Chapter 2
Review of Literature
2. Review of Literature

2.1. Rice bran oil and its components

Interest in rice bran oil (RBO) has been thriving from the health and nutritional aspects as well as its wide application as industrial oil. India is the second largest producer of crude rice bran oil (cRBO) in the world (Usha and Premi 1–2). Crude rice bran oil (cRBO) contains 90–96% of saponifiables including glycolipid and phospholipids and about 4% unsaponifiable matter including antioxidants and micronutrients such as tocopherols, tocotrienols, oryzanol (OZ), sterols and carotenoids (Chen et al. 322–31). RBO has an excellent fatty acid profile with oleic acid (38.4%), linoleic acid (34.4%) and linolenic acid (2.2%) as the unsaturated fatty acids, and palmetic acid (21.5%) and stearic acid (2.9%) as the saturated fatty acids (Rukmini and Raghuram 593–601). The saturated, monounsaturated and polyunsaturated fatty acids are in the ratio of approximately 1:2.2:1.5 that is close to the ideal ratio recommended by American Heart Association (Gopala Krishna 80; Usha and Premi 1).

2.1.1. Oryzanol (OZ)

OZ often identified as the physiologically active component of RBO, is a mixture of ferulate esters of sterols (campesterol, stigmasterol and β-stigmasterol) and triterpene alcohols (cycloartenol, cycloartenol, 24-methylene cycloartanol, cyclobranol). Major portions of OZ include cycloartenyl ferulate, 24–methylene cycloartanyl ferulate and campesteryl ferulate (Fig. 2.1). OZ is 1.5 –2.9 % of RBO and is white or yellowish odorless, tasteless powder with a melting point of 137.5–138°C (Xu and Godber 547–51).

Fig. 2.1: Chemical structures of OZ components (Cho et al. 337–43)
2.1.1.1. Extraction and purification of OZ

Extraction and purification of OZ from RBO have been reviewed extensively. For extraction-based processes, critical process parameters include solid to solvent ratio, temperature, and time (Narayan, Barhate, and Raghavarao 663–70). For crystallization or precipitation based processes, the parameters considered are temperature of the solvent or mixture of solvent along with their proportion, rate of nucleation, and rate of crystal growth (Zullaikah, Melwita, and Ju 299–302). Organic solvent extraction, although is the conventional method for extraction of OZ from RBO, it uses highly toxic and flammable solvents and has problems of waste disposal and leaving toxic residues. Hence, the search for alternative, non-hazardous and environment-friendly extraction technique has led to the emergence of supercritical fluid techniques for the extraction procedures.

Xu and Godber (547) reported that a solvent mixture with 50% hexane and 50% isopropanol (v/v) at 60°C for 45–60 min produced the highest yield (1.68 mg/g of rice bran) of OZ from rice bran. However, the yield (5.39 mg/g of rice bran) of OZ in supercritical fluid extraction under 50°C, 68,901 kPa (680 atm), and 25 min was approximately four times higher than the highest yield of solvent extraction. Moreover, a high concentration of OZ (50–80%) was obtained by collecting the extract after 15–20 min of extraction under optimized conditions.

Zullaikah et al. (299) have recently reported a two-step crystallization process for the isolation of OZ from crude RBO. In the first crystallization step, OZ was concentrated in the liquid phase along with free fatty acid (FFA), monoacylglycerol (MG), squalene, tocols, and phytosterols, whereas the solid phase contained mainly triacylglycerol (TG) and sterol esters. OZ-rich product obtained from the first crystallization was subjected to the second crystallization where the OZ-rich product was kept at room temperature (20.5 ± 1.5°C) for 24 h. Hexane was added as an anti-solvent to the OZ-rich product and kept at 5 ± 1°C for another 48 h, after which white OZ crystals with a purity and recovery of 93-95% and 59%, respectively were obtained.

2.1.1.2. Biological activities of OZ

*In vitro* antioxidant and free radical scavenging activity

One test-tube study showed that at $10^{-4}$ M concentration, OZ was more than four times as effective at stopping tissue oxidation as vitamin E (Hiramitsu and Armstrong 196–203). Xu et al. (645) evidenced significant antioxidant activity for the three major components of OZ (24-methylene cycloartanyl ferulate, cycloartenyl ferulate and campesterol ferulate) in a linolenic
acid model. Xu et al. (2077) further showed that all the three OZ components had higher antioxidant activities against cholesterol oxidation than any of the four vitamin E components (α-tocopherol, α-tocotrienol, γ–tocopherol, and γ-tocotrienol) and that the highest antioxidant activity was evident for the 24-methylenecycloartanyl ferulate.

**In vivo anti-ulcer activity**

Itaya et al. (1001) reported that OZ at 1 to 100 mg/kg s.c. daily for five days, reduced the water-immersion stress ulcer index dose-dependently and slightly prevented the rate of increase in serum 11-hydroxy-corticosterone levels. These effects were prominent in the adrenalectomized as well as sham operated rats, which indicated that the anti-ulcer effect of OZ was due to participation of the autonomic nervous system, and not the hypophysis-adrenal axis.

In male wistar rats, 8-day treatment with 100 mg/kg OZ (s.c.) significantly reduced gastric ulcers. Reduced serum gastrin levels were observed in rats with acetic acid induced gastric ulcers when OZ was administered for 10 days (100 mg/kg; s.c.) (Itaya and Kiyonaga 475–81). The possible mechanism pertaining to OZ’s anti-ulcer action could be the involvement of the monoaminergic neuron system.

Mizuta et al. (285) reported that OZ significantly inhibited tetragastrin-stimulated acid secretion, which might be mediated by the vagus nerve that induces gastrin release.

Another report suggested that gastric lesions in responder mice induced by conditioned emotional stimuli was reduced by twice p.o. administrations at 6 h intervals of OZ at 200 and 500 mg/kg, oxazolam at 2 mg/kg and atropine at 1-10 mg/kg. In addition, the incidence of gastric lesions induced by Rapid Eye Movement (REM) sleep deprivation was also reduced by single administration of OZ at 100 and 200 mg/kg and oxazolam at 5 mg/kg. Additionally, the facilitation of small intestinal propulsive activity in responder mice induced by convulsive electroshock was suppressed by OZ at 100 and 200 mg/kg and atropine at 10 mg/kg (Ichimaru et al. 537–42). Similar modulatory effect on gastrointestinal motility was observed in the dogs as well (Mizonishi and Semba 47–55).

**In vivo anti-hyperlipidemic effects**

Sakamoto et al. (559) showed that oral administration of OZ and cycloartenyl ferulate (10 mg/kg, i.v.) once a day for 12 days along with cholesterol feeding in Sprague Dowley (SD) rats, remarkably reduced serum triglyceride (TG), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol/total cholesterol (HDL-C/TC) ratio, serum non-
esterified fatty acids, aspartate aminotransferase (AST) and serum albumin, which could be due to a direct inhibition of lipid metabolism.

Nakayama et al. (135) demonstrated that OZ (100, 500 or 1000 mg/kg) when administered in combination with high cholesterol diet in SD rats caused about 25% reduction in total cholesterol (TC) level on day 6 for all dose groups. Phospholipids were reduced on day 6 for the maximum dose (MD) and higher dose (HD) group. Serum free cholesterol was around 25% lower (HD only) on day 13. Serum AST was slightly elevated but unremarkable, whereas ALT increased by around 65% (HD group only). Histological examination of the livers found steatosis, but was unremarkable for the OZ-fed animals.

In F1B golden hamsters (16/group) fed with a hypercholesterolemic diet, 1% OZ supplementation for 8 weeks resulted in reduced serum cholesterol (by 28%), sum of intermediate density lipoprotein (IDL), LDL-C and very low density lipoprotein cholesterol (VLDL-C) (by 34%), cholesterol absorption (by 25%), relative to controls (Fujiwara, Sakurai, Sugimoto, et al. 645–52). Body weights, HDL-C, and TG serum levels were unremarkable along with unchanged liver and intestinal cholesterol synthesis.

In a separate experiment, OZ-treated hamsters for 10 weeks reduced plasma TC (44%, \( P < 0.001 \)), Non-HDL-C (57%, \( P < 0.01 \)), and TG (46%, \( P < 0.05 \)) concentrations relative to the control. Despite a 12% decrease in HDL-C (\( P < 0.01 \)), the OZ-treated animals maintained a more optimum non-HDL-C/HDL-C profile (1.1±0.4) than the control (2.5±1.4; \( P < 0.01 \)). Aortic fatty streak formation was reduced by 67% (\( P < 0.01 \)) in the OZ-treated animals (Rong, Ausman, and Nicolosi 303–9).

**In vivo anti-inflammatory effects**

In a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced model of inflammation (ear oedema) in ICR mice, Yasukawa et al. (1072) estimated that relative to OZ, with a median infective dose (ID\(_{50}\)) of 1.4 mg/ear (50% inhibition of oedema), the ID\(_{50}\)s for cycloartenyl ferulate, 24-methylenecycloartenyl ferulate, 24-methylcholesterol ferulate and sitosterol ferulate ranged from 0.2-0.3 mg/ear.

Terada et al. (95) showed that rats administered with OZ (1, 10 and 100 mg/kg/day; route not stated) following adjuvant-induced arthritis (unknown agent used), manifested a significant reduction of paw-edema volume from days 15-19 for the highest dose (HD), from days 17-19 for the medium dose (MD) and on day 19 for the lowest dose (LD).
A recent study by Islam et al. (812) attested the potential of OZ (50 mg/kg/day p.o.) on a mice model of induced colitis with dextran sulphate sodium (DSS). The anti-inflammatory effect was possibly mediated by the inhibition of the inflammatory reactions exerted by tissue myeloperoxidase (MPO), pro-inflammatory cytokines and cyclooxygenase-2 (COX-2), nuclear factor-kappaB (NF-kB) p65 nuclear translocation and inhibitory protein of NF-kappaB-alpha degradation.

Another recent study exhibited the prevention of ethanol-induced liver injury by OZ in c57BL mice as reflected by markedly decreased serum activities of plasma AST, ALT, and significant decreases in hepatic lipid hydroperoxide and thiobarbituric acid reactive substances (TBARS) levels (Chotimarkorn and Ushio 951–8).

Another recent notable finding by Oka et al. (152) revealed that IgE-targeting therapy by intra-dermal OZ injection in SD rats with anti-DNP IgE could provide significant progress in the treatment of allergic inflammation. The mechanism involved might be the attenuation of the passive cutaneous anaphylaxis (PCA) reaction induced by DNP-HAS and inhibition of the degranulation of DNP-IgE sensitized RBL-2H3 mast cells stimulated with anti-DNP-HAS.

### Effects in diabetes

Ohara et al. (130) postulated that OZ might regulate adiponectin secretion by the inhibition of NF-kB activation. Cheng et al. (45) reported that adding OZ to 15 % palm oil increases insulin sensitivity in wistar rats with type 2 diabetes (T2DM) compared to control and palm oil groups. Kozuka et al. (3084) recently demonstrated improved glucose intolerance and prevention of the onset of diabetes in mice with long-term oral administration of OZ.

### Central nervous system (CNS) and behavioral effects

Given orally, the effect of OZ (1000 mg/kg) was insignificant with respect to the seizure onset time and time to death in a pentylenetetrazol (PTZ: 140 mg/kg; s.c.)-induced convulsion model in mice. Cycloartenyl ferulate (10, 30, 100 or 300 mg/kg; p.o.), on the other hand, increased the time to seizure onset in a dose-dependent manner, but had no effect on time to death (Hiraga et al. 715–21). The conflict behavior experiment in mice showed a significant benefit with OZ (30, 100 or 300 mg/kg; single i.p.) during the safe period.

Hirofumi et al. (1402) reported that OZ (50 mg/kg; p.o.) desynchronised electroencephalographic (EEG) activity 10-15 min after administration for 30-40 mins and again from 60 mins post-dose for 120 min in rabbits. In a separate experiment, OZ (50 mg/kg;
p.o.) in rabbits was found to prolong the duration of the amygdaloid after-discharge induced by electrical stimulation of the amygdaloid nucleus, beginning 45 min post-dose.

In cats, EEG activity after a single i.v. administration of OZ (dose not specified) showed low amplitude fast waves starting at 40 mins post-dose, lasting from 12 to 110 mins. Higher dose of OZ increased the duration of the fast wave period (Aikawa and Kobayashi 73–81).

Another study by Kaneta et al. (399) revealed that OZ (100 mg/kg; s.c.) administered once daily for 1, 5 or 10 days increased nor-epinephrine levels in the brain of rats. Hence, it is likely that successive doses of OZ increase brain nor-epinephrine by inhibiting the degradation or release of nor-epinephrine.

**In vivo chromosomal effects**

Tsushimoto et al. (191) observed no cases of mortality in SD rats with OZ given orally at 40, 400 and 4000 mg/kg as a single or repeat-doses (once daily for 5 days), indicating that OZ did not induce chromosome aberration.

**In vivo carcinogenicity studies**

Histopathological findings by Tamagawa et al. (49; 41) indicated that OZ (200, 600 or 2000 mg, p.o.) was not carcinogenic in B6C3F1 mice and Fischer (F344/DuCrj) rats after 78 weeks and 2 years respectively.

Yasukawa et al. (1072), using a two-stage model of skin carcinogenesis with 7, 12-Dimethylbenz[a]anthracene (DMBA) as an initiator, followed topical 12-O-tetradecanoylphorbol-13-acetate (TPA) as a promoter, indicated that topical cycloartenyl ferulate reduced the percentage of tumor bearing mice from 93% to 20% at week 20. The number of tumors/mouse also decreased by a similar factor.

The modifying effects of OZ on the promotion stage of carcinogenesis were investigated using several two stage carcinogenesis models in rats (Hirose et al. 3665–70). In a multi-organ carcinogenesis model, liver adenoma in carcinogen induced male F344 rats decreased from 64% in animals fed with the basal diet to 38% in 1% OZ–treated animals (P < 0.05) after 32 weeks. However, the incidence of lung carcinoma increased from 8% (1 rat) to 54% (8 rats) and multiplicity also increased from 0.1/rat to 0.6/rat. An increase in the multiplicity of lung adenoma was also observed in OZ-treated rats. Esophagus, colon, pancreas, kidney and thyroid lesion development was not influenced by the compound.
Examination of the modifying potential of 1% OZ on mammary carcinogenesis in female SD rats pre-treated with a single intragastric dose of 7,12-dimethylbenz[α]anthracene (DMBA) (50 mg/kg) for 35 weeks revealed no significant differences in the final incidences and multiplicities of mammary tumors, but the average tumor diameter was significantly reduced. The survivorship curve for the OZ-treated rats was >10% higher than controls from week 22 of the experiment and around 50% higher at study end.

A recent study by Kim et al. (935) documented that compared to the control diet without the rice bran ingredient, oral administration for two weeks of OZ in mice with induced tumors significantly reduced the tumor size in a dose-dependent manner by up to 44%. Induction of splenic natural killer (NK) cell activity, activation of macrophages, and inhibition of angiogenesis seemed to contribute to the inhibitory mechanism of tumor regression by OZ.

### 2.1.1.3. Pharmacokinetics

OZ, being a complex multi-constituent substance, data describing the pharmacokinetics of its individual constituents has been reported.

Fujiwara et al. (1011) in their study in rabbits showed that the blood level of radio-labeled OZ was very low following an oral administration (40 mg/kg) and was undetectable in blood 2 h post-dose. The total radioactivity appearing in urine 48 h post-dose was 6.4% of the administered amount. The principle metabolite found in the blood was ferulic acid (FA). However, in urine, FA, vanillic acid, acetovanillone, hippuric acid and the glycine conjugate of vanillic acid were detected. The underlying mechanism might involve the poor absorption, rapid tissue distribution and extensive metabolism of OZ, probably in the liver.

Fujiwara et al. (645) investigated the absorption and metabolism of 14C-labelled ‘gamma oryzanol’ (50 mg/kg) after oral administration in male SD rats. Analyses of the urine and faeces over the next 5 days showed that 9.8% of the ingested radioactivity was excreted in the urine over the first 72 h and faecal excretion was 84.5% for the first 48 h. 94% of the urine and 93% of the faecal radioactivity was excreted within the first 24 h and radioactivity in the blood peaked at 4 h and disappeared by 48 h post-dose. An analysis of the urine metabolites revealed no OZ, but around half of the OZ dose was accounted for as the sulfated form of FA and significant proportions of hippuric acid, m-hydroxyphenylpropionic acid and dihydroferulic acid were also detected along with small quantities of m-coumaric and m-hydroxyhippuric acid.
Fujiwara et al. (973) observed a prominent dose-dependent relationship in FA level in rabbits and beagle dogs with OZ (25, 50 and 100 mg/kg, p.o.) after a 20-24 h fasting. The peak plasma FA levels occurred at 2 h in rabbits and at 1-1.5 h in dogs. The maximum plasma levels of FA were 100 ng/ml and 200 ng/ml in dogs and rabbits respectively. At 24 h post-administration, plasma FA returned to baseline levels in rabbits and dogs.

Tsushimoto et al. (191) reported the maximum plasma concentration (C_{max}) after a single oral consumption of 300 mg \(\beta\)-sitosteryl ferulate, to be around 40 ng/ml in humans.

### 2.1.1.4. Clinical studies

The clinical studies for OZ are summarized chronologically in Table 2.1.

<table>
<thead>
<tr>
<th>Study design (Reference)</th>
<th>Subject details</th>
<th>Treatment details</th>
<th>Endpoints</th>
<th>Key outcomes</th>
<th>Adverse drug reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlled, crossover, 1 day Shimomura et al. (83)</td>
<td>n=7 (both sexes) Aged 20-45 years. Patients with primary hypothyroidism</td>
<td>Oral; 300 mg (single dose)</td>
<td>T4-I, T3 and TSH</td>
<td>25% reduction in serum TSH at 2-4 h post-dose, possibly by a direct action on the hypothalamus</td>
<td>None reported</td>
</tr>
<tr>
<td>Controlled, crossover, 1 day Shimomura et al. (83)</td>
<td>n=7 (both sexes) Aged 20-45 years Patients with primary hypothyroidism</td>
<td>Oral; 300 mg (single dose) and TRH (500 (\mu)g; i.v.)</td>
<td>TSH</td>
<td>No significant differences</td>
<td>None reported</td>
</tr>
<tr>
<td>Controlled, 7 days Shimomura et al. (83)</td>
<td>n=8 (both sexes) Aged 20-45 years Patients with primary hypothyroidism</td>
<td>Oral; 300 mg/day</td>
<td>Serum TSH</td>
<td>Unclear</td>
<td>None reported</td>
</tr>
<tr>
<td>Open labeled, 4 weeks, Arai (71)</td>
<td>n=19 (sex unclear) Aged 25-74 years Patients with chronic gastritis and 62% also neurotic</td>
<td>Oral; 300 mg/day</td>
<td>Serum gastrin, frequency of symptoms</td>
<td>Serum gastrin “75% effective” in symptom reduction</td>
<td>None reported</td>
</tr>
<tr>
<td>Controlled 4 weeks, Arai (85)</td>
<td>n=13 (sex not stated); Patients with chronic gastritis</td>
<td>Oral; 300 mg/day</td>
<td>Serum gastrin, secretin, glucagon, catecholamines, prolactin, cortisol.</td>
<td>Reduced serum gastrin</td>
<td>None reported</td>
</tr>
<tr>
<td>Open labeled, 8 weeks Ishihara et al (243)</td>
<td>n=40 (sex not stated) Xperiencing aging syndromes and with climacteric disturbance</td>
<td>Oral; 300 mg/day</td>
<td>Effect on climacteric disturbance</td>
<td>✓ 90% cases improved ✓ Significantly reduced TC, TG ✓ Increased HDL-C in hyperlipidemia ✓ lipid peroxides recovered</td>
<td>None observed</td>
</tr>
<tr>
<td>Study design (Reference)</td>
<td>Subject details</td>
<td>Treatment details</td>
<td>Endpoints</td>
<td>Key outcomes</td>
<td>Adverse drug reactions</td>
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<tr>
<td>Open labeled, 8 weeks Ishihara (317)</td>
<td>n=40 (females) Aged ≥40 years Patients with climacteric disturbances</td>
<td>Oral; 300 mg/day in 3 divided doses</td>
<td>Serum lipids, serum lipid peroxide, frequency of menopausal symptoms, menopausal index, liver enzymes, haematology</td>
<td>✓ Reduced serum lipid peroxides (31%; week 4) ✓ Reduction in symptoms (75-100%) ✓ Reduced menopausal index (improvement) in 34/40 ✓ Reduced TG (14%)</td>
<td>None observed</td>
</tr>
<tr>
<td>Open labeled, 4 weeks Kawamoto et al. (919)</td>
<td>n=24 (sex not stated) 7 patients with gallstones, 3 with hyperlipidemia and 10 normal subjects</td>
<td>Oral; 600 mg/day</td>
<td>Serum apolipoprotein A-I, lipids</td>
<td>Reduced serum apolipoprotein A-I</td>
<td>Not stated</td>
</tr>
<tr>
<td>Randomized controlled, (13 weeks) Yoshino et al. (543)</td>
<td>n=80 Hypercholesterolemic patients</td>
<td>Oral; 300 mg/day</td>
<td>TC, LDL-C, TG</td>
<td>Reduced TC, LDL-C, TG</td>
<td>None observed</td>
</tr>
<tr>
<td>Open labeled, 16 weeks Sasaki et al. (263)</td>
<td>n=20 (11 males; 9 females) Mean age 51 years, Patients with schizophrenia and dyslipidaemia taking neuroleptic medication</td>
<td>Oral; 300 mg/day in 3 divided doses</td>
<td>Serum lipids (TC, LDL-C, HDL-C, TG, LDL/HDL), apolipoproteins</td>
<td>✓ Reduced TC (6%) at weeks 8 and 14% at week 12 ✓ Reduced LDL-C (19%; week 12 only) ✓ Reduced HDL-C (6%; at week 8 only) ✓ Reduced LDL/HDL (11%; at week 4 only) ✓ Reduced apolipoproteins A-II, B, C-II and B/A-I ratio at weeks 8 and 16 (by 9-15%)</td>
<td>None observed</td>
</tr>
<tr>
<td>Randomized, double blind, placebo controlled 9 weeks Fry et al. (318)</td>
<td>n=22 (males) Aged 19.8 ± 0.87 years Weight-trained subjects</td>
<td>Oral; 500 mg/day and 9 week resistance training programme</td>
<td>Testosterone, β-endorphin, cortisol, estradiol, insulin, hematocrit, albumin, plasma lipids, calcium and magnesium Strength tests, lactate pre- and post-exercise.</td>
<td>No treatment-related effects, no influence on performance or related physiological parameters</td>
<td>None observed</td>
</tr>
<tr>
<td>Randomized, double blind, placebo controlled, cross-over 3.5 weeks Weststrate and Meijer (334)</td>
<td>n=100 (50 males, 50 females) Aged 18-65 years Healthy subjects</td>
<td>Oral; 2.7 g/day</td>
<td>Lipids, liver enzymes, bile acids, sterols, fatty acids, carotenoids.</td>
<td>✓ Reduced TC (8.3%) ✓ Reduced LDL-C (13%) ✓ α + β-carotene (8%)</td>
<td>Not reported</td>
</tr>
</tbody>
</table>
TRH: Thyrotropin-releasing hormone (TRH); TSH: Thyroid-stimulating hormone; T3: triiodothyronine; T4: thyroxine

### 2.1.1.5. Miscellaneous uses

OZ is used as a sunscreen agent on account of its protective role in ultra-violet (UV)-light induced lipid peroxidation. OZ is capable of stimulating hair growth and prevent skin aging by accelerating the cell differentiation and reducing the wrinkles in aged women, thereby protecting them from oxidative damage. Solubilization of OZ into medicinal drinks serve as revitalizing tonics and is achieved by using sucrose fatty acid ester and ethoxylated hydrogenated castor oil. Moreover, OZ is widely employed as an anabolic agent by bodybuilding athletes because of its potential to increase testosterone production and stimulate human growth hormone release (Patel and Naik 569–78).

### 2.1.1.6. Adverse reactions

OZ has been shown to be extremely safe with no major side effects being reported in either animal or human studies (Murray 332–5). Toxicological data for OZ indicates rat median lethal dose (LD₅₀) (oral) as 25000 mg/kg, mouse LD₅₀ (oral) as 250000 mg/kg, and guinea pig LD₅₀ (dermal) as 100 mg/kg (MSDS-Gamma Oryzanol).

### 2.1.1.7. Regulatory scenario

As per the Australia New Zealand Food Authority Annual Report 1999-2000, in the US, unlike ‘vegetable oil phytosterol esters’, OZ does not have a Generally Recognized as Safe (GRAS) 1 status. Furthermore, the database of the Centre for Food Safety and Applied Study design 
(Reference) | Subject details | Treatment details | Endpoints | Key outcomes | Adverse drug reactions |
--- | --- | --- | --- | --- | ---
Randomized 4 weeks Berger et al. (163) | n=30 (males) Aged 40-65 years Total cholesterol 5.1-8.4 mmol/l BMI < 28 kg/m² | Oral; 50 mg/day or 800 mg/day in diet | Serum lipids (TC, LDL-C, HDL-C, LDL/HDL, VLDL-C) | ✓ Reduced TC (6%), LDL-C (11%), HDL-C (8%), LDL/HDL(19%) and VLDL-C (56%) | None observed |
Open labeled, 8 weeks Bucci et al. (104) | n= not reported (males) body builders | Oral; 30 mg/day | body weight and strength | Increase in body weight and strength | Not reported |
Open labeled, 2-6 months Fujiwaki and Furusho (287) | n=20 (8 males; 12 females) Aged 2-15 years Patients with atopic dermatitis In combination with existing medications including steroids | Topical; 0.5% added to bath once a day | Symptom score, IgE, eosinophil count, specific IgE antibodies | ✓ Reduced total symptom score ✓ Reduced eosinophil count ✓ Reduced specific IgE in 2/6 substances tested | None observed |
Nutrition of the US Food and Drug Administration (EAFUS – ‘Everything Added to Food in the United States’) does not contain any listings for OZ. Additionally; OZ does not appear in the US Environmental Protection Agency’s database of existing chemicals, the Toxic Substances Control Act Chemical Substance Inventory. Moreover, since OZ is not approved by the Flavour and Extract Manufacturers’ Association (FEMA), it cannot be used as food additives or pharmaceuticals in the US. However, in the US, OZ is widely used as a sports supplement, as well as for reducing cholesterol (Gamma-Oryzanol-1). Currently, OZ has also been approved in Japan for several conditions, including menopausal symptoms, mild anxiety, stomach upset, and high cholesterol and has been listed as an “antioxidant” under the list of chemical composition of food additives (Gamma-Oryzanol-2).

2.2. Diabetes mellitus

Diabetes mellitus (DM) represents a spectrum of metabolic disorders of multiple etiology, characterized by chronic hyperglycemia, and often comorbidities such as hypertension, dyslipidaemia and inflammation, with the major types being type 1 (T1DM) or type 2 (T2DM). Both forms of diabetes are associated with micro- and macrovascular complications including retinopathy, neuropathy, cardio/cerebrovascular disease and nephropathy which are the major cause of mortality and morbidity in diabetic patients (Sourris and Forbes 180–1).

It is estimated that approximately 366 million individuals worldwide had diabetes in 2011 and by the year 2030 the number will rise to 552 million. India is home to over 61 million diabetic patients - an increase from 50.8 million in 2010. By 2030, India's diabetes burden is expected to cross the 100 million mark (International Diabetes Federation)

2.2.1. Classification (American Diabetes Association S64–71)

Type 1 diabetes mellitus (T1DM)

This form of diabetes also termed as the insulin dependent diabetes, or juvenile-onset diabetes, accounts for about 5–10% of diabetic patients, and results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency.

Type 2 diabetes mellitus (T2DM)

This form of diabetes also termed as the non-insulin dependent diabetes, or adult onset diabetes, accounts for about 90–95% of diabetic patients, and is often a combination of resistance to insulin action and inadequate compensatory insulin secretion.
2.2.2. Pathogenesis (Pittas and Greenberg)

![Fig. 2.2A: Pathogenesis of Type 1 Diabetes](image)

![Fig. 2.2B: Pathogenesis of Type II Diabetes](image)

2.2.3. Management

The initial management of DM is based on dietary therapy with increased physical activity. However, pharmacological agents with different mechanisms of action (oral hypoglycemic drugs like sulphonylureas and biguanides or insulin) are often combined to achieve optimal glycemic control in presence of marked hyperglycemia (Aicher et al. 209–29).

2.2.4. Mechanisms of oxidative stress in diabetes

Oxidative stress is caused by an imbalance between the production of reactive species (RS) and a biological system’s ability to readily detoxify them or easily repair the resulting damage. Reactive oxygen species (ROS) are free radicals containing an oxygen atom and the biologically important ROS are superoxide (•O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (•OH). Reactive nitrogen species (RNS) include free radicals such as nitric oxide (•NO) and nitrogen dioxide (•NO₂⁻), and non-free radicals such as peroxynitrite (OONO⁻) (Erejuwa 218–9). Various mechanisms contributing to diabetes-induced oxidative stress have been proposed (Araki and Nishikawa 90; Tan, Sharma, and de Haan 247) (Fig. 2.3).

The oxidation of glucose in a transition-metal dependent reaction leads to the formation of an enediol radical anion that is converted to reactive ketoaldehydes and •O₂⁻ radicals. •O₂⁻ radicals undergo dismutation to H₂O₂, which if not degraded by catalase or glutathione peroxidase, can lead to the production of extremely reactive hydroxyl radicals in presence of transition metals.

The cytosolic enzyme aldose reductase converts high intracellular glucose concentrations to sorbitol using nicotinamide adenine dinucleotide phosphate (NADPH) derived from the pentose phosphate pathway as a cofactor. During hyperglycemia, consumption of NADPH by
this reaction inhibits replenishment of reduced glutathione, which is required to maintain glutathione peroxidase activity.

Hyperglycemia activates protein kinase C (PKC) in membrane fraction by increasing the de novo diacylglycerol synthesis in vascular cells. Hyperglycemia is reported to stimulate ROS production through PKC-dependent activation of NAD(P)H oxidase.

Hyperglycemia increases non-enzymatic glycation, characterized by the binding of reactive dicarbonyls and amino groups of proteins, thus generating glucose-derived advanced glycation end-products (AGEs). Glycation and oxidative stress are intimately linked, and all steps of glycoxidation generate ROS. In addition, plasma proteins modified by AGE precursors activate AGE receptors on macrophages and induce intracellular oxidative stress by activating NAD(P)H oxidase. Furthermore, site specific and random fragmentation of copper, zinc SOD (Cu, ZnSOD), a key enzyme involved in the detoxification of superoxide radicals, has been observed following the glycation reaction.

Hyperglycemia-induced generation of free radicals from the mitochondrial electron transport chain is considered to be another key event that drives the vicious cycle of oxidative stress in diabetes.

In addition, antioxidant defences are known to be impaired in a hyperglycaemic milieu, shifting the balance away from steady-state levels of ROS towards an environment of oxidative stress.

Fig. 2.3: Proposed mechanism leading to diabetes-induced oxidative stress in cells (Araki and Nishikawa 90–6)
AGE, advanced glycation end-products; DAG, diacylglycerol; GSH, glutathione; GSSG, glutathione disulfide (oxidized glutathione); NADPH, nicotinamide adenine dinucleotide phosphate; PKC, protein kinase C; SOD, superoxide dismutase; TCA, tricarboxylic acid.

### 2.2.5. Antioxidant therapy and diabetes

Antioxidants may act at different levels, inhibiting the formation of ROS or scavenge free radicals, or enhance the antioxidant defense enzyme capabilities. The enzymatic antioxidant systems, such as copper, zinc, manganese superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and catalase may remove the ROS directly or sequentially, preventing their excessive accumulation and consequent adverse effects. Non-enzymatic antioxidant systems consist of scavenging molecules that are endogenously produced such as glutathione, ubichinol, and uric acid or derivatives of the diet such as vitamins C and E, carotenoids, lipoic acid, selenium, etc (da Silva et al. 111).

Mekinova et al. (257) demonstrated that supplementation of streptozotocin (STZ) diabetic rats with vitamins C, E, and beta-carotene for 8 weeks produced a significant reduction of thiobarbituric acid reactive substances (TBARS) levels, reduced glutathione, and glutathione peroxidase, an increase in Cu-SOD, and no change in catalase activity in the kidneys. Treatment with vitamins C and E was also shown to significantly lower malondialdehyde (TBARS) levels and glutathione peroxidase activity, while increasing catalase and SOD activities when compared to unsupplemented diabetic animals (Kedziora-Kornatowska et al. e134–43). A study by Cinar et al. (56) demonstrated that supplementation with vitamin E significantly lowered liver and lung TBARS levels and improved impaired endothelium-dependent vasorelaxation in STZ diabetic rat aorta. Intraperitoneal administration of α-lipoic acid to STZ diabetic wistar rats has been reported to normalize TBARS level in plasma, retina, liver, and pancreas (Obrosova, Fathallah, and Greene 139–46). α-lipoic acid has been observed to normalize diabetes-induced decrease of SOD in rat heart (Maritim, Sanders, and Watkins 288–94) and retina (Obrosova, Fathallah, and Greene 139–46). Another study demonstrated that treatment of STZ diabetic rats with α-lipoic acid reverses SOD induced vasorelaxation, potentially due to the elimination of excess superoxide/hydrogen peroxide and the recovery of basal NO (Koçak et al. 308–18).

In a prospective cohort study, vitamin C intake was found to be significantly lower among incident cases of T2DM. In three prospective observational studies, serum α-tocopherol levels were associated with lower risk of T1 or T2DM. In another prospective study cohort of more
than 4000 non-diabetic subjects over 23 years, vitamin E intake was significantly associated with a reduced risk of T2DM (Bajaj and Khan S267–71).

The Secondary Prevention with Antioxidants of Cardiovascular Disease in End Stage Renal Disease (SPACE) trial in hemodialysis patients with pre-existing cardiovascular disease and diabetes demonstrated a 46% decrease in the primary end point events (myocardial infarction, stroke, peripheral arterial disease or unstable angina) in the vitamin E (800 IU/day for 2 years) group that was mainly due to a 70% reduction in total myocardial infarction (Boaz et al. 1213–8).

The Steno-2 trial in patients with T2DM showed that multi-factorial intensive therapy comprising of daily supplementation of vitamin C (250 mg), E (100 mg), folic acid (400 mg) and chromium picolinate (100 mg) as well as behavior modification resulted in almost 50% decrease in the risk of cardiovascular events compared to that of conventional treatment (Gaede, Vedel, et al. 383–93).

In cultured adipocytes, treatment with α-lipoic acid protected the insulin receptor from oxidative damage, maintaining its functional integrity. A placebo-controlled exploratory study of patients with T2DM indicated that oral administration of lipoic acid significantly increased insulin-mediated glucose uptake, presumably by modulating insulin sensitivity (Jacob et al. 309–14).

2.3. Diabetic nephropathy

Diabetic nephropathy, accounting for approximately one third of all cases of end-stage renal disease (ESRD), is defined as a progressive decline in glomerular filtration rate, accompanied by proteinuria and other end-organ complications such as retinopathy. Approximately 30–40% of patients with T1 and 15% with T2DM will develop evidence of diabetic nephropathy, which represents a continuum from microalbuminuria, to overt nephropathy or macroalbuminuria, and finally ESRD. The progression of the disease is enhanced by hyperglycemia, hypertension and proteinuria, all of which are common in diabetes (S. Siddiqui, Rashid Khan, and W. A. Siddiqui 651; Sourris and Forbes 180). The "Chennai Urban Rural Epidemiology Study" revealed the prevalence of overt nephropathy and microalbuminuria to be 2.2% and 26.9%, respectively, in the urban Indian citizens with diabetes (Unnikrishnan et al. 2019–24).
2.3.1. Pathophysiology

Multiple pathophysiological mechanisms contribute to the development and outcomes of diabetic nephropathy, such as interaction between hyperglycemia induced metabolic and functional/hemodynamic factors in the renal microcirculation that underlies the structural changes of the microvasculature (Fig. 2.4). Hyperglycemia triggers the structural and functional changes such as glomerular hyperfiltration, glomerular and tubular epithelial cell (podocyte) hypertrophy, and microalbuminuria, followed by the glomerular basement membrane thickening, accumulation of mesangial matrix and overt proteinuria, and eventually glomerulosclerosis and ESRD (Vinod 121; Sourris and Forbes 180).

Hemodynamic factors that contribute to the development of diabetic nephropathy include the activation of various vasoactive hormone pathways, such as the renin–angiotensin–aldosterone system (RAAS) and endothelin. In response, secretion of profibrotic cytokines, such as transforming growth factor 1 (TGF-1), is increased and further hemodynamic changes, such as increased systemic and intraglomerular pressure occur. Metabolic pathway involvement leads to nonenzymatic glycosylation that generates advanced glycosylation end products (AGE), increased protein kinase C (PKC) activity, and abnormal polyol metabolism. Findings from various studies support an association between increased secretion of inflammatory molecules, such as cytokines, growth factors and metalloproteinases, and development of diabetic nephropathy (Dronavalli, Duka, and Bakris 444–52).

![Pathways involved in the development of diabetic nephropathy](Vinod 121-6)

2.3.2. Oxidative stress and ROS in diabetic nephropathy

An upregulation of ROS has been implicated in the pathogenesis of kidney injury in diabetes. ROS activate a number of signalling pathways including PKC, p38 mitogen-activated protein kinase (MAPK), p42/p44 MAPK and the transcription factor NF-κB, which leads to the increased activation of growth factors such as TGF-β that contribute to the pathogenesis of diabetic nephropathy. In the diabetic kidney, enhanced glucose uptake in the glomerular epithelial cells, mesangial cells and proximal tubular epithelial cells leads to the excessive production of intracellular ROS, increasing the vulnerability of these cells to the diabetic milieu (Forbes, Coughlan, and Cooper 1446–54).

Hyperglycemia has been shown to elevate 8-hydroxy-2′-deoxyguanosine (8-OHdG), sensitive biomarker of oxidative mitochondrial DNA damage and oxidative stress in diabetic rat kidneys. Intervention by insulin treatment however normalised the renal 8-OHdG level in diabetic rats, clearly linking the diabetic milieu and increased oxidative stress in this pre-clinical model (Kakimoto et al. 1588–95). The urinary 8-OHdG excretion was significantly higher in T2DM patients than in healthy controls and tended to increase with severity of the tubulointerstitial lesion in the kidneys (Kanauchi, Nishioka, and Hashimoto 327–29). In addition, the 24-h urinary content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a product of oxidative DNA damage, strongly predicted the progression of diabetic nephropathy in T2DM patients in a 5-year follow up study (Hinokio et al. 877–82).

Several studies have investigated the pathways through which increased ROS may trigger its damaging effects on glomerular and tubular injury in the diabetic kidney. ROS mediates high glucose-induced activation of PKC-signaled increase in TGF-β expression in glomerular mesangial cells (Studer, Craven, and DeRubertis 918–25). Ha et al. (894) have demonstrated that inhibition of ROS with a series of antioxidants effectively blocked high glucose-induced activation of NFκB and NFκB-dependent monocyte chemoattractant protein-1 (MCP-1) expression in mesangial cells. Furthermore, increased cellular ROS led to accelerated glomerular fibrosis through TGF-β-mediated upregulated expression of plasminogen activator inhibitor-1 (PAI-1) in mesangial cells (Jiang et al. 961–6). Similarly, ROS by acting as act as intracellular messengers, mediate kidney fibrosis in renal cells through the upregulation of the transcription factors NFκB and activator protein-1 (AP-1), that in turn increase MCP-1, TGF-β and PAI-1, resulting in the increased accumulation of extracellular matrix (ECM) in diabetic kidney (Lee et al. S241–5). In another study, antioxidants effectively inhibited the TGF-β-induced cellular ROS, the phosphorylation of Smad2, p38 MAPK and extracellular signal-
regulated kinase (ERK), as well as endothelial-mesenchymal transition (EMT), further elaborating on the critical role of ROS in TGF-β-dependent pathways in renal tubular epithelial cells (Rhyu et al. 667–75).

2.3.3. Treatment options

Based on pathogenesis, achieving the best metabolic control [glycosylated hemoglobin (HbA1c <7%)], treating hypertension (<130/80 mmHg or <125/75 mmHg if proteinuria >1.0 g/24 h and increased serum creatinine), using drugs with blockade effect on the RAAS, and treating dyslipidemia (LDL cholesterol <100 mg/dl) are the currently employed effective strategies for preventing the development of microalbuminuria, in delaying the progression to more advanced stages of nephropathy and in reducing cardiovascular mortality in patients with T1 and T2 diabetes (Choudhury, Tuncel, and Levi 406–15). A recent experimental study in STZ-induced diabetic rats using a multimodal intervention comprising of angiotensin converting enzyme inhibitor, angiotensin receptor blocker, and statin normalized proteinuria and ameliorated diabetic glomerulosclerosis, thus suggesting the importance of targeting various pathogenic processes leading to DN simultaneously (Zoja et al. F1203–11).

2.3.4. Role of antioxidants

Regardless of the benefits obtained from the current therapeutic schemes for diabetic nephropathy, these strategies still provide inadequate protection against renal progression (Navarro-González and Mora-Fernández 433–42). Since implicated as a common product of many of the mechanisms underlying the pathogenesis diabetic nephropathy, inhibition of oxidative stress may constitute a focal point for multiple therapeutic synergies. Some of the other potentially beneficial actions of anti-oxidants that may be involved in the improvement of diabetic nephropathy include amelioration of blocking of intra-cellular as well as extra-cellular formation of AGEs, suppression of circulating levels of glycated proteins, improved glucose disposal, and inhibition of PKC activation (Ha and Kim S228–33).

Bio-flavanoids like quercetin, resveratrol, and curcumin are renoprotective natural antioxidants and have been reported to attenuate oxidative stress-induced renal dysfunction in diabetic rats (Anjaneyulu and Chopra 244; S Sharma, Anjaneyulu, et al. 69, Sharma, Kulkarni, and Chopra 940). Red cabbage (Brassica oleracea) has been noted to activate the defense systems like SOD, catalase and total anti-oxidant capacity in diabetic rats (Kataya and Hamza 281–7). Taurine, a non-essential sulfur-containing anti-oxidant amino acid attenuates proteinuria and glomerulosclerosis in STZ-induced diabetic nephropathy in rats (Trachtman,
The findings are further bolstered by evidence of effective prevention of proteinuria and glomerular TGF-β and fibronectin mRNA expression by taurine and melatonin at doses inhibiting lipid peroxidation in STZ-induced diabetic rats (Ha, Yu, and Kim 944–50). Ginger has been noted to activate anti-oxidant defense systems and reduce lipid peroxidation and nephropathy in diabetic rats (Afshari et al. 148–53). Treatment with vitamin C or vitamin E reverses albuminuria, inhibits the release of glomerular TGF-β and reduces the glomerular size in diabetes (Shirpoor et al. 171–7). Wang et al. (1) recently demonstrated that flavonoid luteolin has a protecting effect against development of diabetic nephropathy by changing the SOD activity, the MDA content, and expression of Heme Oxygenase-1 (HO-1) protein. Brezniceanu et al. (912) demonstrated that renal catalase overexpression in db/db mice attenuated ROS generation, angiotensinogen, proapoptotic gene expression and apoptosis in the kidneys of diabetic mice in vivo.

An 8-month randomized double-masked placebo-controlled crossover trial showed that oral Vit E treatment normalized elevated baseline creatinine clearance in T1DM patients without inducing a significant change in glycemic control (Bursell et al. 1245–51). Gaede et al. (756) reported that Vit E (680 mg/day) and Vit C (1250 mg/day) combination significantly improved renal function in T2DM. Treatment with high-dose benfotiamine has been documented to reduce albuminuria in T2DM patients (Du, Edelstein, and Brownlee 1930–2). HbA1C levels were significantly reduced by high dose Vit E antioxidant supplementation, asserting the benefits of antioxidants in protecting against the complications of T2DM (Akbar, Bellary, and Griffiths 62–8). Bardoxolone methyl, a novel synthetic triterpenoid with antioxidant and anti-inflammatory properties, has been recorded to improve kidney function in patients with advanced diabetic nephropathy already receiving RAS blockers, with few adverse events (Rojas-Rivera, Ortiz, and Egido 321714). In a randomized, double-blind, placebo-controlled, 2-arm parallel trial, silymarin, an herbal drug with antioxidant and anti-inflammatory properties, has been noted to significantly reduce urinary albumin-creatinine ratio, urinary levels of TNF-α and urinary and serum levels of MDA also decreased significantly in the silymarin compared to the placebo group in patients with diabetic nephropathy (Fallahzadeh et al. 896–903).

### 2.4. Diabetic Neuropathy

Diabetic neuropathy is a heterogeneous condition that encompasses a set of clinical syndromes, each being characterized by diffuse or focal damage to peripheral somatic or
autonomic nerve fibers attributable to DM or to factors associated with the disease (J. L. Edwards et al. 1–34). The prevalence rates for diabetic neuropathy range between 8.3 to 45% on account of the heterogeneous nature of the disease (Rani et al. 51–7). Similarly, varying rates (3–26%) of peripheral neuropathy from the Indian subcontinent have been reported (Ramachandran et al. 51; Weerasuriya et al. 439; Pradeepa et al. 407–12).

2.4.1. Pathophysiology

The first pathological change in the microvasculature is a physiological shift favoring vasoconstriction. As the disease progresses, neuronal dysfunction correlates closely with the development of vascular abnormalities, such as capillary basement membrane thickening and endothelial hyperplasia, which contribute to diminished oxygen tension and hypoxia. Indeed, hemodynamic abnormalities, hypoperfusion, and neuronal ischemia are prominent characteristics of diabetic neuropathy (Duby et al. 160–73). The pathophysiology of diabetic neuropathy although complex, hyperglycemia, activation of polyol, AGEs, hexosamine, diacylglycerol/PKC, oxidative stress, nitric oxide, and inflammation are believed to play key roles in diabetic neuropathy (Fig. 2.5) (Hosseini and Abdollahi 168039).

Fig. 2.5: Pathophysiology of diabetic neuropathy (Hosseini and Abdollahi 168039)

AGEs: advanced glycation end products; RAGEs: receptor for advanced glycation end products; NF-κB: nuclear factor kappa B; AD: aldose reductase; SDH: sorbitol dehydrogenase; GSH: glutathione; GSSG: oxidized glutathione; F-6-P: fructose-6-phosphate; UDPGlcNAc: uridine diphosphate-N-acetylglucosamine; PAI-1: plasminogen activator inhibitor-1; TGF-β1: transforming growth factor-β1; DAG: diacylglycerol; PKC: protein
kinase C; ROS: reactive oxygen species; RNS: reactive nitrogen species; PARP: poly ADP ribose polymerase; Mt: mitochondria; MMPs: mitochondrial membrane potentials; Cyc: cytochrome c; NO: nitric oxide; LDL: low-density lipoprotein; LOX1: oxidised LDL receptor 1; TLR4: toll-like receptor 4; FFA: free fatty acids; TG: triglycerides; HDL: high-density lipoprotein; CyK: cytokine.

2.4.2. Oxidative stress and ROS in diabetic neuropathy

Oxidative stress and ROS generated through increased glycolytic process connect the metabolic initiators and physiological mediators implicated in progressive nerve fiber damage, dysfunction, and loss in diabetic neuropathy (J. L. Edwards et al. 1; Yagihashi, Mizukami, and Sugimoto 18). Moreover, mitochondrial damage is attributed to excess formation of ROS and RNS. Hyperglycemia induces mitochondrial alterations such as release of cytochrome C, activation of caspase 3, altered biogenesis and fission, which lead to apoptosis. Reduced mitochondrial action potentials (MMP) with poor energy synthesis of adenosine-5'-triphosphate (ATP) are caused due to excessive entry of glucose, resulting in surplus transport of oxidant electrons into the mitochondria. Neurotrophic support such as neurotrophin-3 (NT-3) and nerve growth factor (NGF) are also reduced by mitochondrial injury. The progression of diabetic neuropathy in a distal–proximal axon length-dependent manner indicates that the damage is initiated in the axons, which are susceptible to hyperglycemic damage due to their direct access to nerve blood supply and their large population of mitochondria (Yagihashi, Mizukami, and Sugimoto 18-32). Oxidative stress in conjunction with hyperglycemia activates poly ADP-ribose polymerase (PARP), which further cleaves nicotinamide adenine dinucleotide (NAD+) to nicotinamide and ADP-ribose residues. This process continues by a link to nuclear proteins, thus altering gene transcription and expression, NAD+ depletion, oxidative stress, and diversion of glycolytic intermediates to other pathogenic pathways such as PKC and AGEs (Fig. 2.5) (J. L. Edwards et al. 1–34). Collectively, the polyol, AGEs, PKC, hexosamine, and PARP contribute to neuronal damage via formation of ROS. The AGEs and polyol pathways openly modify the redox capacity of the cell either through weakening of necessary components of glutathione recycling or by direct construction of ROS. The hexosamine, PKC, and PARP pathways inflict damage through the expression of inflammatory proteins (J. L. Edwards et al. 1–34).
2.4.3. Treatment options

The treatment of diabetic neuropathy can be focused on different strategies. Treatment based on underlying pathogenetic mechanisms includes intensive glycemic control and the use of aldose reductase inhibitors. The symptomatic treatment comprises of standard pharmacological approach involving first-line tricyclic antidepressants, second-line anticonvulsants and third-line opioids. However, only duloxetine and pregabalin are US Food and Drug Administration (FDA)-approved (Tavakoli, Fadavi, and Malik 48–51).

2.4.4. Role of antioxidants

Considering the imperative role of oxidative stress in mediating nerve dysfunction in diabetes, a number of antioxidants have been tested in various animal models (Table 2.2).

Table 2.2: Effect of various anti-oxidants in in vivo models of diabetic neuropathy (Negi et al. 71; Edwards et al. 1)

<table>
<thead>
<tr>
<th>Anti-oxidant</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>Corrects motor nerve conduction velocity (MNCV) and nerve blood flow (NBF) deficits. Improves Nrf2 and HO1 level, decreases NF-κB, iNOS and COX-2 levels</td>
</tr>
<tr>
<td>FeTMPyP and FeTPPS</td>
<td>Corrects MNCV and NBF deficits. Protection against nitrosative stress</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Ameliorates the alterations in MNCV and NBF, significant reduction in DNA fragmentation, Abrogation of NF-κB, iNOS and COX-2 levels</td>
</tr>
<tr>
<td>Edaravone</td>
<td>Protection against MNCV and NBF deficits, restores anti-oxidant enzyme levels</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Attenuation of thermal hyperalgesia, inhibition of TNF-α and NO production</td>
</tr>
<tr>
<td>Trolox</td>
<td>Ameliorates the alterations in MNCV, NBF, hyperalgesia, MDA levels and antioxidant enzymes</td>
</tr>
<tr>
<td>Apocynin</td>
<td>Protection against MNCV and NBF deficits, restores blood glucose, Decreases oxidative stress by inhibition of NAD(P)H oxidase</td>
</tr>
<tr>
<td>Tempol</td>
<td>Corrects MNCV, NBF and SNCV deficits</td>
</tr>
<tr>
<td>DL-α-Lipoic acid</td>
<td>Restores NBF and MNCV deficits</td>
</tr>
<tr>
<td>Probucol</td>
<td>Corrects NBF, normalized MNCV and SNCV</td>
</tr>
<tr>
<td>Eugenol</td>
<td>Improves vascular and neural deficits in STZ-treated rats</td>
</tr>
<tr>
<td>Taurine</td>
<td>Plasma taurine is depleted in diabetic rats and replacement decreases hyperalgesia and other neural and vascular deficits</td>
</tr>
<tr>
<td>Oleuropein</td>
<td>Decreases blood glucose as well as oxidative stress</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Attenuates thermal hyperalgesia and cold allodynia</td>
</tr>
<tr>
<td>Rutin</td>
<td>A polyphenol that may activate the antioxidant response, prevents oxidative stress</td>
</tr>
<tr>
<td>Dimethylthiourea</td>
<td>A hydroxyl radical scavenger, prevents diabetes-induced mechanical and thermal nociceptive sensitivity and nerve blood flow deficits</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>Precursor of reduced glutathione increases tissue reduced glutathione, improves motor nerve conduction velocity and decreases oxidative stress</td>
</tr>
</tbody>
</table>
HO-1: heme oxygenase 1; NF-κB: nuclear factor-kappaB; iNOS: Inducible nitric oxide synthase; COX-2: cyclooxygenase-2; TNF-α: Tumor necrosis factor alpha; NO: nitric oxide; MDA: malondialdehyde; SNCV: sensory nerve conduction velocity; Nicotinamide adenine dinucleotide phosphate (NADPH)

Two 52-week randomized placebo-controlled diabetic neuropathy trials demonstrated significant improvements with the dietary anti-oxidant acetyl-L-carnitine in sural nerve fiber numbers and regenerating nerve fibers (Sima et al. 89–94). The recently published multicenter randomized double masked parallel Neurological Assessment of Thioctic Acid in Diabetic Neuropathy (NATHAN) 1 trial showed that four year treatment with α-lipoic acid, a potent antioxidant could lead to clinically relevant improvement of neurological functions (Firuzi et al. 3871–88). Another recent systematic review and meta-analysis has shown that treatment with α-lipoic acid (300-600 mg/day i.v. for 2-4 weeks) is safe and that the treatment can significantly improve both positive neuropathic symptoms and nerve conduction velocity (Han et al. 465–71). α-lipoic acid is approved in Germany as an agent for the treatment of diabetic neuropathy (Nishikawa and Araki 105–7). A randomized double-blind placebo-controlled study confirms that ubiquinone, a known antioxidant of mitochondrial origin, at 400 mg/kg for 12 weeks improves clinical outcomes and nerve conduction parameters of diabetic polyneuropathy. Furthermore, it reduces oxidative stress without significant adverse events (Hernández-Ojeda et al. 352–8). QR-333 is another antioxidant containing quercetin, a flavonoid with aldose reductase inhibitor effects. A randomized, placebo-controlled, double-blind trial demonstrated that QR-333 can safely offer relief of symptoms of diabetic neuropathy and improve quality of life (Valensi et al. 247–53). Metanx is a product containing L-methylfolate, pyridoxal 5'-phosphate, and methylcobalamin for counteracting oxidative stress in peripheral nerve. Metanx has been shown to improve multiple parameters of peripheral nerve function and increase intraepidermal nerve fiber density in Zucker diabetic fatty rats (Shevalye et al. 2126–33). Metanx is currently undergoing phase IV clinical trial for diabetic neuropathy (Negi et al. 71-8).

2.5. Cancer

Cancer is the second most common disease in India responsible for maximum mortality with about 0.3 million deaths per year (Ali, Wani, and Saleem 56–70). Cancer is a heterogeneous group of diseases that originates through a multistep process. The first stage, the initiation, is caused by the acquisition in a cell of a mutation that can provide a growth advantage and/or
irreversible alterations in cellular homeostasis and differentiation. The next step, the promotion, can be a potentially reversible or interruptible clonal expansion of the initiated cell by a combination of growth stimulation and inhibition of apoptosis. Further progression steps occur upon clonal expansion of the initial cells and accumulation of a sufficient number of mutations and epigenetic alterations to acquire growth stimulus-independency and resistance to growth inhibitors and apoptosis, ultimately leading to an unlimited replicative potential (Vogelstein and Kinzler 138-41).

Since the carcinogenesis is the multistep process, arresting one or several of the steps may impede or delay the development of cancer. One approach to restrain the incidence of cancer is cancer chemoprevention that involves long-term use of a variety of oral natural, synthetic, or biologic substances in pharmacologic doses to reverse, suppress, or prevent the development of cancer (Tsao, Kim, and Hong 150-80). Anti-cancer agents can be preliminarily screened by using in vitro cytotoxicity and apoptosis assays.

2.5.1. *In vitro* cytotoxicity screening

Cytotoxicity assays have been developed which use different parameters associated with cell death and proliferation (Weyermann, Lochmann, and Zimmer 369-76). The basic steps of *in-vitro* cytotoxic screening include: (a) isolation of cells, (b) incubation of cells with drugs, (c) assessment of cell survival and (d) interpretation of the result (Sukhramani, Desai, and Suthar 124–7).

**The MTT assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a sensitive, quantitative and reliable colorimetric test that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue insoluble formazan product by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore accumulates in the healthy cells. The amount of formazan produced is directly proportional to the cell number in a wide range of cell lines (Mosmann 55–63). The assay provides provide important preliminary data to help select natural products with potential anti-neoplastic properties (Cardellina II et al. 25–36). Other commonly employed methods for detection of cytotoxicity or cell viability upon exposure to toxic substances include lactate dehydrogenase (LDH) leakage assay,
sulforhodamine B (SRB) assay, and neutral red assay (Weyermann, Lochmann, and Zimmer 369-76).

2.5.2. *In vitro* assays for cell death determination

2.5.2.1. Apoptosis

The mechanisms of cell death in response to environmental or developmental signals include apoptosis and necrosis. Apoptosis is defined as programmed cell death, characterized by changes including nuclear and cytoplasmic condensation, membrane budding, internucleosomal DNA fragmentation and formation of apoptotic bodies. Necrosis, on the other hand, is the alternative to apoptotic cell death and is considered as a toxic uncontrolled process, where the cell is a passive victim and follows an energy-independent mode of death (Majno and Joris 3–15).

Several studies have reported that many chemopreventive agents can decrease tumor cell growth by induction of apoptosis in various animal models for carcinogenesis and in human chemoprevention trials (Galati et al. 311; S. Y. Sun, Hail, and Lotan 662). Furthermore, apoptosis induction is arguably the most potent defense measure against cancer. The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events (Fig. 2.6).

![Fig. 2.6: Schematic representation of apoptotic events (Elmore 495–516)](image)

MPT: mitochondrial permeability transition; SET: a nucleosome assembly protein

2.5.2.1.1. Apoptosis estimation *in vitro*

Cell viability assay

Calculation of cell viability and the total number of viable cells are widely used methods for estimation effects of apoptosis triggering molecules. The most common assays for estimation
of cell viability are based on cell membrane integrity and among them dye exclusion assay with trypan blue is widely used in routine laboratory work. Blue stained cells are dead cells and the percentage of viable cells is calculated as ratio of viable (unstained) and total number of enumerated cells (dead and viable cells). Cell counting is commonly done using hemocytometer and classic light microscope (Jurisic and Bumbasirevic 49–54).

**Assay for estimation of DNA fragmentation**

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis (Wyllie 555–6). This assay involves extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis resulting in a characteristic “DNA ladder” with each band in the ladder separated in size by approximately 180 base pairs. This methodology is easy to perform, has a sensitivity of $1 \times 10^6$ cells, and is useful for tissues and cell cultures with high numbers of apoptotic cells per tissue mass or volume, respectively.

The TUNEL (Terminal dUTP Nick End-Labeling) method is used to assay the endonuclease cleavage products by specific enzymatic end labeling of DNA fragments (Jurisic and Bumbasirevic 49–54). Terminal transferase is used to add labeled uridine triphosphate (UTP) to the 3’ hydroxyl DNA ends. The dUTP can then be labelled with a variety of probes to allow detection by light microscopy, fluorescence microscopy or flow cytometry. This assay is also very sensitive, allowing detection of a single cell via fluorescence microscopy or as few as ~100 cells via flow cytometry. It is also a fast technique and can be completed within 3 hours (Elmore 495–516).

Other commonly employed apoptosis assays involve cytomorphological alterations, detection of caspases, cleaved substrates, regulators and inhibitors, estimation of cell membrane alterations by Annexin V/propidium iodide (ANNV/PI) assay, detection of apoptosis in whole mounts, and mitochondrial assays (Elmore 495–516).

Western blot technique and appropriate monoclonal antibodies can be utilized to determine various proteins involved in regulation of apoptosis, such as pro-apoptotic Bcl-2 family members (bid, bad, bax, bag, bak, bcl-xs), antiapoptotic bcl-2 members (bcl-xL, Bod, Bim, Mcl-1), apoptosis activators (Cytochrome c, Caspase 9 and apoptosis inhibitory factor) as well as apoptosis inhibitors (Akt, Bap31, Survivin) (Jurisic and Bumbasirevic 49–54).

### 2.6. Colorectal carcinoma

Colorectal carcinoma (CRC) is one of the leading causes of cancer related morbidity and mortality in the world, being the third most common cancer in men (663,000 cases, 10.0% of
the total cancers) and the second in women (570,000 cases, 9.4% of the total cases) worldwide (Ferlay et al. 2893–917). Colon cancer incidence rates are alarmingly increasing in Asia, due to the adaptation of the western lifestyle, dietary habits, and behavior (Sivagami et al. 11–22). Population based time trend studies also show a rising trend in the incidence of CRC in India (Yeole 97–100).

### 2.6.1. Histopathology of development

The digestive surface of the human large intestine is characterized by a monolayer of specialized epithelial cells that forms invaginations called crypts. The first recognizable manifestation of epithelial alteration during colorectal tumor development is the Aberrant Crypt Foci (ACF). These are small hyper- or dysplastic lesions characterized by bigger size than the normal crypts, increased pericryptal space that separates them from the normal crypts, a thicker layer of epithelial cells that often stains darker compared with normal crypts, generally oval rather than circular openings (Cheng and Lai 2642-9). Although only a small number of ACF will ultimately progress to CRC, larger ACF with altered morphology, dysplastic histology and associated gene mutations remain high-risk candidates for adenoma and CRC formation (Hurlstone and Cross 173–81).

Upon increase of birth/loss ratio among epithelial cell, their progressive accumulation results in a benign tumor mass or polyp. Histologically, there are two major types of polyps: the hyperplastic or non-neoplastic polyp and the dysplastic or adenomatous polyp. The adenomatous polyp is represented by abnormal cells with disrupted normal tissue architecture, and hyperchromatic nuclei (Takata et al. 332–9). As an adenoma progresses, more undifferentiated cells appear, with marked pleomorphism and nuclear:cytoplasm ratio close to 1. Moreover, tumor cells show aberrant orientations and grow in disorganized fashion, forming carcinoma in situ. Finally, malignant adenocarcinomas are characterized by the ability to invade the surrounding tissues through the muscularis mucosae and into the stromal compartment, and migrate to distal organs such as the liver, where they can metastasize (Kumar et al. 4849).

### 2.6.2. Molecular mechanisms

Steps that transform normal colonic epithelium into benign neoplasia (adenoma), followed by invasive carcinoma and eventually metastatic cancer are described in the classic tumor progression model (Fig. 2.7) (Vogelstein et al. 525–32).
Fig. 2.7: The canonical Vogelstein Model- the most common analogy for the adenoma carcinoma sequence (Fearon and Vogelstein 759–67).

Loss of APC function is the initial molecular event that leads to adenoma formation. Germline mutations in the APC gene have been identified as the cause of familial adenomatous polyposis (FAP), an inheritable intestinal cancer syndrome (Groden et al. 589–600); and APC is mutated in more than 80% of all sporadic cancers (Powell et al. 235–7). APC belongs to the WNT signaling pathway, where it interacts with other proteins like AXINS and GSK3β to form a complex that down-regulates the cellular levels of β-catenin (Clevers and Nusse 1192–205). Activating mutations in β-Catenin gene have also been observed in more than 10% of CRC (Morin et al. 1787–90). When activated, β-catenin interacts with the transcriptional complex LEF/TCF in the nucleus to induce the expression of growth promoting genes, such as MYC and CYCLIN D1. Additional genetic and epigenetic alterations (KRAS, P53, etc.) follow this early set of molecular changes to sustain the progression of the transformation process till the carcinoma and metastasis stages.

Along with the WNT-β-catenin signaling pathway, other best-studied pathways that are deregulated in CRC are the TGF-β, epidermal growth factor receptor (EGFR)-MAPK, and the phosphatidylinositol 3-kinase (PI3K) pathway (Walther et al. 489-99) and they control the hallmark behaviors of cancer including cell proliferation, differentiation, apoptosis, immortalization, angiogenesis and invasion. Key tumor-suppressor genes that do not necessarily mediate their effects through signal pathway deregulation, such as TP53, and
review cytogenetic aberrations such as 18q loss of heterozygosity (LOH) also affect the malignant transformation of colonic epithelial cells (Vogelstein et al. 525–32).

2.6.3. Involvement of oxidative stress

CRC originates from the epithelial cells that line the bowel. These cells divide rapidly and have a high metabolic rate that is responsible for increased oxidation of DNA (Foksinski et al. 1449–54). Study on primary rat colonocytes has shown that cells from lower crypt sections are more sensitive towards H2O2 damage than differentiated cells at the surface of the crypt (Oberreuther-Moschner, Rechkemmer, and Pool-Zobel 212–8). Since proliferating stem cells and their dividing daughter cells in the colon are based in the lower part of the crypt, they are the putative target cells of CRC. Stem or progenitor cells have been shown to be very sensitive to the redox environment. Their self-renewal and differentiation depend largely on the redox environment in the gut mucosa. Proliferating cells are also exceptionally sensitive against DNA damage because the DNA is present as single strand in the S-phase of the cell cycle and serves as template for the complement strand in daughter cells. DNA damage in single strand could lead to varying mutations in the DNA of daughter cells, which cannot be repaired (Oberreuther-Moschner, Rechkemmer, and Pool-Zobel 212–8). DNA damage can result in either cell cycle arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with CRC (Valko et al. 1–40).

Previous evidence suggests that the generation of ROS may play important role in the the initiation, promotion, and progression stages of carcinogenesis (Valko et al. 44–84). It was found that the human colorectal tumors including adenomas and carcinomas have increased levels of different markers of oxidative stress, such as elevated levels of ROS, NO (Haklar et al. 219–24), 8-oxo-dG in DNA (Guz et al. 170–3), lipid peroxides, glutathione peroxidase, catalase (Rainis et al. 526–30), and decreased methylation of cytosine in DNA (Guz et al. 170–3). Besides, increased leukocyte activation by lipid modifications in the carcinogenic tissue (Rainis et al. 526–30) indicates possible contribution of inflammatory cells to further oxidative stress (Haklar et al. 219–24). Whole-blood levels of ROS have been reported to be higher in patients with history of sporadic CRC in comparison to age- and gender-matched healthy controls (van der Logt et al. 182–7).
2.6.4. Treatment

Surgery, radiation, and systemic therapy form the cornerstone of treatment for patients with CRC. Stage and individual patient characteristics are the key determinants in treatment selection. Systemic therapy relies heavily on 5-fluorouracil (5-FU) combined with leucovorin, with a number of treatment regimens that vary by dose and schedule. Additional systemic options include capecitabine, irinotecan, oxaliplatin, and the monoclonal antibodies cetuximab, panitumumab, and bevacizumab (Mayer 573; “NCCN Clinical Practice Guidelines in Oncology: Colon Cancer”). Systemic therapy is typically administered as adjuvant treatment following surgical resection in order to eliminate any remaining micrometastases (“NCCN Clinical Practice Guidelines in Oncology: Colon Cancer”).

2.6.5. Phytochemicals for chemoprevention

Synthetic non-steroidal anti-inflammatory drugs (NSAIDS) have been identified as potential CRC chemopreventive agents; however, most of these synthetic agents are associated with unwanted and sometimes fatal side effects (Madka and Rao [Epub ahead of print]). The salient features of a candidate compound for chemoprevention are (Ponz 59–69):

- Well-documented biological basis of activity
- Little or non toxic effects in normal and healthy cells
- High efficacy against multiple sites
- Capability of oral consumption
- Affordable cost, and
- Simple way of administration

As the carcinogenic process may be delayed or interrupted in each of its phases, several anticarcinogenic mechanisms may be elucidated (Fig. 2.8). Chemopreventive blockers like indole-3-carbinol, sulforaphane and flavonoids act immediately before or after initiation of carcinogenesis. They prevent carcinogens to hit their cellular targets by enhancing carcinogen detoxification, modifying carcinogen uptake and metabolism, scavenging ROS, and enhancing DNA repair. On the other hand, the chemopreventive suppressors function by interfering with the promotion and progression of carcinogenesis through their effect on cell proliferation, integration and programmed cell death which inhibits translation of initiated cells to cancerous cells (Fig. 2.8) (Johnson 207; M. Russo et al. 61). These agents such as beta-carotene, curcumin, gingerol and resveratrol suppress carcinogenesis by blocking phase 1 enzymes, initiation of phase 2 enzymes, preventing ROS from damaging DNA, suppressing
type 2 cell multiplications generated by carcinogenesis and inhibiting normal cells from transforming to cancer cells (Tanaka, Kohno, and Mori 165–77).

![Fig. 2.8: Classification of chemopreventive agents on the basis of their mechanism of action on cancer multistage (Surh 768–80)](image)

There is mounting evidence in support of the efficacy of naturally-occurring phytochemicals possessing anti-colon carcinogenic effects. A number of studies have investigated the mechanisms underlying the inhibition of colon cancer development by curcumin that possesses antiinflammatory, antioxidant, and antineoplastic potential. The major targets of the signaling pathways regulated by curcumin in CRC include EGFR, AMPK COX-2, MAPK, and Wnt/β-catenin. The mechanisms of chemoprevention proposed for curcumin involve tumor growth inhibition, suppression of carcinogenesis, arresting G2/M phase of cell cycle, and inducing apoptosis in colon cancer cells (Chung, Lim, and Lee 984–93). A non-randomized, open-label phase IIa clinical study reported that oral intake of curcumin (4 g for 30 days) decreased the number of ACF in colon and that curcumin can be used to prevent of colorectal neoplasia (Carroll et al. 354–64).

Supplementation with resveratrol, a naturally occurring phenolic phytochemical in red grapes, has been reported to reduce the levels of dextran sodium sulfate (DSS)-mediated colitis inflammation markers, such as iNOS, COX-2 and TNF-α, in mice (Cui et al. 549–59). Resveratrol also inhibited the 1,2-dimethylhydrazine-induced tumor burden per animal, per group and over the three regimens of colon carcinogenesis (initiation, post-initiation and
entire period) (Sengottuvelan and Nalini 145–53). Resveratrol has been documented to suppress IGF-1-induced human colon cancer cell proliferation by activating p53 signaling pathways. Additionally, resveratrol inhibits human colon cancer cell proliferation and promotes apoptosis by suppressing the pentose phosphate pathway and focal adhesion kinase, a critical protein for cell-extracellular matrix communication (Vanamala et al. 49). Furthermore, resveratrol exerts synergistic anti-cancer effects on chemoresistant HT-29 colon cancer cells by regulating the AMPK signaling pathway (Hwang et al. 441–8). Daily p.o. doses of 0.5 g or 10 g resveratrol for 8 days inhibited tumor cell proliferation by 5% with no resveratrol-related adverse effects in patients with resectable CRC (Patel et al. 7392–9). A phase I pilot study in patients with colon cancer demonstrated that dietary supplementation with resveratrol-containing products is a potential colon cancer preventive strategy and that Wnt/β-catenin is a potential target for resveratrol in normal colonic mucosa (Nguyen et al. 25–37).

p-methoxycinnamic acid (p-MCA), an active antiproliferative phenolic acid of rice bran, has been reported to exert a strong chemopreventive activity against 1,2-dimethylhydrazine-induced colon carcinogenesis in rats by its ability to prevent the alterations in DMH-induced circulatory and tissue oxidative stress and preneoplastic changes (Sivagami et al. 11-22).

2.7. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC), accounting for 70%–85% of primary liver cancer cases, is one of the most frequent tumors representing the fifth commonest malignancy worldwide in men and the seventh in women (Globocan 2008 Cancer Fact Sheet). There were an estimated 694 000 deaths from liver cancer in 2008, and because of its high fatality and extremely poor prognosis, HCC is the third leading cause of cancer-related mortality, exceeded only by cancers of the lung and stomach (Gomaa et al. 4300; Globocan 2008 Cancer Fact Sheet). In India, the mean incidence of HCC in four population-based registries is 2.77% for males and 1.38% for females. The prevalence of HCC in India varies from 0.2% to 1.6% (Annual Report 1987; Jayant et al.). The major risk factors for development of HCC are cirrhosis of liver, chronic hepatitis B and C (HBV and HCV), environmental toxins, and some metabolic and immune-related diseases (Parikh and Hyman 194–202).

2.7.1. The multistep process of hepatocellular carcinogenesis

Hepatic injury induced by risk factors causes necrosis followed by hepatocyte proliferation (Fig. 2.9). Repeated cycles of necrosis-liver regeneration foster a chronic liver disease leading
Cirrhosis is characterized by the formation of hyperplastic liver nodules, surrounded by collagen deposition and scarring of liver, and regenerative nodules. During this process, different subtypes of foci of altered hepatocytes (FAH) develop in the liver, followed by low-grade dysplastic nodules (DNs), and then by high-grade DNs, considered as premalignant lesions, composed of small cells showing mild trabecular disarrays, increased nuclear-cytoplasmic ratio, and tendency to cytoplasmic basophilia. Subsequently, HCC develops which can be further classified into well-differentiated, moderately differentiated and poorly differentiated tumors (Kern, Breuhahn, and Schirmacher 67-112).

Fig. 2.9: Schematic representation of hepatocellular carcinoma (Frau et al. 179–93)

### 2.7.2. Pathogenesis

HCC is phenotypically and genetically heterogenous, possibly in part due to the heterogeneity of etiologic factors implicated in HCC development, the complex functions of the liver cell, and the advanced stage at which HCC usually becomes clinically symptomatic and is diagnosed. Malignant transformation of hepatocytes may occur regardless of the etiologic agent through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation, immune response, and oxidative DNA damage. This may result in genetic alterations that cause activation of cellular oncogenes or inactivation of tumor suppressor genes (Dufour et al. 860–7), possibly in cooperation with genomic instability, including DNA mismatch repair defects and impaired chromosomal segregation, over expression of growth and angiogenic factors, and telomerase activation. Further, epigenetic modifications such as aberrant methylation also seem to be involved in the molecular pathogenesis of HCC (Sanyal, Yoon, and Lencioni 14-22).
During the initiation of the hepatocarcinogenesis process, a progressively increasing genomic instability arises as a consequence of the mutagenic action of carcinogens and oxidative stress. This favors the development of genetic and epigenetic alterations in mature hepatocytes or stem cells, errors in DNA repair, immunologic alterations, and aberrant activation of several signaling pathways, including the EGFR, ERK, phosphoinositol 3-kinase (PI3K)/AKT, c-MET/HGF, WNT, Hedgehog and apoptosis signaling (Meguro et al. 818672). The activation of mitogenic pathways confers growth advantages and favors the selection of cells with somatic mutation and DNA amplifications/deletions. Clones of autonomously proliferating initiated cell then appear in the liver and progress to DNs and HCCs.

While there are evidences that HBV and possibly also HCV may under certain circumstances play an additional direct role in the molecular hepatocarcinogenesis, aflatoxins have been shown to induce mutations of the p53 tumor suppressor gene, thus emphasizing on the contribution of environmental factors to tumor development at the molecular level. Further, in a transgenic mouse model, it has been shown that chronic immune-mediated liver cell injury without environmental or infectious agents is sufficient to cause HCC (Nakamoto, Guidotti, et al. 341; Nakamoto, Kaneko, et al. 1105). Another transgenic mouse model demonstrated that NF-kB may be the link between inflammation and HCC development (Balkwill and Coussens 405; Pikarsky et al. 461). Individual polymorphisms of drug metabolizing enzymes, including various cytochrome P450 oxidases, N-acetyltransferases, and glutathione-S-transferase, may contribute to the genetic susceptibility to HCC development (Chen and Chen 1046–9).

2.7.3. Involvement of oxidative stress

Inflammation, a common response in the human liver, is involved in the development of chronic hepatitis, cirrhosis, steatosis, hepatocarcinogenesis, and in metastasis (Olaya S–20). ROS production, an integral part of the inflammatory processes (Bishayee et al. 753–63), can induce mutations in key cancer genes (Klaunig, Kamendulis, and Hocevar 96–109). Normally, this process is prevented by DNA repair enzymatic systems that maintain sequence fidelity during DNA replication. However, overproduction of free radicals in chronic inflammatory diseases is thought to saturate the ability of the cell to repair DNA damage prior to replications (Hofseth et al. 1887–94).

Compelling evidences suggest that known risk factors for HCC including HBV, HCV, and alcohol consumption lead to the generation of ROS and are associated with oxidative stress (Marra et al. 171). Direct induction of oxidative stress by HCV core protein has been shown to exacerbate liver injury (Wen et al. 230–40). A link between oxidative stress and liver
pathogenesis is also supported by the successful use of antioxidant therapy to treat liver injury caused by chronic HCV infection (Gabbay et al. 5317–23). Ethanol metabolism via the alcohol dehydrogenase pathway and microsomal ethanol oxidizing system contribute substantially to the production of acetaldehyde and generation of ROS (Akbar and Cederbaum 277–84). Hepatitis B virus X protein (HBx) via its association with mitochondria has been shown to induce oxidative stress which in turn leads to activation of a series of transcription factors (Waris, Huh, and Siddiqui 7721–30). Moreover, in addition to direct production of ROS by these pathogens, liver infiltration by activated phagocytic cells provides an additional source of ROS production that promotes oxidative stress via interleukin or NO production that can damage proteins, lipids and DNA (Jüngst et al. 1663–72).

2.7.4. Treatment

Current therapeutic strategies for HCC can be divided into established therapies such as surgical interventions (tumor resection and liver transplantation (LTx)), percutaneous interventions (ethanol injection, radiofrequency thermal ablation), transarterial interventions (embolization, chemoperfusion, or chemoembolization), and experimental strategies such as radiation therapy and systemic treatments including chemotherapy, immunological and hormonal therapies and, more recently, the introduction of new specific molecular target drugs. Sorafenib represents the only primary systemic therapy that has demonstrated, unlike the other treatments previously described, an increase in survival rate in patients affected with advanced HCC during phase III clinical trials (SHARP study). Currently, phase III trials, are directed in order to test the activity and safety of new emerging drugs with targeted activity including sunitinib, gefitinib, cetuximab, bevacizumab and erlotinib (Rossi et al. 348–59).

2.7.5. Phytochemicals for chemoprevention

Accumulating body of evidence suggests that polyphenolic compounds, one of the major classes of phytochemicals, are promising candidate agents for HCC chemoprevention (Table 2.3).
Table 2.3: *In vivo* effects of plant polyphenols on the development and growth of HCC (Stagos et al. 2155–70)

<table>
<thead>
<tr>
<th>Plant polyphenol</th>
<th>Species, strain, sex</th>
<th>Inducing agent</th>
<th>Biomarkers affected</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin 10 mg/kg in water</td>
<td>Male Fischer-344 rats</td>
<td>DEN 200 mg/kg i.p.</td>
<td>↑antioxidant defense system; ↓lipid peroxidation levels; ↑GSH; ↑GSH/GSSG ratio; ↑catalase; ↑GPx</td>
<td>Decreased the total area and number of preneoplastic lesions</td>
</tr>
<tr>
<td>200 mg/kg oral</td>
<td>Male Wistar rats</td>
<td>DEN 20 mg/kg oral</td>
<td>↓MDA; ↓GSH; ↓GPx; ↓GR; ↓mutations in p53 gene</td>
<td>Prevented as shown in histopathological examination</td>
</tr>
<tr>
<td>15, 30, 45 mg/kg oral</td>
<td>Male ICR mice</td>
<td>AFB(1) 0.75 mg/kg oral</td>
<td>↑GSH; ↑SOD; ↑MDA</td>
<td>Protected from pro-oxidant liver damage by promoting antioxidative defense systems and inhibiting lipid peroxidation</td>
</tr>
<tr>
<td>1% in diet</td>
<td>Rats</td>
<td>Initiation IQ 100 mg/kg i.; promotion PB (0.05% in diet) and a single dose of D-galactosamine (100 mg/kg, i.p.)</td>
<td>–</td>
<td>Reduced the number of GST-P positive foci in liver</td>
</tr>
<tr>
<td>0.75, 1, 1.5% in diet</td>
<td>Male Fischer-344 rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion 0.05% PB</td>
<td>–</td>
<td>Reduced the evolution of preneoplastic foci into persistent and hyperplastic nodules as well as the number of carcinomas</td>
</tr>
<tr>
<td>EGCG</td>
<td>Male Fischer-344 rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion PB 0.05% in water</td>
<td>–</td>
<td>Decreased the number and total volume of GST-P positive foci in liver cancer</td>
</tr>
<tr>
<td>0.05–0.1% in water</td>
<td>C3H/HeNCrj mice</td>
<td>–</td>
<td>–</td>
<td>Reduced the incidence of hepatoma</td>
</tr>
<tr>
<td>Luteolin 50, 200 ppm in diet</td>
<td>Male BALB/c athymic nude mice</td>
<td>–</td>
<td>–</td>
<td>Inhibited xenografted tumor growth in liver</td>
</tr>
<tr>
<td>Silibinin 80, 160 mg/kg gavage</td>
<td>Nude mice</td>
<td>–</td>
<td>Apoptosis; G1 arrest; ↓[NF]-B; ↓Rb phosphorylation; ↓survivin; ↓ERK; ↓Akt</td>
<td>Reduced development of hepatoma Huh-7 xenografts</td>
</tr>
<tr>
<td>Silymarin 1000 ppm in diet</td>
<td>Male Wistar albino rats</td>
<td>DEN 0.01% in water</td>
<td>↑antioxidant defense system; ↓lipid peroxidation levels; ↑GSH; ↑SOD; ↑catalase; ↑GPx; ↑GR</td>
<td>Reduced the number of liver nodules</td>
</tr>
<tr>
<td>Trans-resveratrol 50–300 mg/kg in diet</td>
<td>Female Sprague–Dawley rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion PB 0.05% in water</td>
<td>↑Bax; ↓Bcl-2</td>
<td>Reduced the incidence, total number and multiplicity of visible hepatocyte nodules. Decreased cell proliferation and increased apoptosis in liver</td>
</tr>
<tr>
<td>Plant polyphenol</td>
<td>Species, strain, sex</td>
<td>Inducing agent</td>
<td>Biomarkers affected</td>
<td>Outcome</td>
</tr>
<tr>
<td>---------------------------------</td>
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<td>----------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Trans-resveratrol 50–300 mg/kg in diet</td>
<td>Female Sprague–Dawley rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion PB 0.05% in water</td>
<td>↓ lipid peroxidation; ↓ protein oxidation; ↓ nitric oxide synthase; ↓ 3-nitrotyrosine; ↑ Nrf2</td>
<td>Attenuation of oxidative stress; suppression of inflammatory response</td>
</tr>
<tr>
<td>Trans-resveratrol 50, 100, 300 mg/kg in diet</td>
<td>Female Sprague–Dawley rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion PB 0.05% in water</td>
<td>↓ Hsp70; ↓ COX-2; ↓ NF-κB</td>
<td>Anti-inflammation in liver</td>
</tr>
<tr>
<td>Curcumin 10, 100, 200 lmol/0.2 ml corn oil</td>
<td>Male BALB/c mice</td>
<td>DHPN 0.1% in water</td>
<td>–</td>
<td>Inhibited multiplicity and incidence of HCC</td>
</tr>
<tr>
<td>0.2% in diet</td>
<td>Male C3H/HeN mice</td>
<td>DEN i.p.</td>
<td>↓ p21, ↓ PCNA, ↓ CDC2</td>
<td>Inhibited multiplicity and incidence of HCC</td>
</tr>
<tr>
<td>100 mg/kg oral</td>
<td>Male Wistar rats</td>
<td>Initiation DEN 200 mg/kg i.p.; promotion PB 0.05% in water</td>
<td>–</td>
<td>Inhibited the development of altered hepatic foci</td>
</tr>
<tr>
<td>3000 mg/kg oral</td>
<td>Male Wistar rats</td>
<td>–</td>
<td>↓ VEGF; ↓ COX-2</td>
<td>Exerted anti-angiogenic effects on implanted HepG2 cells</td>
</tr>
<tr>
<td>Mice</td>
<td>Male Balb/c nude mice</td>
<td>–</td>
<td>–</td>
<td>Exerted anti-angiogenic effects on implanted HepG2 cells</td>
</tr>
<tr>
<td>Caffeic acid 2% in diet</td>
<td>Male Fischer-344 rats</td>
<td>–</td>
<td>–</td>
<td>Inhibited the development of naturally occurring GST-P positive foci</td>
</tr>
<tr>
<td>CAPE 20 mg/kg in diet</td>
<td>Male Wistar rats</td>
<td>200 mg/kg DEN i.p.; after 1 week 20 mg/kg per day 2-AAF for 3 days</td>
<td>↓ NF-κB</td>
<td>Reduced the increase of GGT+ foci and the expression of both GGT and GST mRNA in liver</td>
</tr>
<tr>
<td>Ellagic acid 0.4% in diet</td>
<td>Female Sprague–Dawley rats</td>
<td>Initiation DEN 90 mg/kg i.p.; Promotion PCB 300 lM/kg i.p.</td>
<td>–</td>
<td>Decreased the size of GST-P positive foci</td>
</tr>
<tr>
<td>0.005% in diet</td>
<td>Male Wistar rats</td>
<td>AFB(1) 25 lg/kg oral intubation</td>
<td>–</td>
<td>Reduced the number of GGT + foci in liver</td>
</tr>
<tr>
<td>400 ppm in diet</td>
<td>Male AC1/N rats</td>
<td>FAA 200 ppm in diet</td>
<td>–</td>
<td>Reduced GGT + foci and HCC neoplasms</td>
</tr>
<tr>
<td>Male C3H mice</td>
<td>DEN 4 mg/kg i.p.</td>
<td>–</td>
<td>–</td>
<td>Decreased the number of adenomas</td>
</tr>
</tbody>
</table>

The immune system is a remarkably versatile defense system that has evolved to offer protection against invading pathogenic microorganisms and cancer. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders (Goldsby, Kindt, and Osborne 3–26).

The less specific component of the immune system termed as the innate immunity, provides the first line of defense against infection. It primarily constitutes of a chemical response system called complement, and the endocytic and phagocytic systems, which involve roaming “scavenger” cells, such as macrophages, that detect and engulf extracellular molecules and materials, clearing the system of both debris and pathogens. In contrast, the specific component, adaptive immunity responds to the challenge with a high degree of specificity as well as the remarkable property of “memory.” The major agents of adaptive immunity are lymphocytes and the antibodies (Goldsby, Kindt, and Osborne 3–26).

**2.8.1. Cell mediated immunity**

It constitutes of the specialized immune mechanisms that involve the activation of phagocytes, natural killer cells (NK), antigen-specific cytotoxic thymus (T)-lymphocytes, and the release of various cytokines like TNF-β and interferon (INF)-γ in response to an antigen (Abbas and Lichtman 113-29).

**2.8.2. Humoral immunity**

The humoral immunity refers to the immunity that can be conferred upon a non-immune individual by administration of serum antibodies from an immune individual. It is mediated by the interaction of B cells with antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination (Goldsby, Kindt, and Osborne 3–26)

**2.8.3. Immunomodulators**

Immunomodulators may be defined as substances, biological or synthetic, which can stimulate, suppress or modulate the humoral or cellular aspects of the immune response (Wagner 1217–22). From a clinical perspective, immunomodulators can be classified into following three categories.
Immunoadjuvants

These agents are used for enhancing efficacy of vaccines and, therefore, could be considered specific immune stimulants. One of the best known examples is Freund’s adjuvant (Agarwal and Singh).

Immunostimulants

These agents are inherently non-specific in nature and can act through innate as well as adaptive immune response. In healthy individuals, immunostimulants are expected to serve as prophylactic and promoter agents i.e. as immunopotentiators by enhancing basic level of immune response, and in individuals with impairment of immune response as immunotherapeutic agents (Agarwal and Singh).

Immunosuppressants

These are a structurally and functionally heterogeneous group of agents, which are often concomitantly administered in combination regimens for control of pathological immune response in autoimmune diseases, graft rejection, graft versus host disease, hypersensitivity immune reaction (immediate or delayed type), and immune pathology associated with infections (Agarwal and Singh).

2.8.4. Effect of specific natural compounds on immune function

The effects of flavonoids on immune responses may derive from their different mechanisms of action such as protein binding, active site interference, or antioxidant effects. Dietary flavonoids seem to modulate the inflammatory response and have primarily inhibitory effects on T lymphocytes (Middleton, Kandaswami, and Theoharides 673–751). Cocoa flavonoids modulate the cellular immune response in vitro, inhibiting the production of oxygen reactive species by lymphocytes and granulocytes. Cocoa liquor phenols inhibit the proliferation of lymphocytes and the production of immunoglobulins, and also modulate the secretion of pro-inflammatory cytokines by myeloid cells (Lamuela-Raventós et al. 159–76). Quercetin suppresses antigenic stimulation of cytotoxic T-lymphocytes and inhibits natural killer (NK) cell mediated cytolysis (Nichenametla et al. 161–83).

The immunomodulatory effects produced by the anthocyanin have been attributed to cell mediated and humoral antibody mediated activation of T and B cells, increased WBC count and increased phagocytosis (Gomathi et al. 1665–8).
Punarnavine, an alkaloid from *Boerhaavia diffusa*, enhanced natural killer cell activity, antibody-dependent cellular cytotoxicity, and antibody-dependent complement mediated cytotoxicity in tumor-bearing mice (Manu and Kuttan 569–86). In contrast, pro-inflammatory cytokines were significantly reduced by punarnavine (Manu and Kuttan 569–86). The extract (cepharanthin) of *Stephania cepharantha* Hayata containing bisoclarine alkaloids activate macrophages directly or indirectly through T cell-associated effects (Furusawa and Wu 1073–9).

Terpenes extracted from *Zanthoxylum rhoifolium*, a South American tree, significantly improved NK cell cytotoxicity *in vitro* and *in vivo* in tumors (da Silva, Figueiredo, and Yano 180–8). Terpenes in Ginkgo biloba such as ginkgolides and bilobalide reduce the production of some pre-inflammatory cytokines (Li 33–40).

### 2.8.5. Antioxidants and immune system

The protective function against external pathogens carried out by the immune system is by itself a source of ROS, since activated neutrophils, produce free radicals (Fialkow, Wang, and Downey 153–64). Moreover, during the inflammatory process, activation of phagocytes through the interaction of proinflammatory mediators, or bacterial products with specific receptors results in the assembly of the multicomponent flavoprotein NADPH oxidase which catalyzes the production of large quantities of the superoxide anion radical (O$_2^-$) (Behe and Segal 1100–3). Activated neutrophils and monocytes release the hemoprotein MPO into the extracellular space, where it catalyzes the oxidation of Cl- by H$_2$O$_2$ to yield hypochlorous acid (HClO) that reacts rapidly with a variety of biological compounds (Malle et al. 838–54). Apart from their direct toxic effects, neutrophil-derived oxidants may promote tissue injury indirectly by altering the protease/antiprotease equilibrium that normally exists within the intestinal interstitium (Rice and Weiss 178-81). The immune cells are also sensitive to external ROS, due to their high polyunsaturated fatty acids (PUFA) content. Immune cells are atypical, as compared to other somatic cells, in that they contain high levels of antioxidant vitamins, presumably providing protection against lipid peroxidation and immunosuppression, both of which are well known risks posed by high PUFA content (Bendich 615–20).

Moreover, it has been demonstrated that a micronutrient deficiency can be the cause of suppression of immune function affecting both innate T cell-mediated immune response and adaptive antibody response. Therefore, an adequate intake of vitamins and antioxidant elements seems to be essential for an efficient function of the immune system (Wintergerst, Maggini, and Hornig 301–23). For example, administration of vitamin E supplement to
healthy elderly patients produced an increased antibody titer to both hepatitis B and tetanus vaccine (Meydani et al. 1380–6), thus enhancing T-cell mediated functions.

Several classes of regulatory T cells, such as Th2, CD25+ and NK T cells, are implied in autoimmune pathologies. In an animal model of a Th2-dominated autoimmune syndrome, the administration of the antioxidant N-acetyl-cysteine induced a decrease in mast-cell expression of both immunoglobulin E (IgE) and IL-4 (Wu, Turner, and Oliveira 267–73). Intriguingly, the combined treatment with wheat germ and vitamin C profoundly inhibited metastasis formation in Lewis lung carcinoma, B16 melanoma and human colon carcinoma xenografts (HCR25) (Hidvégi et al. 2353–8). Dietary antioxidants, in particular polyphenols such as caffeic acid and curcumin, have been shown to increase the expression of cytoprotective and antioxidant enzyme HO-1 in different in vitro systems (Scapagnini, Colombrita, et al. 395; Scapagnini, Foresti, et al. 554).

2.9. Pharmaceutical technologies for enhancing oral bioavailability of poorly soluble drugs

Oral ingestion is the most convenient and commonly employed route of drug delivery due to its ease of administration, high patient compliance, cost-effectiveness, least sterility constraints and flexibility in the design of dosage form. However, the major challenge with the design of oral dosage forms lies with their poor bioavailability. The oral bioavailability depends on several factors including aqueous solubility, drug permeability, dissolution rate, first-pass metabolism, pre-systemic metabolism and susceptibility to efflux mechanisms (Vieth et al. 224; Wenlock et al. 1250). The most frequent causes of low oral bioavailability is attributed to poor solubility and low permeability (Vieth et al. 224–32). The novel technologies to achieve enhanced oral bioavailability of drugs with poor aqueous solubility include the use of micronization, nanosizing, crystal engineering, solid dispersions, cyclodextrins, solid lipid nanoparticles and other colloidal drug delivery systems such as microemulsions, self-emulsifying drug delivery systems, selfmicroemulsifying drug delivery systems and liposomes (Fahr and Liu, 2007; Gomez-Orellana, 2005).

2.9.1. Liposomes

Liposomes or lipid based vesicles were first described by Bangham and coworkers in 1965 at Cambridge University (Bangham, Standish, and Watkins 238–52). Among the various colloidal drug delivery systems, liposomes or lipid based vesicles are microscopic
(unilamellar or multilamellar) vesicles that are formed as a result of self-assembly of phospholipids in an aqueous media resulting in closed bilayered structures (Fig. 2.10) (Bangham and Horne 660–8). The assembly into closed bilayered structures is a spontaneous process (D. Lasic 302–4) and usually needs energy in the form of physical agitation, sonication, heat etc. Since lipid bilayered membrane encloses an aqueous core, both water and lipid soluble drugs can be successfully entrapped into the liposomes. The lipid soluble or lipophilic drugs get entrapped within the bilayered membrane whereas water soluble or hydrophilic drugs get entrapped in the central aqueous core of the vesicles (Gregoriadis and Florence 15–28).

Fig. 2.10: Basic liposome structure (Blomme 21)

2.9.1.1. Structural components

**Phospholipids: Bilayer formers**

The most common phospholipid used is phosphatidylcholine (PC), often referred to as lecithin. Phosphatidylcholine is an amphipathic molecule with a hydrophilic polar head group - phosphocholine, a glycerol bridge, and a pair of hydrophobic acyl hydrocarbon chains. The molecules of PC are not soluble in water. In aqueous media, they align themselves closely in planar bilayer sheets in order to minimize the unfavorable action between the bulk aqueous phase and the long hydrocarbon fatty chain. They orient themselves so that the fatty acid chains face each other, and the polar heads face the aqueous phase, thus reducing the instability which exists when the molecules exist alone. Such unfavorable interactions are completely eliminated when the sheets fold on themselves to form closed sealed vesicles. PC confers membrane rigidity to the liposomes (New 301).
Membrane additives (Sterols)

Cholesterol by itself does not form a bilayer structure. However, cholesterol acts as a fluidity buffer, i.e. below the phase transition temperature, it makes the membrane less ordered and slightly more permeable; while above the phase transition temperature it makes the membrane more ordered and stable. Cholesterol inserts into the membrane with its hydroxyl groups oriented towards the aqueous surface and aliphatic chain aligned parallel to the acyl chains in the center of the bilayer (New 301).

2.9.1.2. Mechanism of liposome formation

Many potential mechanisms have been suggested for the formation of liposomes, and some of them are more complex than others (D. D. Lasic 1–11). One approach considers the self-closing of a bilayer into a liposome to be a competition between two effects, the bending or curvature energy and the edge energy of a bilayer (D. D. Lasic 35-41). For a flat lamellar fragment, in a hydrophilic surrounding, there will be a high surface tension at the rim of the lamellar sheet. Bending can reduce this edge energy but it also implies an energy penalty due to the induced curvature. To further minimize the edge energy, a higher curvature is required and finally a closed sphere will be formed, where the edge energy is reduced to zero. The bending energy, on the other hand, has now reached its maximum and the excess free energy per liposome, regardless of the radius, is then 8K, where K is the bending rigidity. Thus, larger liposomes are energetically favored, while entropy would favor many small ones.

2.9.1.3. Classification of liposomes

2.9.1.3.1. According to size and shape (Akbarzadeh et al. 102)

Multilamellar Vesicles (MLVs): These liposomes with more than one lamella can vary in size between 100 to 1000 nm.

Small Unilamellar Vesicles (SUVs): These liposomes are < 0.1 µm with a single lamella.

Large Unilamellar Vesicles (LUVs): The size of these liposomes varies from 0.1 µm to 1000 nm. They have a single lamella.

2.9.1.3.2. According to composition

Conventional liposomes (New 301)

These liposomes are composed of natural phospholipids (which may be neutral or negatively charged) and cholesterol.
**pH-sensitive liposomes** (Sharma and Sharma 123–40)

The membranes of these liposomes are composed of either cholesterol hemisuccinate (CHEMS), phosphatidyl ethanolamine (PE), oleic acid (OA) or dioleoylphosphatidyl ethanolamine (DOPE). These liposomes fuse with cells when the pH is low, thus releasing its content into the cell cytoplasm. These liposomes are ideal for the delivery of macromolecules and weak bases.

**Cationic liposomes** (New 301)

Cationic lipids make up the membrane of these liposomes with dimethyl-dioctadecyl ammonium bromide (DDAB), dioctadecyldimethyl ammonium chloride (DOGS), 2,3-dioleoyloxy- N - ( 2 (spermine carboxamido) - ethyl) - N, N-dimethyl – 1 - propanaminium fluoracetate (DOSPA), 1,2 dioleoyloxy-3-(trimethylammonio) propane (DOTAP), 1,2dimrystyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE), and 1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DORIE) combined with dioleoylphosphatidyl ethanolamine (DOPE). These liposomes tend to be toxic in high doses with a short lifespan, thus restricting them to local administration. They are most often used for the delivery of macro molecules that have a negative charge, including the delivery of DNA and RNA.

**Long-circulating liposomes** (Sharma and Sharma 123–40)

These liposomes use cholesterol and neutral lipids with high transition temperature. These liposomes have a long circulation half life of up to 40 h.

**Immuno-liposomes** (Sharma and Sharma 123–40)

These liposomes are conventional liposomes or long circulating liposomes with antibody or other recognition sequences attached to the surface. They bind to specific cells and release the drug in that area, thus acting as a targeted delivery system.
2.9.1.3.3. According to production method (Fig. 2.11)

Fig. 2.11: A simplified illustration of the production methods of liposomes (Muller, Benita, and Bohm 396)

2.9.1.4. General method of preparation of liposomes (Akbarzadeh et al. 102)

All the methods of preparing the liposomes involve four basic stages:

- Drying down lipids from organic solvent
- Dispersing the lipid in aqueous media
- Purifying the resultant liposome
- Analyzing the final product

2.9.1.4.1. Mechanical dispersion methods

These methods basically involve drying lipids onto a surface and then adding the aqueous phase. The lipid is then moved from the surface using mechanical methods when an aqueous phase is added. Methods in this class include the following (New 301):

- Sonication
- French pressure cell: extrusion
- Freeze-thawed liposomes
- Lipid film hydration by hand shaking, non-hand shaking or freeze drying
- Micro-emulsification
- Membrane extrusion
2.9.1.4.2. Solvent dispersion methods

These methods involve dissolving the lipids and other constituents of the liposome’s membrane in an organic solution. The resulting solution is then added to the aqueous phase. The aqueous phase normally contains the material which is to be entrapped. Methods in this category include the following:

- Ethanol injection
- Ether injection
- Reverse phase evaporation vesicles/Water-in-organic phase (New 301)

2.9.1.4.3. Detergent solubilization methods

Methods in this class employ an intermediary detergent soluble in both aqueous and organic solutions when adding the phospholipids to the aqueous phase. This helps to bring the phospholipids in close contact with the aqueous phase, but still protects the hydrophilic part of the phospholipid, thus creating micelles. The specific methods in this class are as follows:

- Bile salt preparation
- Alkyl glycoside dialysis
- Triton X-100 solubilized Sendai virus particles (New 301)

2.9.1.4.4. Interactions of liposome with cells

Intermembrane transfer

This type of interaction occurs when the lipid components of liposomes interact with cell membranes. The components such as the PC, and cholesterol can exchange freely from one membrane to the other without disrupting the liposome integrity. This interaction may not even disturb the liposome’s aqueous interior (New 301). This is an approach often used for cells that are not actively phagocytic.

Contact release

When the liposomes come in close proximity of the cells, the permeability of the liposomal membrane drastically increases leading to the release of the content. This causes a very high dosage of the drug in the cells’ vicinity. The effect seems to be more pronounced in liposomes that have a cholesterol concentration above 30 mol % (Van Renswoude and Hoekstra 540–6).
Adsorption

Cell adsorption occurs when the liposome attaches to the surface of a cell either by nonspecific weak hydrophobic or electrostatic forces or by specific interactions with cell-surface components. The content of the liposome is not necessarily released into the cell, neither the lipid nor aqueous components. This is attributed to the attraction between the membranes. Specific surface receptors are thought to play a role in this interaction. This interaction is the first step to take place before pinocytosis or phagocytosis (Betageri, Jenkins, and Parsons 135).

Fusion

The liposomes come into close proximity of the cells, from where the fusion can take place. The liposome content is completely introduced into the cytoplasm of the cell. Fusogens including lysolecithin, surfactants and detergents can be used to help this type of interaction, but these chemicals often cause toxic effects, as they disturb the cell membrane even after the interaction has been completed (Betageri, Jenkins, and Parsons 135). This method does not occur often, because phagocytosis takes place faster and liposomes are thus rapidly removed from circulation by the RES (New 301).

Phagocytosis or endocytosis

Cells of the RES with phagocytic ability invaginate the liposome through the cell membrane and into a sub-cellular vacuole. Lysosomes then attach to this internalized vacuole which contains the liposome. The lysosomes introduce lysosomal enzymes that break down the lipids of the liposomes to fatty acids and release the solutes contained in the liposome. The solute can slowly leak into the cell if it is not highly charged at a low pH (Betageri, Jenkins, and Parsons 135). Endocytosis can also occur, which then gives the liposomes access to other cell organelles like the Golgi apparatus. This interaction is dependent on the interactions the liposome have with the surface receptors of the cell. If transferin is added to the outer surface of the liposome, the liposomes activate cell receptors that cause endocytosis instead of phagocytosis (New 301).
2.9.1.5. Characterization of liposomes

Table 2.4: Liposome characterization and commonly used methods (Sharma et al. 1–16)

<table>
<thead>
<tr>
<th>CHARACTERIZATION PARAMETERS</th>
<th>ANALYSIS METHODS/INSTRUMENTATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Characterization</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>Barlett/Stewart assay, HPLC</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Cholesterol oxidase assay, HPLC</td>
</tr>
<tr>
<td>Drug</td>
<td>Method as in individual monograph</td>
</tr>
<tr>
<td>Phospholipid Peroxidation</td>
<td>UV absorbance, TBA, iodometric, GLC</td>
</tr>
<tr>
<td>Hydrolisis</td>
<td>HPLC, TLC, Fatty Acid Conc.</td>
</tr>
<tr>
<td>Cholesterol auto-oxidation</td>
<td>HPLC, TLC</td>
</tr>
<tr>
<td>Ant-oxidant degradation</td>
<td>HPLC, TLC</td>
</tr>
<tr>
<td>pH</td>
<td>pH meter</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>Osmometer</td>
</tr>
<tr>
<td>Physical Characterization</td>
<td></td>
</tr>
<tr>
<td>Vehicle Size &amp; Surface</td>
<td>TEM, Freeze fracture electron microscopy</td>
</tr>
<tr>
<td>morphology</td>
<td></td>
</tr>
<tr>
<td>Size distribution</td>
<td>DLS, Zetasizer, TEM, FPR, gel permeation, exclusion</td>
</tr>
<tr>
<td>Physical Charge</td>
<td>Free flow electrophoresis</td>
</tr>
<tr>
<td>Electric surface potential &amp; pH</td>
<td>Zeta potential measurement, pH probes</td>
</tr>
<tr>
<td>Lamellarity</td>
<td>SAXS, 1H-NMR, Freeze fracture EMI</td>
</tr>
<tr>
<td>Phase behavior</td>
<td>Freeze fracture EMLDSC</td>
</tr>
<tr>
<td>% Entrapment Efficiency</td>
<td>Mini-column centrifugation, gel exclusion, ion exchange, protamine aggregation, radiolabelling</td>
</tr>
<tr>
<td>Drug release</td>
<td>Diffusion</td>
</tr>
<tr>
<td>Biological Characterization</td>
<td></td>
</tr>
<tr>
<td>Sterility</td>
<td>Aerobic or anaerobic cultures</td>
</tr>
<tr>
<td>Pyrogenicity</td>
<td>LAL test</td>
</tr>
<tr>
<td>Animal toxicity</td>
<td>Monitoring survival rates, Histopathology</td>
</tr>
</tbody>
</table>

2.9.1.6. Stability (Lasic and Papaioannou 9; Zuidam and Crommelin 1113)

Physical stability

The stability of a pharmaceutical product is usually defined as the capacity of the delivery system to remain within defined or pre-established limits during the shelf life of the product. There is no established protocol for either accelerated or long-term stability studies for the liposomal formulation. Classical models from colloidal science can be used to describe liposome stability. Liposomes exhibit both physical and chemical stability characteristics. Generally, the physical characteristic describes the preservation of liposome structure and the chemical characteristic refers to the molecular structure of the liposomal components to ensure protection against hydrolysis and oxidation of phospholipid. Physically stable formulations preserve both liposome size distribution and the amount of material encapsulated. The stability problem is overcome by using appropriate techniques like freezing, lyophilization and osmification. It is also prevented by using fresh solvents and freshly purified lipid, using inert nitrogen gas, avoiding high temperature and including antioxidants such as α, β, γ and δ-tocopherol.
Plasma Stability

Although liposomes resemble biomembranes, they are still foreign objects for the host. Therefore, liposomes are recognized by the mononuclear phagocytic system (MPS) after interaction with plasma proteins. As a result, liposomes are cleared from the bloodstream. These stability problems are solved by using synthetic phospholipids, gangliosides, polymerization, coating liposomes with chitin derivatives, freeze drying, microencapsulation and particle coated with amphipathic polyethylene glycol.

2.9.1.7. Advantages of liposomes (Anwekar, Patel, and Singhai 945–51)

- Provides selective passive targeting to tumor tissues (liposomal doxorubicin)
- Increased efficacy and therapeutic index (Actinomycin-D)
- Increased stability of encapsulated drug
- Biocompatible, completely biodegradable, non-toxic, flexible and nonimmunogenic for systemic and non-systemic administrations
- Ability to protect their encapsulated drug from the external environment and to act as sustained release depots (Propranolol, Cyclosporin)
- Can be formulated as a suspension, as an aerosol, or in a semisolid form such as gel, cream and lotion, as a dry vesicular powder (proliposome) for reconstitution or they can be administered through most routes of administration including ocular, pulmonary, nasal, oral, intramuscular, subcutaneous, topical and intravenous
- Can encapsulate not only small molecules but also macromolecules like superoxide dismutase, haemoglobin, erythropoietin, IL-2 and IFN-γ
- Reduction in toxicity of the encapsulated agent (Amphotericin B, Taxol)
- Reduced exposure of sensitive tissues to toxic drugs
- Site avoidance effect (avoids non-target tissues)
- Improved pharmacokinetic effects (reduced elimination, increased circulation life times)
- Flexibility to couple with site-specific ligands to achieve active targeting (Anticancer and Antimicrobial drugs)
2.9.1.8. Liposomal products on market and in clinical development

A number of liposomal products are currently on the market, and many more are in various phases of clinical development (Allen and Cullis 36–48) (Table 2.5).

Table 2.5: Liposomal products on market and clinical development (Allen and Cullis 36–48)

<table>
<thead>
<tr>
<th>Product</th>
<th>Drug</th>
<th>Indications</th>
<th>Year approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil (Johnson &amp; Johnson)</td>
<td>Doxorubicin</td>
<td>Kaposi’s sarcoma</td>
<td>1995</td>
</tr>
<tr>
<td>DaunoXome (Galen)</td>
<td>Daunorubicin</td>
<td>Kaposi’s sarcoma</td>
<td>1996 (Europe), 1996 (USA)</td>
</tr>
<tr>
<td>Mycex (Cephalon)</td>
<td>Doxorubicin</td>
<td>Breast cancer + cyclophosphamide</td>
<td>2000 (Europe)</td>
</tr>
<tr>
<td>Ampeox (QDI)</td>
<td>Amphotericin B</td>
<td>Multiple myeloma + Velcade</td>
<td>2007 (Europe, Canada)</td>
</tr>
<tr>
<td>DepoCyt (Fycompa)</td>
<td>Cytosine sulfite</td>
<td>Pain following surgery</td>
<td>2004</td>
</tr>
<tr>
<td>Liposoxil (Taiwan)</td>
<td>Docosanol</td>
<td>Meningitis</td>
<td>1996</td>
</tr>
<tr>
<td>Liposone (Taiwan)</td>
<td>Vincristine</td>
<td>Acute lymphoblastic leukemia</td>
<td>2012 (USA)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Approved product</th>
<th>Drug</th>
<th>Indications</th>
<th>Year approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>Ampiphospholipid</td>
<td>Fungal infections, Leishmaniasis</td>
<td>1999 (Europe), 1997 (USA)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Daunorubicin</td>
<td>Kaposi’s sarcoma</td>
<td>1996 (Europe), 1996 (USA)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Doxorubicin</td>
<td>Breast cancer + cyclophosphamide</td>
<td>2000 (Europe)</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Amphotericin B</td>
<td>Invasive aspergillosis</td>
<td>1996</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Daunorubicin</td>
<td>Kaposi’s sarcoma</td>
<td>1996 (Europe), 1996 (USA)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Doxorubicin</td>
<td>Breast cancer + cyclophosphamide</td>
<td>2000 (Europe)</td>
</tr>
<tr>
<td>Liposoxil (Taiwan)</td>
<td>Docosanol</td>
<td>Meningitis</td>
<td>1996</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Vincristine</td>
<td>Acute lymphoblastic leukemia</td>
<td>2012 (USA)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Product</th>
<th>Drug</th>
<th>Indications</th>
<th>Year approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPX-351 (Celator)</td>
<td>Cytarabine/daunorubicin</td>
<td>Acute myeloid leukemia</td>
<td>Phase II</td>
</tr>
<tr>
<td>CPX-1 (Celator)</td>
<td>Carboxy maculostatin</td>
<td>Colorectal cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>M880 (Mersana)</td>
<td>Opti-1</td>
<td>Gastric and pancreatic cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>MM-302 (Mersana)</td>
<td>Ethyl-2-succinyl-2-nitroso-N-</td>
<td>Gastric cancer and gastro-endothelial junction</td>
<td>Phase II</td>
</tr>
<tr>
<td>M8 (Mersana)</td>
<td>Topotecan</td>
<td>Relapsed solid tumors</td>
<td>Phase I</td>
</tr>
<tr>
<td>Bicyclomycin (Taloz)</td>
<td>Topotecan</td>
<td>Relapsed solid tumors</td>
<td>Phase I</td>
</tr>
<tr>
<td>Liposud (Regenon)</td>
<td>Docetaxel</td>
<td>Non-small cell lung cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Lipidex (Medgene)</td>
<td>Docetaxel</td>
<td>Non-small cell lung cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Thermosent (Gerson)</td>
<td>Thermosensitive doxorubicin</td>
<td>Primary hepatocellular carcinoma</td>
<td>Phase II</td>
</tr>
<tr>
<td>Enol (Medgene)</td>
<td>Cationic liposomal palmitoyl</td>
<td>Pancreatic cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>AMLN-1 (Arihara)</td>
<td>RNA targeting thymidase</td>
<td>Triple negative breast cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>AMLN-2 (Arihara)</td>
<td>RNA targeting thymidase</td>
<td>Colorectal liver metastases</td>
<td>Phase II</td>
</tr>
<tr>
<td>TK-1 (Nycomed)</td>
<td>RNA targeting polo-kinase 1</td>
<td>Liver tumors</td>
<td>Phase I</td>
</tr>
<tr>
<td>SRM (Ficias)</td>
<td>Anti-MUC1 cancer vaccine</td>
<td>Non-small cell lung cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Expel (Facies)</td>
<td>Bupivacaine</td>
<td>Nerve block</td>
<td>Phase II</td>
</tr>
<tr>
<td>Chapter 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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2.9.1.9. Natural product-based liposomal drug delivery systems

A variety of natural product-based liposomal formulations has been studied which are summarized in Table 2.6 (Ajazuddin and Saraf 680–9).

Table 2.6: Natural product-based liposomal formulations (Ajazuddin and Saraf 680–9)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Active ingredients</th>
<th>Applications of liposome formulations</th>
<th>Biological activity</th>
<th>Method of preparation</th>
<th>% Entrapment efficiency</th>
<th>Route of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin liposomes</td>
<td>Quercetin</td>
<td>Reduced dose, enhance penetration in blood brain barrier</td>
<td>Antioxidant, Anticancer</td>
<td>Reverse evaporation technique</td>
<td>60%</td>
<td>Intranasal</td>
</tr>
<tr>
<td>liposomes encapsulated silmarin</td>
<td>Silmarin</td>
<td>Improve bioavailability</td>
<td></td>
<td>Reverse evaporation technique</td>
<td>69.22 ± 0.6%</td>
<td>Buccal</td>
</tr>
<tr>
<td>Liposomes Artemisia absencens</td>
<td>Artemisia absencens</td>
<td>Targeting of essential oils to cells, enhance penetration into cytoplasmatic barrier</td>
<td>Antiviral</td>
<td>Film method and sonication</td>
<td>60–74%</td>
<td>In vitro</td>
</tr>
<tr>
<td>liposome Amelopin</td>
<td>Amelopin</td>
<td>Increase efficiency</td>
<td>Anticancer</td>
<td>Film-ultrasound method</td>
<td>62.30%</td>
<td>In vitro</td>
</tr>
<tr>
<td>Paclitaxel liposome</td>
<td>Paclitaxel</td>
<td>High entrapment efficiency and pH sensitive</td>
<td>Anticancer</td>
<td>Thin film hydration method</td>
<td>94%</td>
<td>In vitro</td>
</tr>
<tr>
<td>Curcumin liposome</td>
<td>Curcumin</td>
<td>Long-circulating with high entrapment efficiency</td>
<td>Anticancer</td>
<td>Ethanol injection method</td>
<td>88.27 ± 2.18%</td>
<td>In vitro</td>
</tr>
<tr>
<td>Gallicin liposome</td>
<td>Gallicin</td>
<td>Increase efficiency</td>
<td>Lungs</td>
<td>Reverse-phase evaporation method</td>
<td>90.77%</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids liposomes</td>
<td>Quercetin and rutin</td>
<td>Binding of flavonoids with Hb is enhanced</td>
<td>Hemoglobin</td>
<td>Solvent evaporation</td>
<td>-</td>
<td>In vitro</td>
</tr>
<tr>
<td>liposomes with β-CD</td>
<td>Urocanic acid</td>
<td>Increase solubility and localization with prolonged-release profile</td>
<td>Antimicrobial</td>
<td>Hydration of a thin lipid film method with sonication</td>
<td>99.3%</td>
<td>In vitro</td>
</tr>
<tr>
<td>Wogonin liposome</td>
<td>Wogonin</td>
<td>Sustained release effect</td>
<td>Anticancer</td>
<td>Film dispersion method</td>
<td>81.20 ± 4.20%</td>
<td>In vivo</td>
</tr>
<tr>
<td>Colchicine liposome</td>
<td>Colchicine</td>
<td>Enhance skin accumulation, prolong drug release and improve site specificity</td>
<td>Antigout</td>
<td>Rotary evaporation sonication method</td>
<td>66.3 ± 2.2%</td>
<td>Topical</td>
</tr>
<tr>
<td>Catechins liposomes</td>
<td>Catechins</td>
<td>Increased permeation through skin</td>
<td>Antioxidant and chemopreventive</td>
<td>Rotary evaporation sonication method</td>
<td>93.0 ± 0.1%</td>
<td>Transdermal</td>
</tr>
<tr>
<td>Bryopinoliposomes</td>
<td>Bryopinoliposomes</td>
<td>Sustained delivery of bryopinoliposomes</td>
<td>Cardiovascular diseases</td>
<td>Double emulsification process</td>
<td>87.9 ± 3.1%</td>
<td>Intramuscular</td>
</tr>
</tbody>
</table>

2.9.1.10. Liposomes as oral drug delivery systems

The use of liposomes as oral drug delivery system is challenging due to the poor stability of the vesicles under the physiological conditions typically encountered in the gastro-intestinal tract (Rogers and Anderson 421–80). Nevertheless, several studies and recent publications indicate the potential application of phospholipid-based liposomal formulations as oral drug delivery systems to improve the bioavailability of poorly soluble and low-bioavailability drugs, including peptides and proteins.

Cyclosporin A, an immunosuppressive agent formulated in lecithin vesicles, was found to be equivalent to the marketed formulation Sandimmun Neoral® in rabbits after oral administration (Guo, Ping, and Chen 17–21). Subsequent bioavailability studies in rats
revealed that the absorption constant for the liposomal Cyclosporin A was nine times higher than for free drug solution and four times higher than for a marketed sample of microemulsion (Shah et al. 2967–73).

Another recent study promoting the suitability of liposomes to administer larger peptides and proteins via oral route showed that the area above the blood calcium concentration-time curve (AAC) after oral administration of calcitonin-loaded chitosan-coated liposomes to rats increased about 11-fold compared to calcitonin in solution (Werle and Takeuchi 26–32). A further study with self-assembling mucoadhesive pectin-liposome nanocomplexes of oral calcitonin, demonstrated an improved pharmacological effect over calcitonin solution and calcitonin-loaded standard liposomes with an enhanced and prolonged reduction in plasma calcium concentration. The ability of the pectin-coated liposomes to adhere to the mucus layer and prolong retention in the intestinal mucosa could possibly contribute to this action (Thirawong et al. 236–45).

A similar approach was employed for the oral delivery of oxymatrine, a natural quinolizidine alkaloid, used clinically for treating hepatitis B. *In vivo* pharmacokinetic studies in rats indicated that following oral administration, the area under the plasma concentration-time curve of N-trimethyl chitosan-coated oxymatrine containing multivesicular liposomes was about 3.26 times that of a simple drug solution (Cao et al. 1339–47).

Experimental reports from previous studies indicate that blood glucose level may be significantly reduced by orally administered insulin-loaded liposomal systems (Değim et al. 2945–9).

Another multivesicular liposomal formulation of human epidermal growth factor (rhEGF) following oral administration in rats significantly enhanced the gastric ulcer healing effect compared to that of rhEGF in aqueous solution and was comparable to that of cimetidine (Li et al. 988–94).

Another mixed-micellar proliposomal formulation of progesterone showed an increased transport of the drug across Caco-2 cells and everted rat intestinal sacs compared to control by enhancing the extent of dissolution and membrane transport of progesterone (Potluri and Betageri 227–32).
Bile salt-containing liposomal formulations of poorly water-soluble anti-hyperlipidemic drug, fenofibrate also manifested a 3 to 5-fold higher bioavailability than micronized fenofibrate, as evident from in vivo pharmacokinetic studies in Beagle Dogs (Chen et al. 153–60).

The oral bioavailability of N3-o-toluyl-fluorouracil, the pro-drug of fluorouracil with tumor-inhibiting properties, upon encapsulation in soybean lecithin liposomes, demonstrated significant improvement in comparison to an aqueous dispersion of the drug in mice (Zou et al. 90–8).

In rabbits, the bioavailability of proliposomes containing vinpocetine, a seed extract from perivincle (Vinca major) used for the treatment of cerebrovascular disorders and age-related memory impairment, was more than 3.5 times higher than the vinpocetine suspension (Xu et al. 61–8).

Liposome encapsulated curcumin, a food additive with potential anti-metastatic properties, when administered orally to rats, evidenced a high bioavailability with a faster rate and greater extent of absorption as compared to the other forms. The plasma antioxidant activity following oral liposome administration was also significantly higher compared to the other treatments, suggesting the utility of liposome encapsulated of curcumin as a novel nutrient delivery system (Takahashi et al. 9141–6).

More recently, in vivo, oral administration of the liposomal encapsulation of a combination of curcumin and resveratrol, a naturally occurring polyphenol with manifold biological effects, has been reported to synergistically improve their bioavailability and enhance their anti-tumor effect against prostate cancer in prostate-specific phosphatase and tensin homolog (PTEN)-knockout mice (Narayanan et al. 1–8).

Another recent approach involving the coupling of folic acid as the mediator of uptake to the surface of liposomes containing poorly absorbable peptidomimetic drugs resulted in an enhanced oral bioavailability of cefotaxime in rats along with an increased peak plasma concentration compared to the folic acid-free liposomes (Ling et al. 445–9).