Chapter 1
Introduction
1. Introduction

In recent years, *Oryza Sativa* (Rice) bran oil (RBO) is gaining surmount importance on account of its balanced fatty acid profile and rich source of commercially and nutritionally important antioxidative and disease-preventative phytochemicals such as, ferulic acid, its esterified derivative (oryzanol), and unsaponifiable components such as tocopherol, tocotrienol, and vegetable sterols (Jariwalla 17–26). Literature reports have suggested that RBO constituting of bioactive polyphenols exhibit anti-oxidative, hypocholesterolemic, antiatherogenic, antidiabetic, cancer chemopreventive and immune potentiation properties (Jariwalla 17; Sierra et al. 509; Chen and Cheng 1472).

The isolation of bio-active principles from crude RBO (cRBO) has been carried out by preparative HPLC, calcium ion induced precipitation of anionic micellar aggregates and silica-based continuous chromatography combined with multistage of crystallization. However, some serious limitations of these techniques have been exposed, including low productivity, use of chlorinated or aromatic toxic solvents like benzene, multi-stage processes and non-reproducibility of the methods to production scale (Zullaikah, Melwita, and Ju 299–302).

Oryzanol (OZ), often identified as the physiologically active constituent of RBO, is a mixture of ferulic acid esters of triterpene alcohols such as cycloartenyl ferulate, 24-methylene-cycloartanyl ferulate and campesteryl ferulate (Metwally, Habib, and Khafagy 68; Norton 269). A number of therapeutically useful biological activities have been reported for OZ, including the antioxidant/free radical scavenging activity, cholesterol-lowering action, modulation of pituitary and gastric secretion, decreasing platelet aggregation, inducing thyroid stimulating hormone release, reducing menopausal symptoms, increasing muscle mass, anti-inflammatory, anti-carcinogenic, and neuroprotective effects (Patel and Naik 569; Yasukawa et al. 1072; Islam et al. 812; N. Ismail et al. 9692). The antioxidant activity of OZ is primarily based on hydrogen donation from the ferulic acid hydroxyl group, which enables it to counteract the severe oxidative stress generated due to the imbalance between the production of reactive oxygen/nitrogen species (RONS) and/or a reduction in antioxidant defenses responsible for their metabolism (Nystrom et al. 947–54). As a potent antioxidant, OZ directly scavenges the free radicals (Akiyama et al. 295–7) and exhibits a strong inhibitory effect on lipid peroxidation (Saenjum et al. 1070–7). Studies have also demonstrated that OZ inhibits lipopolysaccharide-induced nitric oxide synthase, tumor
necrosis factor-α (TNF-α) expression and nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) activity (Islam et al. 812–24). Moreover, OZ has been shown to be extremely safe with no major side effects being reported in either animal or human studies (Murray 332–5).

Accumulating evidence suggests that oxidative stress plays a pivotal role in the etiology of diabetes and its complications. Implication of oxidative stress in the pathogenesis of diabetes is suggested, not only by oxygen free-radical generation, but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes, lipid peroxides formation, and decreased ascorbic acid levels (Moussa 225-36). The overproduction of superoxide anion radical in the mitochondrial electron transfer chain activates the classical metabolic events evidenced in course of diabetes, such as: increased polyol pathway activity; increased formation of advanced glycation end products (AGEs); protein kinase C (PKC) and nuclear transcription factor κB (NF-κB) activation; and increased hexosamine pathway flux. Thus, hyperglycemia plays a critical role in providing a favorable cellular environment for increased RONS production (Bandeira et al. 3265–84).

Although a wide range of oral anti-diabetic agents are currently available, many of them meet some but not all patients' needs (Hung et al. 580–606), and suffer from various adverse effects such as liver problems, lactic acidosis, diarrhea and high rates of secondary failures (Inzucchi 360–72). Another drawback is that all glucose lowering medications can lose their effectiveness after a few years of use (Kahn et al. 2427–43). Hence, the search for more affordable agents from natural origin that retain the therapeutic efficacy and minimize the adverse effects of free radicals by enhancing the antioxidant defenses has currently become the major focus of investigation (Saxena and Vikram 369–78). There have been copious evidences confirming the hypoglycemic potential of OZ which is mediated by its regulation of adiponectin and insulin secretion and hepatic glucose-regulating enzyme activities (S. H. Lee et al. 827; Ohara et al. 130; Son et al. H7). However, no systematic scientific investigation has been carried out so far to verify the claims on the anti-diabetic activity of OZ against diabetogenic agents like streptozotocin (STZ), and its relationship with its antioxidant properties.

An important role of hyperglycemia-mediated oxidative stress for the development and progression of diabetic nephropathy is suggested by observations that 1) lipid peroxides and 8-hydroxydeoxyguanosine, indices of oxidative tissue injury, increase in the kidneys of
diabetic rats with albuminuria; 2) high glucose directly increases oxidative stress in glomerular mesangial cells and target cells of diabetic nephropathy; 3) oxidative stress induces mRNA expression of transforming growth factor beta 1 (TGF-β1) and fibronectin, which are the genes implicated in diabetic glomerular injury, and 4) inhibition of oxidative stress ameliorates all the manifestations associated with diabetic nephropathy (Ha and Kim 147–51). In addition, accumulating evidences postulate that elevated levels of cholesterol and low-density lipoproteins play an important role in the development of glomerulosclerosis and deterioration of renal functions in diabetics (Arora, Reddy, and Balakumar 137–44). The most effective current clinical treatments to arrest the progression of diabetic nephropathy such as strict blood glucose control and anti-hypertensives have failed to prevent new cases (Sourris and Forbes 180–1). However, several studies have implicated an amelioration of diabetic nephropathy and concurrent improvement of the antioxidant system of the body with the consumption of vitamins C, E, beta-carotene, quercetin, curcumin, and resveratrol via attenuation of oxidative stress (Katyal et al. 252–63).

There is also a growing body of evidence to support the notion that oxidative stress is the biochemical trigger for sciatic nerve dysfunction and reduced endoneurial blood flow in diabetic rats. In addition, diminished activities of Cu-Zn superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione content, coupled with elevated lipid peroxidation products such as malondialdehyde or conjugated dienes have been reported in diabetic sciatic nerves. Enhanced oxidative stress in turn activates redox-sensitive transcription factor NF-κB, which up-regulates genes such as cytokines, adhesion molecules, endothelin-1 and tissue factor (Kamboj, Vasishtha, and Sandhir 77–91). Several drugs such as antidepressants, topical capsaicin, nonsteroidal anti-inflammatory drugs, anticonvulsants and opioid receptor agonists are currently under investigation in the management of diabetic neuropathy. However, the treatment is limited by their partial effectiveness, development of tolerance, and potential toxicity (Anjaneyulu and Chopra 766–9). Treatment with antioxidants such as α-lipoic acid, glutathione, erythropoietin, transition metal chelators like deferoxamine and trientine, vitamin C, vitamin E, and docosahexaenoic acid have been nevertheless associated with improved nerve function (Kamboj, Vasishtha, and Sandhir 77–91). But the physiological role of the potent free radical scavenger, OZ (Akiyama et al. 295–7) with regard to chronic diabetic complications in experimental animal models has also not been fully instituted and therefore requires extensive research.
Cancer continues to be one of the major causes of death worldwide and only a modest progress has been made in reducing the morbidity and mortality of this dreadful disease (Ramasamy et al. e34793). Oxidative stress induces a cellular redox imbalance in various cancer cells which may be linked to oncogenic stimulation. Permanent modification of genetic material resulting from oxidative damage represents the first step involved in mutagenesis, carcinogenesis, and ageing. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in various tumors. ROS-induced DNA damage involving single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links, can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with carcinogenesis (Valko et al. 1; Marnett 361). Radical mediated damage to cellular biomembranes also results in lipid peroxidation that generates a variety reactive electrophiles such as epoxides and aldehydes that are mutagenic and carcinogenic in nature (Dix and Aikens 2–18). Declined antioxidant defense comprising SOD, catalase, the glutathione system to counter free radicals could further represent a systemic marker for cancer lesions (Glass and Gershon 531–7).

For many years, cancer chemotherapy has been dominated by potent drugs that either interrupt the synthesis of DNA or destroy its structure. Unfortunately, their toxicity is not limited to cancer cells but normal cells are also harmed. Therefore, efforts to develop less toxic drugs that affect the antioxidant system, and malignant cells by virtue of a mechanism-based approach are necessary for the prevention and therapy of cancer (Sivalokanathan, Ilayaraja, and Balasubramanian 264–7). Besides, most of the anticancer drugs currently used in chemotherapy cause immunotoxicity which affects not only tumor development, but also prolongs patient’s recovery. Thus, the discovery and identification of new anticancer drugs with low side effects on immune system has also become an essential goal in many immunopharmacology studies (Subhadra Devi et al. 1–4).

Considering that conventional antineoplastic therapies have unwarranted side-effects related to oxidative stress, dietary antioxidants may be a valuable adjuvant therapy in cancer. Dietary antioxidants can overcome the inefficiency of the endogenous antioxidants of normal cells against ROS challenge and can influence the response to chemotherapy as well as the development of adverse effects resulting from treatment with antineoplastic agents (Manda, Nechifor, and Neagu 342; Conklin 1). Moreover, modulation of cell signaling pathways by antioxidants could help to prevent cancer by (i) preserving normal cell cycle regulation; (ii)
inhibiting proliferation and inducing apoptosis; (iii) inhibiting tumor invasion and angiogenesis; (iv) suppressing inflammation; and (v) stimulating phase II detoxification enzyme activity. It has been demonstrated that activation of redox-regulated NF-κB by nearly all stimuli can be blocked by antioxidants, including l-cysteine, N-acetyl cysteine (NAC), thiols, green tea polyphenols, and Vitamin E (Valko, Leibfritz, et al. 44–84). The beneficial effects of tea polyphenols, curcumin, genistein, resveratrol, lycopene, pomegranate, and lupeol against cancers of the skin, prostate, breast, lung, and liver have also been reported previously (Khan, Afaq, and Mukhtar 475–510). Findings from a large-scale randomized clinical trial have further revealed a significant reduction of esophagus and stomach cancers using a combination of dietary antioxidants such as beta-carotene, vitamin E, and selenium (Blot et al. 1483–92).

Moreover, uncontrolled proliferation is a universal property of tumor cells. Investigation of the cellular growth control mechanisms has contributed to the understanding of carcinogenesis and identification of compounds with specific antitumoral activities. A reduction in cell growth and induction in cell death are two major ways to inhibit tumor growth (Cardellina II et al. 25–36). Several bio-active constituents of the rice bran including Vitamin E, phytic acid, ferulic acid, and phytosterols such as β-sitosterol, campesterol and stigmasterol have been previously documented to manifest anti-proliferation and apoptosis induction in various forms of cancer cells (Leardkamolkarn et al. 978–85). However, more significant constituents in rice bran and cRBO that protect against cancer are waiting to be discovered.

The adenoma–carcinoma sequence is the basis for the development of colorectal carcinoma (CRC) with corresponding accumulation of genetic changes that cause increased rate of cell proliferation (Arnold et al. 2035–47). It has been suggested that these genetic events are enhanced by oxidative stress, resulting in an overall increase in cellular levels of ROS (Skrzydlewski et al. 213–22). A host of exogenous processes including environmental agents such as non-genotoxic carcinogens can also directly or indirectly induce ROS in cells (Rice-Evans and Burdon 71–110), eventually leading to persistent DNA damage (Klaunig and Kamendulis 239–67) and accumulation of lipid peroxidation products (Nelson et al. 341–7). ACF, considered the standard biomarker of colon carcinogenesis, are the putative pre-neoplastic lesions of colonic neoplasia that appear in the early stages and sequentially develop into polyps, adenomas and eventually into carcinomas (Cheng and Lai 2642–9).
A recent epidemiological study showed that rice consumption is associated with a decreased risk of distal colorectal cancer (Uchida et al. 1223–31). Several phytochemical components or extracts of RBO have protective effects on colon carcinogenesis in vitro (Hudson et al. 1163; Kong et al. 1487) and in vivo (Sunagawa et al. 45; Shih et al. 562) by virtue of their anti-oxidative and anti-inflammatory attributes. Likewise, OZ has also been considered as a potential therapeutic and/or preventive agent for colonic inflammation and the anti-inflammatory effect could be possibly mediated by inhibition of NF-κB activity, which could be at least partly due to its antioxidant effect (Islam et al. 812–24). OZ has been previously reported to possess anti-carcinogenic potential in carcinogen-treated mouse mammary glands and two-stage model of skin cancer (Patel and Naik 569; Yasukawa et al. 1072). Nonetheless, the mechanism by which OZ exerts its in vivo chemopreventive effects against carcinogen-initiated colonic neoplasia remains largely unknown. Relatively little is also elucidated about the in vitro cytotoxicity and the effect of OZ on cell proliferation and apoptosis during carcinogenic events, which are the critical effects for successful cancer treatment.

Considering the limited treatment and grave prognosis, chemoprevention has been considered as the best strategy in lowering the current morbidity and mortality associated with hepatocellular carcinoma (HCC) (Yates and Kensler 1331–42). Oxidative stress serves as a critical predisposing factor to hepatocarcinogenesis and is a major driving force of HCC in chronic liver diseases (Kawanishi et al. 365–72). It is widely acknowledged that inflammation is one of the biological responses driven by oxidative stress. Therefore, modulation of oxidative damage as well as inflammation is considered to be a significant protective measure against hepatocarcinogenesis and could offer substantial benefit in cancer prevention (Marra et al. 171). Several biologically active natural compounds have been evaluated for their potential as liver protectants against chemical carcinogen-induced hepatotoxicity in experimental animals (Ramakrishnan et al. 104–14). It has been shown that OZ exerts a protective action on liver injury induced by chronic ethanol ingestion in mice (Chotimarkorn and Ushio 951–8). Besides, OZ has potent antioxidant (Xu and Godber 645–9) and anti-inflammatory (Terada and Haruta 95–9) properties, which might play an important role in protecting the liver against carcinogen-induced neoplasia in vivo. However, an experimental validation of this premise and the precise mechanism(s) by which OZ exerts a chemopreventive action against liver tumor development has not been completely elucidated. Therefore, our studies seek to address the hypothesis that OZ exerts its effects in CRC and HCC, in part, through its tumor suppressive and strong antioxidant property,
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coupled with cell proliferation inhibition and apoptosis induction. Given its wide use in the treatment of gastrointestinal cancers (J. Wang et al. 1353–60), 5-fluorouracil (5-FU), a pyrimidine analog, was employed as a positive control for both the studies.

Tumor mediated immunosuppression, including the suppression of T and B lymphocytes in spleen, bone marrow and thymus, is the greatest challenge in cancer treatment (Hakim 1–6). Most of the commonly used antineoplastic agents suppress both cellular and humoral immunity (Devita, Lawrence, and Rosenberg 301–5). In clinical practice, bone marrow toxicity accompanied by leucopenia, with a high rate of infection; thrombocytopenia and granulocytopenia is the most dangerous form of toxicity for many of the antineoplastic drugs such as anti-metabolites and alkylating agents like cyclophosphamide (CP). Thus, harnessing the immune system to treat cancer is a major goal of immunotherapy (Waldmann 65–81). Many of the presently available immunomodulators such as levamisole, glucans, telerones, L-fucose as well as Corynebacterium parvum bacterium, are not free from side effects, which include fever, neutropenia, leucopenia and, at times, allergic reactions (Ghule et al. 311–5). Hence, there is an urgent need to evaluate the potential of natural products as adjuvants to counteract the side effects of modern therapy.

Nutrition and nutritional status can have important implications on immune functions, resistance to infection and autoimmunity via provision of antioxidants (Chandra and Kumari 1433S–5S). Immune cells like T-cells, NK cells and T- helper cells are characterized by excessive levels of ROS which are employed, in part, to kill ingested pathogens. In addition, immune cell membranes are enriched with poly-unsaturated fatty acids which are susceptible to ROS-mediated damage (Chew and Park 257S–61S). Therefore, supplementation of nutrients with anti-oxidant properties such as carotenes, vitamin E, vitamin C, zinc, and selenium, may quench these free radicals and influence several components of the immune system (Erickson, Medina, and Hubbard S5–10). Although, RBO-enriched diets have been reported to significantly potentiate the immune response (Sierra et al. 509–16), there is paucity of scientific reports on the potential effects of OZ on cell mediated and humoral immune responses in vivo.

Despite the multitude of promising biological activities and favorable safety profile, the therapeutic usefulness of OZ has been limited because of its unfavorable physicochemical properties, especially its poor water-solubility (Juliano et al. 146) and low oral bioavailability (Kim et al. 368). The underlying mechanisms contributing to the low plasma levels of OZ
following an oral administration appear to be poor absorption, rapid tissue distribution and extensive metabolism, probably in the liver (Fujiwara et al. 1011). To improve the existing limitations, various novel delivery drug approaches have been employed so far, including, first, liposomal OZ with enhanced anti-oxidant activity and lower cytotoxicity in normal human foreskin fibroblast (NHF) cells (Viriyaroj et al. 665); second, niosomal entrapment of OZ for stability improvement along with improved anti-oxidant activities and skin hydration enhancement (A. Manosroi et al. 2269); third, the microencapsulated OZ for protection against the unfavorable gastric environment and to increase the aqueous solubility and oral bioavailability (Kim et al. 368); and fourth, cycloartenol ferulate-cyclodextrin complexation to elevate OZ aqueous solubility and improve its gastric absorption (Inagaki et al. 1986). To enhance the bioavailability and functionality of OZ, we focused our attention on the feasibility of using liposomes as an efficient oral carrier for OZ.

Liposomes are biodegradable, colloidal and concentric bi-layered vesicles in which an aqueous compartment is completely enclosed by a membranous lipid bi-layer predominantly composed of naturally derived or synthetic phospholipids (Abe et al. 136–41). On account of their unique properties, liposomes are able to enhance the performance of products by improving the ingredient solubility, bioavailability, bio-distribution, in vitro and in vivo stability, altering the pharmacokinetics and enhancing intracellular uptake. Liposomes as a drug delivery system can enhance the therapeutic efficacy and safety of drugs, primarily by delivering them to their site of action and by maintaining the therapeutic drug levels for a prolonged period of time (Ajazuddin and Saraf 680; Devi, N. Jain, and Valli 27). Evidence of liposomes enhancing the bioactivity and bioavailability of polyphenolic compounds has been previously reported for quercetin, silymarin, curcumin, and resveratrol (Ajazuddin and Saraf 680-9). Similarly, our study attempted to provide evidence of liposomal bioavailability enhancement of the oral delivery of OZ by evaluating the plasma pharmacokinetics of OZ concentrations from the most stable liposome encapsulated OZ (LEO) formulation after oral administration in rats.

1.1. Statement of Problem

Presently, there is a dearth of information on the comprehensive physicochemical characterization and quantitative estimation of crude RBO indigenous to the subcontinent, particularly in India. Although the extraction and purification of OZ from RBO have been reviewed, the purity and yield have not been fully specified, which makes it difficult to
interpret the results and draw conclusion. Besides, the techniques currently employed for the isolation of OZ from RBO has some serious limitations as mentioned earlier.

Considering the serious impact of the increasing global prevalence of diabetes, cancer, and tumor mediated immunosuppression, exacerbated by the absence of effective interventions and various adverse effects of the existing medications, the search for more affordable agents from natural origin that retain the therapeutic efficacy and are devoid of side effects has now become the major focus of scientists and researchers.

Despite possessing several health benefits and manifesting a favorable safety profile, OZ, because of its poor water solubility and low bioavailability, insufficiently exerts its biological effects \textit{in vivo} that eventually limits its therapeutic usefulness. Although several bioavailability enhancement approaches for OZ have been attempted, there is still a lacuna and further elaborate studies are warranted.

1.2. Aim of the study

The present study aims to isolate a bio-active principle from crude \textit{Oryza sativa} bran oil using an optimized technique, followed by its pharmacological investigation, strategic formulation development and subsequent \textit{in vivo} pharmacokinetic evaluation.

1.3. Objectives of the study

The following objectives were explored as a part of the current work.

1) To characterize cRBO based on physico-chemical parameters

2) To isolate and identify OZ from cRBO using an optimized technique

3) To investigate various pharmacological activities of OZ using experimental animal models such as:

i) To study the effects of OZ on STZ-induced diabetes and its chronic complications (diabetic nephropathy and neuropathy) in rats

ii) To study the effects of OZ on carcinogenesis

a) To carry out \textit{in-vitro} cytotoxicity screening of OZ in human colorectal and hepatocellular carcinoma cell lines

b) To study the effects of OZ on 1,2-dimethyl hydrazine (DMH)-induced colorectal carcinoma in mice
c) Effects of OZ on N-nitrosodiethylamine (NDEA)-induced hepatocellular carcinoma in mice

iii) To elucidate the mechanism of action of OZ for its effect in various pathophysiological conditions by virtue of its antioxidant potential

iv) To study the effects of OZ on immunomodulatory potential in rats

a) Effects on cellular immunity
   o Carbon clearance assay
   o Delayed-type hypersensitivity (DTH) response
   o Cyclophosphamide-induced myelosuppression

b) Effects on humoral immunity
   o Haemagglutinating antibody (HA) titre assay

4) To develop and optimize an appropriate liposome encapsulated OZ (LEO) formulation using different proportions of lipid, cholesterol and OZ by reverse evaporation phase (REV) method

5) To evaluate the optimized LEO formulation by in vivo pharmacokinetic studies in rats

1.4. Scope of the work

Oxidative stress, implicated in the etiology of various pathological conditions, can lead to deleterious consequences including damage to DNA, lipids, proteins, disruption in cellular homeostasis and accumulation of damaged molecules. It is therefore biologically plausible that antioxidant therapy could represent a promising therapeutic avenue. The present study, based on the available materials and literature reports, attempts to explore several pharmacological properties of a bio-active antioxidant oryzanol, which have not been reported earlier. The study clarifies the role of free radicals in the etiopathogenesis of chronic diabetic complications, carcinogenesis and immunomodulation and addresses new avenues pertaining to the impact of long term oryzanol supplementation in experimental animal models. Based on the suitability of data obtained from the pharmacological studies, the present investigation further focuses on a novel liposomal formulation approach to overcome the limitations of absorption and oral bioavailability with oryzanol. The findings of the study would be of immense benefit to the researchers since it attempts to reduce the lacunae in the existing literature and offers new insights on the spectrum of potent pharmacological responses elicited by OZ, thus supporting its ongoing research and development as a
preventive and disease-modifying agent using novel delivery strategies. Following successful completion of the studies, OZ can be further extended to industrial and social applications.

### 1.5. Outline of the thesis

- **Chapter 2: Review of literature**: It critically appraises the existing knowledge on oryzanol as a key bio-active constituent of rice bran oil, the role of free radicals, oxidative stress and dietary antioxidant strategies in diabetes, chronic diabetic complications, colorectal carcinoma, hepatocellular carcinoma, and immunomodulation. It further focuses on liposome as an effective pharmaceutical technology for enhancing the oral bioavailability of poorly soluble drugs.

- **Chapter 3: Report on present investigation**: It explores all the experimental setups, and procedural aspects adopted in the research work.

- **Chapter 4: Results and discussion**: It focuses on the presentation of results and observations and a comprehensive discussion of the observations made and the inference drawn. It also includes the scope for possible future work of the present investigation.

- **Chapter 5: Summary and conclusion**: It encompasses the summary and concluding remarks on the research work.

- **Chapter 6: Appendices**: It constitutes of the list of instruments, drugs and chemicals used in the present study along with the approval from the Institutional Animal Ethics Committee (IAEC) for carrying out the animal experiments. It also includes a pictorial representation of the retro-orbital blood collection from rats.

- **Chapter 7: References**

- **Chapter 8: List of publications in national or international journals**

- **Chapter 9: List of presentations at various national and international conferences**