioavailability they may not show the same efficiency in ex vivo and hence in vivo systems. Hence it was our proposal to evaluate the physicochemical properties of these agents and evaluate the scope of developing suitable delivery systems for these agents to overcome the limitations of their physicochemical nature e.g., aqueous solubility, log P etc.

Further, the antimutagenicity studies performed in the TA102 strain of S. typhimurium using the Ames test indicated that the in vitro antioxidant activity can be extended to in vivo systems as shown by the good correlations between the antioxidant and the antimutagenic activities exhibited by these agents. It may however be observed that the difference in relative activity between these agents though sometimes not apparent in the in vitro system was magnified several fold in the in vivo systems. In vitro scavenging can thus be used as a preliminary test for selecting potent antioxidants. This was one of the important findings of the study.
Summary

Analysis of the results of antioxidant and antimutagenicity studies showed catechin to be the most active agent, but catechin has been extensively studied for its anticancer activity and its formulations are also developed (Carlson and Thompson, 1998; Dorothy et al., 2002; Lambert and Yang, 2003). Although the flavonoids quercetin and rutin show good in vitro activity, but the reports on their prooxidant activity (Gaspar et al., 1994) discouraged us. Further, the antimutagenic activity of these two agents did not show good dose activity correlations. Sesamol and NDGA hitherto less explored agents (only a handful (Avis et al., 1996; Kapadia et al., 2002; Nakamura et al., 2003) of reports being available for the anticancer studies activity of these agents) showed good antimutagenic activities. The in vitro antioxidant activity of sesamol was found to be better than vitamin C and NDGA showed a better activity than vitamin E. Hence these agents were selected for further studies.

The developed HPLC method described herein for both the drugs is simple and rapid. The short retention times indicate that a single analysis can be completed within 5 min for each drug. So the method can be used to process large number of samples and at the same time detect low concentrations of drugs in plasma or tissue samples (LOD-10 and 20 ng/ml for sesamol and NDGA respectively). This was probably possible due to the selection of an isocratic solvent system. A retention time of 22 min is reported for NDGA with the mobile phase which was a 30 min gradient from water and acetonitrile (80:20) to a 20:80 system, in plasma of mice (Lambert et al., 2002). The drug peak did not interfere with any plasma or other homogenate artifacts. The method was accurate and precise as evident from low percentage R.S.D. values in recovery studies and inter- and intra-day variations. In our method, direct deproteinization of plasma gave an extraction efficiency of more than 98%, which circumvents not only the need for an internal standard but also a multistage extraction procedure. It also augments the accuracy of the method and helps to reduce the time and money involved in sample preparation. The inherent property of sesamol and NDGA and their high molar extinction coefficient at 294nm and 290nm also impart a high sensitivity to the assay procedure.

We may thus claim that the developed HPLC method of analysis is simple, reproducible, selective and rapid. Furthermore, the method has been successfully
employed, as discussed later (Part IV), in the study of pharmacokinetics of these agents in the rat animal model by both i.v. and oral routes of administration. The study can be extended for further investigation of disposition of these agents in humans also. The UV method was also validated for linearity, accuracy and precision and was used for the analysis of diffusion and dissolution samples of the drugs and their formulations.

Only drugs with appropriate physical properties for absorption can be used effectively for pharmacodynamic effects. A certain extent of water solubility and an optimum lipophilicity is required for passive diffusion through membrane barriers. Four parameters namely molecular weight, log P, the number of H-bond donors and acceptors are thought to be associated with solubility and permeability. The ‘rule of five’ proposed by Lipinski and his colleagues states that poor absorption or permeation are more likely when there are more than five H-bond donors; the molecular weight is over 500; the log P is over 5; and there are more than 10 H-bond acceptors (Lipinski et al., 2001). Identifying potential drug molecules among the hundreds of lead molecules and getting a safe and more efficacious drug molecule to the therapeutic arena is the goal of drug discovery process. The salient findings of the physicochemical and gastrointestinal permeability characteristics of sesamol and NDGA are: Sesamol shows good aqueous solubility (~38.8 mg/mL), with a pKa of 9.79. It has a partition coefficient of 1.29 and distribution coefficient >1. The distribution coefficient decreases as the pH increases. Sesamol is well absorbed throughout the GIT, especially the stomach (~85% absorption). NDGA shows a low solubility (45 ± 0.06 µg/mL) at pH<7. The determined pKa value of NDGA is 9.14. The n-octanol-water partition coefficient is found to be 1.72 and the distribution coefficient did not change with pH of the buffer (log D varies from 1.20-1.29). It is better absorbed from ileum and poorly absorbed from stomach and proximal parts of the intestine. A suitable log P (< 5.0), molecular weight (< 500) and solubility ensures these molecules to have a good permeability and bioavailability confirmed by gastrointestinal permeability studies in rats as per the Lipinski’s rule of five and as also.

The floating beads of sesamol and NDGA were prepared successfully with desired profiles by using sodium alginate and HPMC as controlled release polymers and calcium
carbonate as a gas forming agent. To provide an effective local action from a gastroretentive system, ideally it would be required to release significant amount of drug in initial stages to obtain the desired concentration and then more slowly to replace the drug lost by gastric emptying. The mechanism of drug release from the floating beads seems to be diffusion controlled. The study clearly shows that the amount of gas forming agent has a profound effect on floating ability, release rate and mechanical strength of beads. Also, varying the proportion of polymer composition influenced the release profile of drugs. These floating beads were found to be suitable for oral controlled and localized delivery of sesamol and NDGA.

A high flux of almost $38.92 \pm 0.62 \mu g/cm^2/h$ shown by sesamol solution indicates it to be a highly permeable molecule which will be lost into the systemic circulation with low skin retention. Incorporation of sesamol into SLN and subsequent SLN into o/w cream, resulted in a considerable decrease in flux value; $23.06 \pm 0.17 \mu g/cm^2/h$ for SLN and $15.76 \pm 0.63 \mu g/cm^2/h$ for sesamol SLN in cream. Low flux value, retention of a high amount of drug in the skin (750 $\mu g$ for SLN in cream base in comparison to 200 $\mu g$ when sesamol solution of equivalent strength is used), for a considerably longer time indicated a better penetration and retention of sesamol in the skin when incorporated into SLN. NDGA, however showed a lower permeability ($20.82 \pm 0.23 \mu g/cm^2/h$) through skin probably because of its lipophilicity, eventhough it was applied as a solution in PEG-400. But the flux value and skin retention improved when it was applied as SLNs ($65.32 \pm 0.41 \mu g/cm^2/h$) and incorporated into a cream base ($48.39 \pm 0.21 \mu g/cm^2/h$). Thus, it may be concluded that incorporation of sesamol into SLN cream considerably decreases the rate of flux through the skin and is able to provide a sustained and targeted effect for a longer period of time. NDGA formulated as SLNs improved its permeability and skin retention; the later in turn is expected to reduce its systemic toxicity since lesser amount of drug would then enter into the systemic circulation. Further, a slower release rate (98.09 $\mu g/h$ for NDGA solution which was reduced to 26.55 and 19.07 $\mu g/h$ for SLNs and SLN in cream) would ensure the absence of any hypersensitivity reactions which have earlier been reported for the marketed topical formulation (Lambert et al., 2002) of NDGA.

275
The stability studies of floating beads indicate them to be highly stable at the accelerated stability testing conditions for 3 months, fulfilling the requirements of ICH guidelines. SLNs though indicating serious changes during accelerated stability studies were found to be quite stable when stored at room temperature for upto 6 months. Although the increase in particle size was considerable, the drug loss from SLNs was within the acceptable limits as per ICH guidelines.

Information on the bioavailability and disposition of sesamol and NDGA are important for understanding their biological effects. To our knowledge, this is the first report on the absorption, distribution and elimination of sesamol and NDGA in rats. When single dose of the drugs was administered as a solution orally, the amount of drug entering into the plasma was high and the tissue distribution was more. But, when the drugs were given orally in the form of floating beads, the amount of drug entering into the plasma and tissues was reduced significantly. In case of SLNs also, more amount of drug was retained in the skin and the release of drug was prolonged (lower release rate). Thus there was an improved bioavailability at the site of action when the drug was administered as floating beads or SLNs than when applied as a solution. In case of NDGA, the amount of drug entering into the liver and kidney was reduced when applied as floating beads and SLNs. Since NDGA is reported to cause liver and nephrotoxicity (Goodman et al., 1970; Lambert et al., 2002) it may thus be concluded that development of suitable dosage forms which release the drug slowly and over a prolonged period of time can considerably reduce the chances of tissue toxicity. The histopathology studies indicate the absence of any necrotic effect on the liver and kidney of rats administered the developed floating beads of NDGA at a dose of 20 mg/kg body weight, twice daily for 16 weeks.

NFκB and VEGF inhibitory activity of NDGA conducted with BJAB-K1 cells (these cells constitutively express K1 protein showing a substantially high NFκB promoter activity and produce high levels of VEGF), showed that NDGA inhibits both NFκB and VEGF activities, while sesamol showed a significant VEGF inhibition only. Nuclear factor-kappa B (NFκB) and vascular endothelial growth factor (VEGF) though involved in the regulation of pro-inflammatory genes and mediation of angiogenesis are indirectly
or directly implicated in the promotion of tumours. Very promising results with NDGA and a considerable inhibition of VEGF achieved with sesamol prompted us to perform apoptotic studies (because of the reported ability of NFκB to protect transformed cells from apoptosis; VEGF maintains the viability of tumours by helping in the survival of immature blood vessels supplying the tumours) and subsequently anticancer studies in animal models with these two agents.

DNA fragmentation is an important method for the determination of apoptotic cell death. While MTT assay is used to study cell viability and proliferation in cell populations. Leukemia cell lines, molt-4 and HL-60, were chosen as representative human tumour cell lines which would grow as colonies in a relatively simple medium. Sesamol and NDGA showed a high (50-60%) antiproliferative activity in MTT assay with NDGA showing significant DNA fragmentation in Molt-4 and HL-60 cell lines while sesamol did not show any activity in Molt-4 cell lines. Eventhough a significant DNA fragmentation in HL-60 cell lines and dose related apoptotic effect of upto 60.43% at 100 μg, in MTT assay, was observed.

Since conventional chemotherapeutic and surgical approaches have not been able to control the incidence of most of the cancer types. Therefore there is an urgent need to develop mechanism-based approaches for the management of cancer (Aziz et al., 2003). Many naturally occurring agents have shown cancer chemopreventive potential in a variety of bioassay systems and in animal models, having relevance to human disease. Sesamol and NDGA, though pure compounds, are of natural origin. Present study indicates their potential in the control of cancer as discussed above. Further, an effective and acceptable chemopreventive agent should have (a) little or no toxic effects in normal and healthy cells; (b) high efficacy against multiple sites; (c) capability of oral consumption; (d) known mechanism of action; (e) low cost; (f) acceptance by human population (Aziz et al., 2003). The test agents (sesamol and NDGA) seem to qualify most of these characteristics. The LD50 of both the compounds is sufficiently high. In vitro antioxidant studies indicate them to be effective in scavenging a multiplicity of free radicals and ROS. The development of floating beads helps in a slow and controlled release thus overcoming any local irritating effects (sesamol) and systemic toxicity if
any (NDGA), because low concentrations are reached in blood plasma and important organs (as indicated by pharmacokinetic studies; Chapter IV). A free radical scavenging effect (confirmed in our study) and VEGF inhibition in cancer cell lines are proposed as the mechanism of anticancer effects of these agents. Since, the agents are of a natural origin, so they are expected to be cost effective and will also be accepted well by the public, considering the proposed effectiveness in folklore.

Hence, we can say that both these agents fulfill the criteria for successful chemotherapeutic agents.

We believe that continued efforts are however needed; especially well-designed pre-clinical studies in the animal models that closely mimic/represent human disease is the first step and has partly been established by us.

There is however a need for multidose long term animal studies followed by human clinical trials in appropriate cancer types in suitable populations.


