ORGANOPHOSPHOROUS INSECTICIDES INDUCED HYPERGLYCEMIA AND ITS ROLE IN DIABETIC PROGRESSION: POSSIBLE AMELIORATION BY SELECTED PHYTOCHEMICALS

A Thesis

Submitted to the Department of Biochemistry
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In fulfillment of the requirement for the degree of

Doctor of Philosophy

By

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DECLARATION

I hereby declare that the thesis entitled **"Organophosphorous insecticides induced hyperglycemia and its role in diabetic progression: possible amelioration by selected phytochemicals"** *submitted to the* **University of Mysore** *for the award of the degree of* **Doctor of Philosophy in Biochemistry** *is the result of research work carried out by me under the guidance of Dr. P.S.Rajini, Scientist, Food Protectants and Infestation Control Department, Central Food Technological Research Institute, Mysore during the period of 2004-2007.*

I further declare that these results have not been submitted for any other degree or fellowship.

Place: Mysore
Date: -06-2007

Vasudeva Kamath G

Dr. P. S. Rajini,
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Date: -06-2007

CERTIFICATE

This is to certify that the thesis entitled **"Organophosphorous insecticides induced hyperglycemia and its role in diabetic progression: possible amelioration by selected phytochemicals"** *submitted by* **Mr. Vasudeva Kamath G** *is the result of research work carried out by him at Food Protectants and Infestation Control Department, Central Food Technological Research Institute, Mysore under my guidance during the period of 2004-2007.*

Place: Mysore
Date: -06-2007

P. S. Rajini
(Guide)

Dedicated to

My Grandmother

My Father

My Mother

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LIST OF SYMBOLS AND ABBREVIATIONS

AChE	Acetylcholinesterase	μg	Microgram
BSA	Bovine serum albumin	μM	Micromolar
b.w.	Body weight	μmol	Micromole
cm	Centimeter	μl	Microliter
° C	Degree celsius	M	Molar
d	Days	Mol	Mole
DPPH	diphenyl-β-picrylhydrazyl	ml	Milliliter
DMSO	Dimethyl sulfoxide	min	Minutes
DM	Diabetes mellitus	OS	Oxidative stress
DNA	Deoxyribonucleic Acid	OD	Optical density
EDTA	Ethylenediamine tetraacetic acid	GSH	Reduced glutathione
GAE	Gallic acid equivalent	OFR	Oxygen free radicals
g	Gram	PBS	Phosphate buffered saline
h	hour	ROS	Reactive oxygen species
HPLC	High performance liquid chromatography	rpm	Revolutions per minute
HCl	Hydrochloric acid	STZ	Streptozotocin
H_2O_2	Hydrogen Peroxide	SE	Standard Error
H & E	Haematoxylin & Eosin	SD	Standard deviation
i.g	Intra gastric	TBARS	Thiobarbituric acid reactive substances
i.p.	Intraperitoneal	TCA	Trichloroacetic acid
IDDM	Insulin dependent diabetes mellitus		
kg	kilogram	TLC	Thin layer chromatography
LDH	Lactate dehydrogenase	v/v	Volume/volume
LPO	Lipid peroxidation	w/v	Weight/volume
MDA	Malondialdehyde	w/w	Weight/weight

ABSRACT

The main objectives of the thesis were to address three fundamental questions viz., (a) the impact of organophosphorous insecticides (OPI) in pancreatic dysfunction and ensuing hyperglycemia and the mechanism/s involved (b) the role played of OPI in inducing / exaggerating diabetic condition (c) dietary modulation of the OPI-induced hyperglycemia employing selected antioxidant phytochemicals. Our experimental approach involved biochemical studies employing both *in vitro* (rat pancreatic homogenate, isolated rat pancreatic islet culture, *C.elegans*) and *in vivo* (experimental rats) model systems. Initially we screened a few selected OPI for their toxicity and oxidative stress (OS) inducing potential. Results of our study revealed that OPI in general, Dimethoate (DM) and Dichlorvos (DDVP) in particular, have the potential to induce OS *in vitro.* Our results also clearly demonstrated that both DM and DDVP possessed the potential to alter blood glucose levels in rats, inhibit AChE and induce OS in pancreas. Altered glucose homeostasis induced by both DM and DDVP was associated with biochemical and oxidative impairments in pancreas as well as derangement in carbohydrate metabolism. Based on the results of our study, it could be surmised that rats pre-treated with OPI (DDVP) could be at a risk of developing diabetes if exposed to even a sub-diabetogenic dosage of a diabetogen (Streptozotocin). We also demonstrated that 'epicatechin-rich' ethanolic extract of cashew nut skin (CSE) is a powerful natural antioxidant and it had the propensity to offset DM and DDVP induced oxidative damage in rat pancreatic tissue *in vitro.* Our findings also established the ameliorative effect of CSE against OPI induced toxicity with special reference to pancreatic oxidative damage and altered glucose homeostasis in rats. In conclusion, the data obtained from the present series of investigations provide evidence on the effects of OPI on pancreatic dysfunction and their likely role in the development/ progression of metabolic disorder like diabetes. Our studies also demonstrated that OPI-induced pancreatic damage and hyperglycemia could be attenuated by antioxidant-rich phytochemicals.

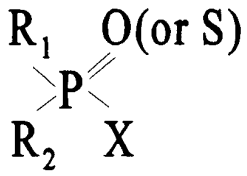
ORGANOPHOSPHOROUS INSECTICIDES (OPI)

The hazards of pesticides have increased due to the rise in their use in agriculture, industry, by households and governments. It was estimated that 3,000,000 cases of severe poisoning and 220,000 deaths are caused globally every year due to pesticides (WHO, 1997). Their easy availability makes them a suicidal poison in rural areas of many developing countries. Organophosphorous (OP) pesticides have gained popularity worldwide in preference to organochlorines (OC), which are persistent and more damaging to the environment. The OPs breakdown more rapidly than do OCs in the environment, they have milder impact, and they are safer and less persistent (Sullivan and Blose, 1992; Schenker et al., 1992). OPI are now one of the most important classes of insecticides with 150 different compounds used for protecting crops, livestock, and human health (Eto, 1974; Ware, 2000; Tomlin, 2003). The increased use of OPs has led to a different range of ecotoxicological problems associated with their high acute toxicity. Since OPI are extensively used in widespread applications, exposure to OPI is one of the occupational hazards and OPI poisoning is a major cause or morbidity and mortality in many countries.

OP compounds are usually esters, amides or thiol derivatives of phosphonic acid and form a large family (>50 000 compounds) of chemical agents with biological properties that have important, and sometimes unique applications for the benefit of mankind. The principal use of these is as pesticides in agriculture, mainly as insecticides. Some formulations are used in veterinary and human medicine, particularly as antiparasitics, e.g. against ticks, lice and fleas. In commerce, OP compounds have been used as lubricants, plasticisers and flame-retardants. The development and use of some of these compounds as very potent agents of chemical warfare is of global significance.

Chemistry and Classification

The OPI are all characterized by the general formula:



Where X is a leaving group of variable structure and R_1 and R_2 are alkoxy, amino, thioalkyl, phenyl or other substituent groups (Gallo and Lawryk, 1991; Chambers, 1992). OPs have been categorized based on the nature of the atoms immediately surrounding the central phosphorus atom (Chambers, 1992). Phosphates are the prototype for the entire class and are those compounds where all four atoms surrounding the phosphorus are oxygen (e.g. dichlorvos, mevinphos). Sulfur containing OP compounds (phosphorothioates; phosphorothiolates; phosphorodithioates; phosphorodithiolates) are far more numerous than phosphates and include well recognized OPI (e.g. parathion, diazinon, chlorpyrifos). Other organophosphate groups contain nitrogen (phosphoramides; phosphorodiamides); nitrogen and sulfur (phosphoramidothionates; phosphoramidothiolates); carbon (phosphonates; phosphinates); or, carbon and sulfur (phosphonothionates; phosphonothionothiolates; phosphinothinates).

Residues

Presence of pesticide residues in fruits and vegetables has become a global phenomenon. Frank et al., (1987) reported residues of OC and OP insecticides along with fungicides and herbicides in fruits from Ontario, Canada. Recent studies of Kumari et al., (2006) revealed the extent of pesticide residues in Indian fruits and vegetable samples. Fruit samples processed to determine residues of some commonly used OPI viz. monocrotophos, methyl parathion, chlorpyrifos and quinalphos revealed that among the tested OPI, quinalphos was a dominant contaminant with guava containing maximum residues. The

probable reason of more frequent residues of quinalphos was attributed to its use on the tested fruits for controlling fruit fly attack close to harvest stage. However all OPI detected were well within their respective maximum residue limit (MRL) values. Presence of OPI residues in vegetables and fruits is indicative of change in usage pattern of insecticides in India where a shift has taken place from OC to the easily degradable groups of these insecticides in the last decade.

Site of action

All OP pesticides exert toxicity via a common mechanism of action — binding to and phosphorylation of the enzyme acetylcholinesterase (AChE) (Mileson et al., 1998). This causes AChE inhibition and a buildup of the neurotransmitter acetylcholine at the synapses in central and peripheral nervous system resulting in over stimulation at muscarinic and nicotinic cholinergic synapses. Over stimulation at muscarinic synapses results in hypersalivation, excess lacrimation, miosis, intestinal cramps, vomiting, diarrhea, urinary and fecal incontinence, bronchorrhea and bronchoconstriction while, over stimulation at nicotinic synapses results in muscle cramps, fasciculation, weakness, paralysis and pallor. Central nervous system (CNS) effects include anxiety, restlessness, dizziness, confusion, ataxia, convulsions and respiratory and circulatory depression (Ballantyne and Marrs, 1992). Symptoms are more or less severe depending on the compound, the dose, the route, frequency and duration of exposure, and the time of observation relative to the time of peak toxic effect. Effects may be immediate or delayed by hours or days.

OPs vary considerably in their toxic potency due to a number of factors. Binding affinity of the parent compound, and or its metabolic products for AChE is a major determinant of potency. Binding affinity is determined by structural characteristics such as lability of the P–X bond and overall hydrophobicity and steric characteristics of the molecule (Gallo and Lawryk, 1991; Maxwell and

Lenz, 1992). P-O compounds are generally more potent than P-S compounds because the greater electronegativity of the oxygen substituent weakens the P–X bond thereby enhancing binding to AChE; compounds with longer n-alkyl R groups are generally more potent than those with shorter n-alkyl groups because their greater hydrophobicity enhances binding to AChE; and compounds with less bulky R or X groups are generally more potent than those with bulkier groups which hinder interaction with AChE (Gallo and Lawryk, 1991; Maxwell and Lenz, 1992). OPs also bind to a wide variety of other esterases present in blood and or tissues — the specific esterase(s) involved and the affinity of binding being dependent upon the species and specific OP (Sultatos, 1988; Chambers et al., 1990; Sultatos et al., 1991; Zhang and Sultatos, 1991; Chambers and Carr, 1993; Butler and Murray, 1997). Such binding is not directly related to the neurotoxic consequences of these compounds but may affect their overall potency. Potency is also affected by AChE binding reversibility.

Neurotoxicity

OP-induced delayed polyneuropathy (OPIDP) is a rare toxicity resulting from exposure to certain OP esters. It is characterized by distal degeneration of some axon of both the peripheral and central nervous systems occurring 1-4 weeks after single or short-term exposures. Neuropathy target esterase (NTE) is thought to be the target of OPIDP initiation. The ratio of inhibitory power for acetylcholinesterase and NTE represents the crucial guideline for the etiological attribution of OP-induced peripheral neuropathy (Lotti and Moretto, 2005).

Toxicokinetics

The toxicokinetics and toxicodynamics of OP poisoning vary not only with the route and extent of exposure, but also the chemical structure of the agent. The mechanism of toxicity is the inhibition of AChE, resulting in an accumulation of the neurotransmitter acetylcholine and the continued

stimulation of acetylcholine receptors. The standard treatment consists of reactivation of the inhibited AChE with an oxime antidote and reversal of the biochemical effects of acetylcholine with atropine. Patients who receive treatment promptly usually recover from acute toxicity but may suffer from neurologic sequelae (Kwong, 2002).

Recovery of AChE activity occurs in some cases via hydrolytic removal of the inhibiting phosphoryl moiety. Like the rate of binding, the rate of hydrolytic removal varies depending upon the specific compound. Some phosphorylated–AChE complexes are characterized by a process termed 'aging' in which the rate of reactivation declines after exposure due to dealkylation of the phosphorylated–AChE (Ballantyne and Marrs, 1992). When this happens, reactivation of phosphorylated AChE is not possible. De novo synthesis of the AChE enzyme itself, which occurs within 12 and 24 h depending on the anatomical location, also leads to recovery of AChE activity (Chambers, 1992).

These toxicodynamic factors alone, however, are not the only determinants of OP potency. Also important are their toxicokinetic characteristics. For most OPs, absorption is rapid and complete, or nearly complete, via dermal, oral or inhalation exposure, and distribution is usually extensive. Metabolic reactions, which both detoxify and toxify these compounds, however, are often highly complex and variable. Elimination half-lives, which vary from minutes to days, are also highly variable. The overall balance between metabolic generation and elimination of more versus less potent toxic derivatives is increasingly recognized as a major factor determining OP potency (Chambers et al., 1990, 1994; Chambers and Carr, 1993; Ma and Chambers, 1995; Pond et al., 1995; Atterberry et al., 1997). Differences in overall rates of detoxification contribute to the greater sensitivity of female rats to some OP (parathion, methyl parathion, EPN, chlorpyrifos) compared to male rats (Chambers et al., 1994; Ma and Chambers, 1994; Neal and DuBois, 1965),

and to the greater sensitivity of young animals to some OPs (parathion, methyl parathion, EPN) compared with adults (Neal and DuBois, 1965; Zhang and Sultatos, 1991; Atterberry et al., 1997). Additionally, differences in the overall balance between generation and elimination of more versus less potent toxic derivatives following oral exposures compared to inhalation exposures, due to first pass hepatic metabolism, can contribute to differences in potency for the same compound when administered by different routes.

Metabolism

The metabolic fate of OPI is basically the same in insects and animals. Following absorption, the distribution of OPs is variable. Blood half-lives are usually short, although plasma levels are in some cases maintained for several days. OPs undergo extensive biotransformation, which is complex and involves several metabolic systems in different organs, with simultaneous oxidative biotransformation at a number of points in the molecule, utilizing the cytochrome P-450 isoenzyme system. Metabolism occurs principally by oxidation, hydrolysis by esterases, and by transfer of portions of the molecule to glutathione. Oxidation of OPI may result in more or less toxic products. In general, phosphorothioates are not directly toxic but require oxidative metabolism to the proximal toxin. Most mammals have more efficient hydrolytic enzymes than insects and, therefore, are often more efficient in their detoxification processes. Hydrolytic and transferase reactions affect both the thioates and their oxons. Numerous conjugation reactions follow the primary metabolic processes, and elimination of the phosphorus-containing residue may be via the urine or faeces. Some bound residues remain in exposed animals. Binding seems to be to proteins, principally, since there are limited data showing that incorporation of residues into DNA (Eto, 1974).

Chronic toxicity

There is a paucity of data on the possible deleterious effects of chronic exposure to OPI in occupational and/or environmental settings. In general, the literature brings out three types of 'non-acute' OP poisoning: occupational exposure with reductions in ChE levels; occupational exposure with no reduction in ChE levels; and environmental exposure (Costa, 2006). However, the relationships between chronic exposure, ChE inhibition and symptoms do not, as yet, seem to be well established. Available evidence suggests that there is a possibility of adverse effects occurring below OP concentrations that are generally considered to be safe based on measurements of ChE inhibition; i.e. these effects are not clearly related to the inhibition of cholinesterases (Ames et al., 1989; Popendorf, 1990; Salvi et al., 2003; Singh and Sharma, 2000). Studies on health hazards to agricultural workers, who handle, store and use OPI have documented a range of non-specific, self-reported symptoms that have been attributed to chronic exposure.

Mechanisms of chronic toxicity

Chronic toxicity to OPI may be related to the rate of regeneration of AChE and the speed at which pesticide metabolites are hydrolysed and eliminated from the body. This 'fast' or 'slow' enzymatic hydrolysis status seems to be determined by gene polymorphisms of hydrolases such as paraoxonase (Lee et al., 2003). However, in some situations where there is chronic exposure to OPs, there seems to be poor correlation between evidence of toxicity and the degree of AChE inhibition (Ray and Richards, 2001; Singh and Sharma, 2000). It may well be that toxicity in these situations is mediated more by other mechanisms, such as '*oxidative stress*' through OP-induced generation of free oxygen radicals leading to lipid peroxidation (Bebe and Panemangalore, 2003; Gultekin et al., 2000), rather than inhibition of AChE.

Effects on other enzymes

OP compounds are known to inhibit other enzyme systems, the consequences of which are as yet unknown. A variety of tissue carboxyesterases abound in serum, liver, intestine and other tissues. Although inhibition of one specific carboxyesterase, i.e. NTE, has toxic sequelae, no direct deleterious effects of inhibition of other carboxyesterases have been demonstrated. However, carboxyesterases may contribute markedly to the metabolic degradation of OPI such as malathion, and inhibition of these enzymes may potentiate the toxicity of some OP compounds (Casida and Quistad, 2004).

Other candidate targets

There are also additional OP targets *in vitro* or *in vivo* of varied or unknown toxicological relevance. Malathion and malaoxon have been shown to inhibit lysyl oxidase in homogenates of *Xenopus* embryos, suggesting that they alter posttranslational modification of collagen with resultant morphological defects in connective tissue (Snawder and Chambers, 1993). Chlorpyrifos and its oxon are reported to activate '*Ca^{2+}/cAMP response element binding*' (CREB) protein in cultured rat neurons, which has been suggested to be the possible mechanism for its neurotoxicity (Schuh et al., 2002). Paraoxon is reported to induce apoptotic cell death in a leukemia cell line by disruption of mitochondria, leading to activation of caspase-9 (Saleh et al., 2003). Fenitrothion has been demonstrated to act as an androgen receptor antagonist *in vitro* (Tamura et al., 2003) and also inhibit the development of androgen-dependent tissues *in vivo* with potency comparable to the pharmaceutical antiandrogen, flutamide (Tamura et al., 2001). ATPases are found to be inhibited by varying levels of OPs (Barber et al., 2001; Blasiak, 1995). Blood clotting factors (thrombin, plasmin, and kallikrein) and digestive enzymes (R-chymotrypsin, trypsin, and elastase) have also been found to show moderate to low sensitivity to OPs (Pruett et al., 1994; Quistad and Casida, 2000).

The genotoxicity of numerous OPI has been evaluated (Eastmond and Balakrishnan, 2001). The mutagenicity of dichlorvos to bacteria is reported to be due to methylation of DNA (Wright et al., 1979) while chlorpyrifos inhibits DNA synthesis in cultured cells (Qiao et al., 2001). Chlorpyrifos and its trichloropyridinol hydrolysis product both inhibit neuronal differentiation in PC12 cells by a mechanism independent of AChE inhibition (Das and Barone, 1999). Parathion and TOCP inhibit the mitochondrial transmembrane potential which may be a possible target for their cytotoxicity in cultured cells (Carlson and Ehrich 1999). Several OP pesticides, both phosphates and phosphorothionates displace binding at the *N*-methyl-D-aspartate receptor of rat brain (Johnson and Michaelis, 1991). Dichlorvos also decreases the activities of murine splenic natural killer, cytotoxic T lymphocyte, and lymphokine-activated killer by a proposed mechanism involving serine protease (granzyme) inhibition (Li et al., 2002). Developmental neurotoxicity and numerous *in vivo* effects have been reported for chlorpyrifos in rats (Slotkin, 2004).

Other effects of organophosphorus insecticides

Effects on the CNS

Eyer (1995) concluded that neuropsychological effects can occur after OP poisoning and that the most frequently reported symptoms include impaired memory and vigilance, reduced information processing and psychomotor speed, memory deficit, linguistic disturbances, depression, anxiety and irritability. There is some concern at present that exposure to OP agents may precipitate psychosis and that chronic psychiatric effects of varying intensity may persist for years (Fiedler et al., 1997).

Effects on Immune system

OP pesticides have been demonstrated to elicit a variety of immunotoxic effects in laboratory animals, wildlife, and humans through both cholinergic and non-cholinergic pathways (Rodgers, 1995; Galloway and Handy, 2003). Bellin

and Chow (1974) suggested that OP agents might have an effect on the human immune system. OP-induced immunosuppression was associated with severe cholinergic stimulation, either from a direct action of acetylcholine on the immune system or secondary to the toxic chemical stress associated with cholinergic poisoning.

Effects on cardiovascular system

Cardiac complications often follow OP poisoning and the disorders reported range from hypotension or hypertension to arrhythmias and cardiac arrest (Saadeh et al., 1997).

Effects on reproductive system

In experimental animals, OP poisoning during pregnancy causes pre- and postnatal death and congenital abnormalities such as vertebral deformities, limb defects, polydactyly, intestinal herniae, cleft palate and hydroureter (Hayes, 1975). Dichlorvos causes damage to seminiferous tubules (Krause and Homola, 1974) while phosphamidon appears to affect the principal cells in the caput epididymis through its toxic effect on the Leydig cells (Akbarsha and Sivasamy, 1998).

Effects on Endocrine system

In animals, cholinesterase inhibitors have been shown to modify the pituitary –thyroid and pituitary-adrenal axes, and to affect prolactin levels. Direct effect of OPI on endocrine changes in patients with acute organophosphate poisoning was reported by Guven et al., (1999). Experimental evidences strongly suggest the effect of OPI on the endocrine system. Exposure to low levels of OPI such as diazinon resulted in impairment of hepatic metabolism of corticosterone. Plasma levels of corticosterones were also elevated in these animals, but without a concomitant increase in adrenal steroidogenesis (Cramer et al., 1978). Spassova et al., (2000) demonstrated that the effect of

methamidophos on adrenal cortex was mediated by pituitary gland, while the effect of acephate on adrenal gland did not involve the pituitary gland. It was also observed by these authors that these endocrine effects of acephate and methamidophos differed from their cholinergic effects, and were not proportional to the amount of pesticides in the tissue. Recently, chlorpyrifos has been implicated as a putative endocrine disrupting chemical since it had profound effect on hypothalamic gonadotropin-releasing hormone (GnRH), altering GnRH gene expression, cell survival and neurite outgrowth (Gore, 2001). A parallel effect on mRNA GnRH expression in female rats raised the possibility that neurological and reproductive effects could be mediated through effects of chlorpyrifos on the hypothalamus. Impaired thyroid function, also under hypothalamic control, was detected in a segment of male pesticide formulators exposed to mixtures of pesticides including chlorpyrifos (Zaidi et al., 2000).

Other sundry effects

Very few effects other than those described in the sections above have been noted, except those arising from ill-health due to severe anticholinesterase effects. Thus, impaired growth rate is commonly associated with a rapid depression in AChE levels to less than 50%, but much lower levels can be tolerated without ill-effects (Worek et al., 2005). Various changes in glucose metabolism, in serum enzymes, and in other clinical chemical variables have been reported after single, acute, or repeated doses of various pesticides at from one-tenth to one-quarter of the LD_{50}, daily (Dimov and Kaloyanova, 1967; Enan et al., 1982).

OP poisoning

OPI are the most commonly associated with serious human toxicity. OPI may cause serious poisoning either accidentally or by deliberate ingestion (Bardin et al., 1994). According to a recent survey, suicidal and occupational OP poisoning in agricultural workers was prevalent in developing countries,

whereas accidental OP poisoning was prevalent in developed countries (Dharmani and Jaga, 2005). Children accounted for 35% of the OP poisoned victims. The most common symptoms observed were nausea and the most common sign was increased bronchial secretions. Laboratory abnormalities were similar to those previously reported in the literature: luekocutosis, hyperglycemia and hypokalemia. Preventing environmental OP exposure and increasing the awareness of pesticide toxicity would reduce OP poisoning.

OP compounds produce significant pesticide-related illness in developing countries. Thus there is a need to determine exact extent of the problem and to develop appropriate strategies to manage these cases with available resources in these countries. Results of some studies reported that the many deaths are due to OPI and occur in the young economically active age group (Eddleston et al., 1998; Van der Hoek et al., 1998; Eddleston et al., 2003). Intoxication by OPs, occasionally leading to death, represents up to 80% of pesticide-related hospital admissions (Taylor, 2001). There are few published studies of agrochemical poisoning in developed countries. The study by Singh and Unnikrishnan (2006) revealed that the maximum cases of self-poisoning due to OPI in South India, which is different from the results of North Indian studies. This may be because of easy availability, and uncontrolled sale and use of these agents. In another study, Singh and Unnikrishnan (2006) showed that the 87% of the mortality was due to pesticides and among which 65% was due to OPIalone.

OPI and hyperglycemia

Multiple evidences indicate that, OPI affect glucose homeostasis and blood glucose levels are severely increased in OP poisoning (Seifert, 2001; Hayes et al., 1978; Meller et al., 1981; Namba et al., 1971; Teichert-Kuliszenwska et al., 1981). Induction of hyperglycemia and alterations in blood glucose homeostasis has been demonstrated in several studies employing rat as animal model following exposure to several OPI (**Table 1**).

Table 1 Studies demonstrating that organophosphorous insecticides (OPI) affect glucose homeostasis in rats

OPI	Exposure	Effect	Reference
Acephate	Subchronic	Depletion of liver glycogen, ↑ blood glucose level	*Deotare & Chakrabarti, 1981*
Dichlorvos	Acute	↓ Hepatic glucokinase, ↑ hepatic glucokinase mRNA	*Romero-Navarro et al., 2006*
	Chronic	Depletion in brain glycogen content, ↑ GP, ↓ HK, PFK, and LDH	*Sarin & Gill ,1999*
Dimethoate	Subchronic	Hyperglycemia	*Reena et al., 1989*
		↑ Blood glucose level, pancreatic damage	*Hagar et al., 2002*
Malathion	Acute	↑ Blood glucose level	*Rodrigues et al., 1986*
		Hyperglycemia, ↓ cerebral glycogen, ↑GP and HK	*Matin & Husain, 1987c*
	Subchronic	Hyperglycemia, ↑hepatic GR and PEPCK	*Abdollahi et al., 2004a*
		↑ Blood glucose and insulin levels, ↑muscle PFK-1 and muscle GP	*Pournourmohammadi et al., 2005*
		↓ Hepatic GP ; ↑ HK, ↑ glycogen storage in liver	*Rezg et al., 2006*
	Acute and subchronic	↑ Blood glucose and insulin levels	*Panahi et al., 2006*
Soman	Acute	↑Blood glucose level	*Shih & Scremin ,1992*

Sarin and Gill (1999) found that dichlorvos administration decreased the activities of Hexokinase I, phosphofructokinase and lactate dehydrogenase in rat brain. They also observed an increase in the activity of glycogen phosphorylase accompanied by a significant depletion in brain glycogen content. Chronic exposure to dichlorvos was also found to cause a significant depletion in the brain glycogen content accompanied by an increase in the activity of GP (Sarin and Gill, 2000). Teichert-Kuliszenwska and Szymcyc (1979) found that a single dose of dichlorvos (50% LD_{50}) increased the activity of glycogen phosphorylase, while it decreased UDP-glucose pyrophosphorylase activity. Acute intraperitoneal treatment of rats with diazinon resulted in depletion of brain glycogen and increase in the activities of glycogen phosphorylase (GP) (Matin and Husain, 1987a, b). Treatment of rats with acute dose of diazinon also resulted in hyperglycemia, depletion of glycogen from brain and peripheral tissues accompanied with increased activity of GP in the brain and liver, and increased activity of the hepatic gluconeogenic enzymes, phophoenolpyruvate carboxykinase (PEPCK) (Matin et al., 1990).

Increased blood glucose levels and decreased hepatic glycogen content were also observed in several other animal models (**Table 2**). In *Macrobrachium lamarrei*, a freshwater prawn, as well as in fresh water fish (*Clarias batrachus, Saccobranchus fossilis, and Mystus vittatus*) dichlorvos exposure significantly decreased liver glycogen content and affected glucose homeostasis (Omkar and Shukla, 1985; Verma et al., 1983). Sublethal single dose of dimethoate has been shown to increase the hepatic and muscle GP activity of Indian catfish, *Clarias batrachus* (Begum and Vijayaraghavan, 1995, 1999).

Table 2 Studies demonstrating that organophosphorous insecticides (OPI) affect glucose homeostasis in other animal models

OPI	Animal	Exposure	Result	Reference
Diazinon	Mice	Acute	Hyperglycemia	*Seifert, 2001*
Dimethoate	Catfish (*Clarias batrachus* L.)	Acute	↓ Glycogen, ↓ LDH, and ↓ GP	*Begum & Vijayaraghavan, 1995*
Dursban	Snail (*Biomphalaria Alexandria*, *Bulinus truncates*)	Acute	↓ glycolysis, ↑ glucose utilization and gluconeogenesis	*Sharaf et al., 1975*
Fenitrothion	European eel (*Anguilla anguilla*)	Acute	↑ Blood glucose,	*Sancho et al., 1997*
Fenitrothion	Fresh water fish (*Sarotherodon mossambicus*)	Acute	↑ Blood glucose, ↓hepatic glycogen	*Koundinya & Ramamurthi, 1979*
Fenthion	Fresh water teleost (*Heteropneustes fossilis)*	Acute	Hyperglycemia	*Srivastava & Mishra, 1983*
Malathion	Chick embryo	Sub acute	Hyperglycemia	*Arsenault et al., 1975*
Methidathion	Carp	Acute	↑ Blood glucose	*Nemcsok et al., 1987*
Methyl parathion	Fresh water fish (*Brycon cephalus*)	Acute	↓ hepatic glycogen, gluconeogenesis	*De Aguiar et al., 2004*
Monocrotophos	Broiler chick	Chronic	↑ Blood glucose	*Garg et al., 2004*
Phorate	Fresh water fish (*Clarias batrachus*)	Acute	↑ Blood glucose	*Jyothi & Narayan, 1999*
Quinalphos	Rosenged parakeet	Sub acute	↑ Blood glucose	*Anam & Maitra, 1995*

Several investigations have reported that Dichlorvos exposure affects polysaccharide metabolism (Sarin and Gill, 1999; Omkar and Shukla, 1985; Verma et al., 1983). DDVP reportedly decreases UDP-glucose pyophosphorylase activity (Teichert-Kuliszenwska and Szymcyc, 1979) an enzyme that participates in glycogen synthesis and it is also reported to increase the activity of glycogen phosphorylase (Sarin and Gill, 1999; Teichert-Kuliszenwska and Szymcyc, 1979), a key enzyme in glycogen breakdown. Studies on the effect of DDVP administration during the oral glucose loading test in rabbits revealed that 1h after administration of Dichlorvos, animals exhibited hyperinsulinemia (Teichert-Kuliszenwska et al., 1981). The cholinergic effects of DDVP may account for these effects (Kobayashi et al., 1986; Ehrich et al., 1997;)since acetylcholine increases glucose-induced insulin secretion by a rapid mechanism which involves a rise in free cytosolic Ca^{2+} concentration and a protein kinase C-mediated increase in Ca^{2+} exocytosis (Gilon and Henquin, 2001). It has been reported that acetylcholine is a potent secretagogue of both insulin and glucagon (Gilon and Henquin, 2001; Duttaroy et al., 2004); therefore, the cholinergic effects of Dichlorvos may perhaps involve changes in the insulin /glucagon ratio also (Teichert-Kuliszenwska et al., 1981).

OPI and oxidative stress

Free radicals play an important role in the toxicity of pesticides and environmental chemicals (Abdollahi et al., 2004b). Pesticides stimulate peroxidation of cellular membranes by different mechanisms; direct initiation by free radicals produced by metabolism of the chemical, indirect initiation by the production of reactive forms of oxygen during their metabolism, inhibition of enzymatic systems of defence involved in the control of reactive oxidizing entities, and destruction of natural antioxidants (Parashar and Singh, 1987; Dikshith, 1991; Gupta et al., 1992; Comporti, 1993; Datta et al., 1994; Yarsan,1998; Prasanthi et al., 2005 a, b) .

Numerous studies have demonstrated enhanced oxidative stress (OS) due to by OPs in rats and humans although the most prominent clinical effects of poisoning with OPs result due to inhibition of AChE. Ranjbar et al., (2002) studied OS status and AChE activity in blood samples obtained from OP formulatory pesticide workers with a minimum history of 1 year in the age range of 23–55 years. Their results showed marked inhibition of AChE activity and increased TBA reactive substances indicating lipid peroxidation. Subchronic exposure of malathion has been reported to enhance antioxidant enzymes which were also accompanied by elevated levels of MDA in rat erythrocytes and liver (Akhgari et al., 2003; Abdollahi et al., 2004b). Malathion has also been reported to increase plasma MDA content Yarsan et al., (1999) and enhance microsomal TBARS in rats (Pedrajas et al., 1995). Studies of Shadnia et al., (2005) also demonstrated that chronic exposure of humans to OPI was associated with increased activities of antioxidant enzymes such as, CAT, SOD, and GSP-Qx in erythrocytes.

Gromov et al., (1993) studied the influence of dichlorvos on the activity of antioxidant enzymes such as SOD and CAT in brain and blood of female rats and found that CAT activity was lowered in brain 3 h after administration. Also Altuntas et al., (2002a, b) investigated the effects of methidathion on lipid peroxidation and antioxidant enzymes and ameliorating effects of a combination of vitamin E and C against the toxicity. Their results demonstrated that treatment of rats with methidathion increased the lipid peroxidation and decreased antioxidant enzymes in erythrocytes.

Pancreas and OPI toxicity

Pancreas is both an exocrine and an endocrine organ and the major hormones it secretes – insulin and glucagon – play a vital role in carbohydrate and lipid metabolism, since they are absolutely essential for maintaining normal blood concentrations of glucose. The bulk of the pancreas is composed of exocrine tissue and embedded within this are '*islets of Langerhans*', which are

the endocrine cells. The pancreatic β cells and its secretory product, insulin is central in the pathophysiology of diabetes (Guyton and Hall, 1996). Active oxygen metabolism is reported to play a key role in the normal functioning of the β cells of pancreas. Diminished rate of secretion of insulin by the β cells of the islets of Langerhans in almost all instances causes Diabetes mellitus and all forms of diabetes are characterized by chronic hyperglycemia (Brownlee, 2001). Further, increased risk of pancreatic cancer has been reported among diabetic patients and after exposure to OC pesticides (Slebos et al., 2000). However, basic understanding regarding the possible association of these risk factors with the alterations found in pancreatic damage is limited.

Occupational and environmental diseases of pancreas have been under emphasized relative to the other causes of pancreatitis. The usual causes of pancreatitis include alcohol, biliary tract disease, drugs and infectious agents (Vikas and Jamie, 2001). Environmental toxins cause an unknown proportion of so-called idiopathic pancreatitis. The foremost toxins include venom of scorpion (Gallagher et al., 1981), anticholinesterase insecticide (Lee, 1989; Dagii and Shaikh, 1983), organic solvents, pentachlorophenol (Cooper and Macaulay, 1982) and diethyl glycol (O'Brien et al., 1998). Few studies have however demonstrated some evidence for damage in pancreatic exocrine function after anticholinesterase insecticides intoxication (Panieri et al., 1997; Dressel et al., 1979; Lankisch et al., 1990).

Acute pancreatitis as a complication of OP intoxication is not an uncommon condition. Its incidence in adults is 12.7 % (Sahin et al., 2002) and is higher in children (Weizman and Sofer, 1992). In a study conducted by Rubi et al., (1996), out of 506 cases admitted due to OPI poisoning, 3 cases were reported with pancreatitis. In previous case reports, severe acute pancreatitis with pseudo cyst formation has been described in an adult woman following accidental ingestion of an OPI (Dressel et al., 1979). Furthermore, OPs such as ecothiophate, which inhibit both the cholinesterase isoenzymes

(butyrylcholinesterase and acetylcholinesterase) in human pancreas, are known to increase pancreatic sensitivity to acetylcholine (Oguchi et al., 1989; Liu et al., 1990). However, pancreatitis secondary to OPI toxicity is rare and is believed to follow a sub clinical, uneventful course (Panieri et al., 1997). Pancreatic ductal hypertension and stimulation of exocrine pancreatic secretion secondary to cholinergic stimulation are considered to be responsible for development of pancreatitis (Kandalaft et al., 1991; Weizman and Sofer, 1992; Goodale et al., 1993; Moritz et al., 1994; Dressel et al., 1982). Based on studies conducted on dogs, cats, and guinea pigs subjected to a single dose of Diazinon (75mg/kg) and on the histochemical examination of the acinar tissue, it was suggested that pancreatic tissue-fixed butyrylcholinesterase is the target enzyme of OP toxicity (Frick et al., 1987).

The association between pancreatic dysfunction and OPI are still not widely recognized. However, since both acute and chronic exposure to OPI appear to have a significant impact on pancreatic function and in turn on blood glucose, comprehensive studies are warranted to elucidate the spectrum of biochemical / functional alterations induced by OPI in experimental animals. Data obtained from such studies would certainly enable us to develop suitable and efficient intervention or corrective strategies to minimize or overcome the specific toxic effects of xenobiotics in general and OPI in particular.

OPI and antioxidants

Experimental evidences suggest that lipid peroxidation (LPO) is one of the molecular mechanisms involved in OP-induced cytotoxicity (Akhgari et al., 2003; Ranjbar et al., 2002; Abdollahi et al., 2004b). It has been suggested that OP-induced hyperglycemia is associated with oxidative stress and antioxidants can be protective agents in such a condition (Milani et al., 2005; Rahimi et al., 2005). Several recent studies have demonstrated the protective effects of antioxidants against OPI-induced toxic effects (**Table-3**).

Table 3 Beneficial effects of antioxidants on experimentally-induced OPI toxicity

OPI	Antioxidants	Protective effect	Reference
Chlorpyrifos	Vitamin A, E & C	↓ tissue OS (R)	*Verma et al., 2007*
	Black tea extract	↓ OS in liver (M)	*Khan et al., 2005*
Chlorpyrifos-ethyl	Vitamin C, E & melatonin	↓ lung toxicity	*Karaoz et al., 2002*
Diazinon	Vitamin E & C	Decrease pancreatic damage (R)	*Gokalp et al., 2005*
		↓ OS in heart (R)	*Akturk et al., 2006*
	Vitamin E	↓ Hepatotoxicity (R)	*Kalender et al., 2005*
		↓ Myocardial MDA (R)	*Ogutcu et al., 2006*
		Protection to psuedocholinesterase and haematological indices (R)	*Kalender et al., 2006*
Dichlorvos	Vitamin E & C	↓ Endometrial damage	*Oral et al., 2006*
Dimethoate	Vitamin E	↓ OS, ↓ LPO (R)	*John et al., 2001*
Fenthion	Interleukin	↓ Pancreatic damage (R)	*Ikizceli et al., 2005*
Methidathion	Vitamin E & C	Amelioration of nephrotoxicity (R)	*Sulak et al., 2005*
		Reduced hepatic toxicity (R)	*Sutcu et al., 2006*
		↓ liver damage	*Altuntas et al., 2002a*
		↓ LPO in erythrocytes	*Altuntas et al., 2002b*

R: Rat ; M: Mice
OS: oxidative stress ; LPO: lipid peroxidation

Supporting the role of OS, it has been reported that the antioxidant, tocopherol reduces diazinon-induced hepatic biochemical and ultrastructural toxicity following chronic administration (Kalender et al., 2005). Recent study showed that administration of tocopherol and N-acetylcysteine decreased malathion-induced oxidative stress in blood and ChE inhibition following a subchronic regimen (Shadnia et al., 2006). Antioxidant compounds react directly with radicals reducing OS and exerting protective effects against cellular damage (Gaetke and Chow, 2003).

Diabetes, hyperglycemia and impaired glucose tolerance

Diabetes is by far the most common metabolic disorder that is associated with significant morbidity / mortality and with a worldwide prevalence. All forms of diabetes, both inherited and acquired, are typified by chronic *'hyperglycemia'*, a relative or absolute lack of insulin secretion and / or action associated with disturbances of carbohydrate, lipid, and protein metabolism leading to the development of diabetes-specific microvascular pathology in the retina, renal glomerulus and peripheral nerve (Schmidt and Stern, 2000). When fully expressed, diabetes is characterized by fasting hyperglycemia, but the disease can also be recognized during less overt stages and before fasting hyperglycemia appears, most usually by the presence of '*glucose tolerance*' (Bennett, 1994). '*Impaired glucose tolerance*' is a category that permits classification of individuals whose glucose tolerance is above the conventional normal range but lower than the level considered diagnostic of diabetes. Such persons do have a high risk of developing diabetes mellitus. Impaired glucose tolerance has been attributed to a wide variety of causes, including certain drugs and toxins (WHO, 1980).

Diabetes is rapidly emerging as a major problem in the world (Zimmet et al., 2001, Onkamo et al., 1999). The prevalence of diabetes is rising due to the westernized life style, which include excessive energy intake and physical inactivity (Zimmet et al., 2001). However, several drugs (Ferner, 1992) and environmental factors also account for the development of diabetes (Ferner, 1992; Yoon et al., 1987; Department of Veterans Affairs, 2001). The increasing use of OPI with their adverse effects on glucose metabolism may well be an additional factor in the speedy expansion of diabetes in the world.

Diabetes and oxidative stress

There are convincing experimental and clinical evidences which suggest that generation of reactive oxygen species is increased in both insulin dependent (type 1) and non-insulin-dependent diabetes (type 2) and that the onset of diabetes is closely associated with oxidative stress (Rosen et al., 2001; Johansen et al., 2005). Hyperglycemia increases oxidative stress via an over production of oxygen free radicals which are formed disproportionately through processes such as: glucose autooxidation, polyol pathway and non-enzymatic glycation of proteins (Wolff and Dean, 1987; Lee et al., 2002; Obrosova et al., 2002). While on one hand hyperglycemia engenders free radicals, on the other hand it also impairs the endogenous antioxidant defense system in many ways (Saxena et al., 1993). Abnormally high levels of free radicals and simultaneous decline of antioxidant defense systems can lead to damage of cellular organelles and enzymes, increased lipid peroxidation and development of complication of diabetes mellitus (Maritim et al., 2003).

OS produced under diabetic condition is likely to be involved in progression of pancreatic β-cell dysfunction (Kajimoto and Kaneto, 2004). Also, because of the relatively low expression of antioxidant enzymes such as catalase and superoxide dismutase, pancreatic β-cells may be vulnerable to ROS attack when the system is under OS situation (Lenzen et al., 1996; Tiedge et al., 1997). Therefore, once β-cells face oxidative stress, they may be rather

sensitive to it, and subsequent OS may in part mediate the toxic effect of hyperglycemia. It is generally believed that the number of functionally intact β-cells in the islets is of decisive importance in the development, course and outcome of diabetes mellitus (Hardikar et al., 1999).

Diabetes, antioxidants and phytochemicals

In recent years, dietary plants with antioxidative property have been the center of focus. It is believed that certain plants can prevent or protect tissues against damaging effect of free radicals (Osawa and Kato, 2005). Further, dietary supplementation with natural antioxidants such as, vitamins C and E, melatonin and flavonoids significantly attenuated the oxidative stress and diabetic state induced by STZ (Kaneto et al., 1999; Coskum et al., 2005; Montilla et al., 1998). Although animals have their own antioxidant defense systems, the defense can be externally strengthened. This might be especially true for the pancreas, since it has a relatively weak intrinsic defense system against OS (Tiedge et al., 1997).

Antioxidant vitamins

Numerous studies have demonstrated that antioxidant vitamins and supplements can help lower the oxidant stress markers and lipid peroxidation in diabetic subjects and animals. A number of studies have reported vitamin C and E and beta-carotene deficiency in diabetic patients and experimental animals (Nazirogilu and Butterworth, 2005; Penckofer et al., 2002).

Phytochemicals

Data from both scientific reports and laboratory studies show that plants contain a large variety of substances that possess antioxidant activity (Chanwitheesuk et al., 2005). Natural antioxidants occur in all higher plants and in all parts of the plant (wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen, and seeds) (Chanwitheesuk et al., 2005). The occurrence of oxidative

mechanisms in plants may explain why an abundance of antioxidant compounds have been identified in plant tissue (Larkins and Wynn, 2004). Therefore it seems that plants particularly those with high levels and strong antioxidant compounds have an important role in improvement of disorders involving oxidative stress such as diabetes mellitus. There are many investigations that have studied the effects of these plants and their antioxidant ingredients on diabetes and its complications and achieved good results (**Table 4 & Table 5).**

Table 4 Plants with protective effects in experimental diabetes due to their antioxidant properties

Plant material	Protective effect	Reference
Dandelion (*Taraxacium offinal*)	Prevents diabetic due to LPO in STZ diabetic rats	*Cho et al., 2002*
Gangronema latifolium	↓ OS in liver of STZ diabetic rats	*Ugochukwu et al., 2003*
Grapefruit seed extract	↓ Pancreatitis	*Dembinski et al., 2004*
Momordica charantia	Renewal of β cells in STZ-diabetic rats	*Ahmed et al., 1998*
Peumus boldus	↓ STZ induced oxidative tissue damage	*Jang et a., 2000*
Potato peel	Prevents diabetic complication in STZ diabetic rats	*Singh et al., 2005a*
	↓ STZ induced oxidative tissue damage	*Singh et al., 2005b*
Tamarindus indica seed extract	Antidiabetic activity in STZ-induced rats	*Maiti et al., 2004*
Teucrium polium	Normalizing oral glucose tolerance test, regeneration of pancreatic islets, ↑ Glucokinase	*Vessal et al., 2003*

STZ: streptozotocin ; OS: oxidative stress ; LPO: lipid peroxidation

Table 5 Plants with protective effects in experimental diabetes due to their antioxidant properties: active components identified

Plant	Active ingredient	Mechanism of action	Reference
Allium cepa	S-methyl cysteine sulfoxide	Scavenging free radical	*Kumari & Augusti, 2002*
Allium sativum	S-allyl cysteine sulfoxide	Scavenging free radical, ↓ LPO	*Augusti & Sheela, 1996*
		↑ GST, ↑ SOD in liver and kidney, ↓ LPO	*Anwar & Meki, 2003*
Eugenia jambulana	Flavonoids	Scavenging free radical	*Ravi et al., 2004*
Ferula asafetida	Ferulic acid	Scavenging free radical, ↓ LPO	*Ohnishi et al., 2004*
Jugulans regia	Pedunculagin	↓ biomarkers of OS	*Fukuda & Yoshida, 2003*
Trifolium alexandrinum	Flavonoids	Scavenging free radicals, ↓ LPO, ↑ hepatic GSH	*Amer et al., 2004*
Viburnum dilatatum	Cyanidin 3-sambubioside	Scavenging free radical, ↓ LPO	*Iwai et al., 2004*

OS: oxidative stress; LPO: lipid peroxidation

AIM AND SCOPE OF THE PRESENT INVESTIGATION

Organophosphorous insecticides (OPI) are primarily recognized for their ability to induce toxicity in mammals through inhibition of acetylcholinesterase leading to accumulation of acetylcholine and subsequent activation of cholinergic, muscarinic and nicotinic receptors. Toxicity of OPI results in various toxic manifestations in many organs particularly the nervous system while other systems that could also be affected by OP intoxication include liver, immune system and kidney. OP may induce oxidative stress on acute exposure in humans while it has also been postulated that exposure to chronic low levels of OP can adversely affect the health of individuals. Although a large number of studies have been reported on detrimental effects of OPI, their damaging effect on the endocrine function of pancreas have not been comprehensively elucidated. However, acute pancreatitis has been often reported following OP intoxication in human subjects.

Several environmental insults have been implicated in the etiopathogenesis of both insulin-dependent and insulin-independent diabetes. These factors are known to alter the β cell function and disturb its homeostasis i.e., qualitative and quantitative changes in its activity. Compelling experimental and epidemiological evidence indicates that at least in some forms of insulin-dependent diabetes mellitus (IDDM), environmental factors such as chemical toxins play an important role in the etiology of this disorder. The association between pancreatic dysfunction and OPI are still not widely recognized. However, since both acute and chronic exposure to OPI appear to have a significant impact on pancreas and its function and in turn on blood glucose, comprehensive studies are warranted to elucidate the spectrum of biochemical alterations induced by OPI in experimental animals. Data obtained from such studies would certainly enable us to develop suitable and efficient intervention or corrective strategies to minimize or overcome the specific toxic effects of xenobiotics in general and OPI in particular.

It has been suggested that β cell dysfunction or death occurs by oxyradical-mediated processes. It has been demonstrated that reduced oxidative stress by supplementation with antioxidants may prevent the loss or even restore the β-cell activity from environmental diabetogenic insults. In recent years, various plant components have been demonstrated to be useful for the therapy of diabetic hyperglycemia and have been widely investigated. Under oxidative stress conditions, several phenolics exhibit a powerful protective effect on β-cells *in vitro*.

Despite these findings, the ameliorative effect of phytochemicals on xenobiotic-induced pancreatic damage / hyperglycemia has not received much attention. Hence, in this proposal, it is hypothesized that "OPI may specifically damage pancreas and cause hyperglycemia, either permanent or transient and render the organism susceptible to diabetes" (**Fig. 1**). Thus the proposal basically addresses three fundamental questions viz., (a) the role played by OPI in pancreatic dysfunction and ensuing hyperglycemia and the basic mechanism/s involved in the process (b) the interactive role of OPI and diabetic condition in exaggerating the disease condition (c) the dietary modulation of the xenobiotic-induced hyperglycemia employing selected phytochemicals.

The data obtained from these investigations will provide (i) a comprehensive overview on the effects of OPI on pancreatic function and their contribution towards the development of metabolic disorders (ii) experimental evidences that offer a conceptual framework to formulate better avenues for the treatment of pancreatic damage/ hyperglycemia induced by OPI (iii) evidences on the role of antioxidant phytochemicals in alleviating the oxidative damage impact on the pancreas.

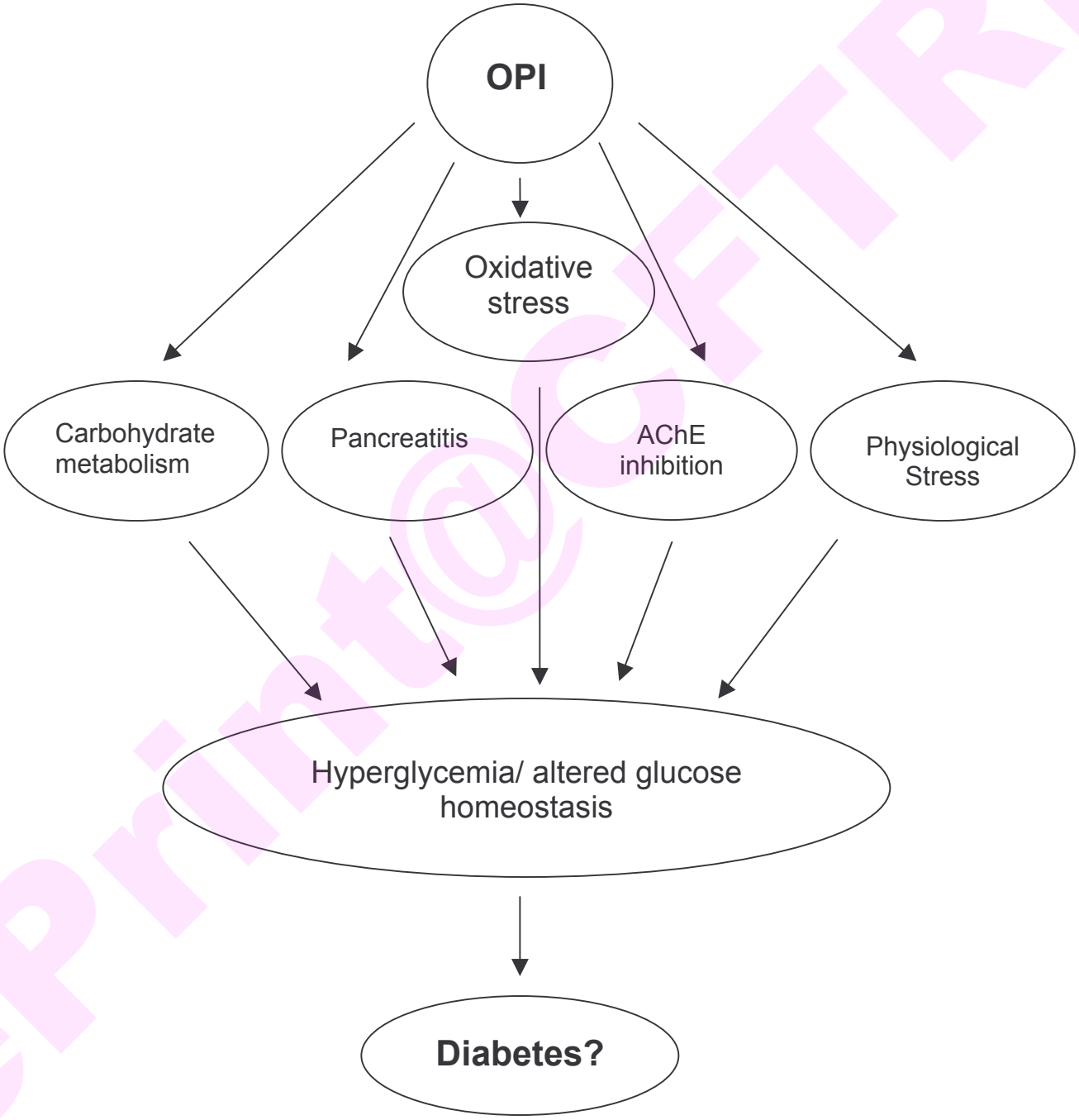


Fig. 1 Mechanisms likely to be involved in OPI-induced hyperglycemia

CHAPTER I

OXIDATIVE STRESS MEDIATED TOXICITY OF ORGANOPHOSPHOROUS INSECTICIDES

PREFACE

The major aim of this study was to determine whether oxidative stress plays a role in OPI-induced pancreatic dysfunction and the ensuing altered glucose homeostasis. Prior to this, we planned to investigate the ability of OPI to induce oxidative stress *in vitro* as well as *in vivo,* and then investigate the potential of those OPI that induce OS, to impair pancreatic function and alter glucose homeostasis in experimental animals.

This Chapter has been presented under two sections.

Section A focuses on the potential of selected OPI to induce oxidative stress *in vitro* in rat pancreatic homogenate system. Initially, selected OPI such as chlorpyrifos (CP), ethion (ET), dimethoate (DM), dichlorvos (DDVP), monocrotophos (MC), malathion (MAL) and quinalphos (QP) were screened for their oxidative stress inducing potential. We selected DM and DDVP for our further mechanistic studies in other model systems: rat pancreatic islet cultures and *Caenorhabditis elegans* cultures.

Section B describes the *in vivo* investigations related to alterations in blood glucose levels in rats exposed to single / multiple oral doses of DM and DDVP.

SECTION A

ORGANOPHOSPHOROUS INSECTICIDE INDUCED OXIDATIVE STRESS AND TOXICITY IN SELECTED *IN VITRO* MODEL SYSTEMS

1.0 INTRODUCTION

Organophosphorous (OP) compounds are cholinesterase-inhibiting chemicals used as pesticides. Exposures to OPs cause a significant number of poisonings and deaths each year (Hsiao et al., 1996; Rubi et al., 1996; Panieri et al., 1997; Sahin et al., 2002). Hyperglycemia is one of the side effects in poisoning by organophosphorous insecticides (OPI) in humans (Hayes et al., 1978; Meller et al., 1981) and the ability of OPI to cause hyperglycemia has also been confirmed in laboratory animals (Matin and Siddiqui, 1982; Fletcher et al., 1988). The mechanism of OPI-induced hyperglycemia has not yet been identified but the different mechanisms likely to be involved have been recently reviewed (Rahimi and Abdollahi, 2007), and they include physiological stress, oxidative stress, inhibition of paraoxonase, nitrosative stress, pancreatitis, inhibition of cholinesterase and stimulation of adrenal gland.

Generation of free radicals has been implicated in several insecticide toxicities (Bagchi et al., 1995; Yamano and Morita, 1995; Pitrowski et al., 1996; Banerjee et al., 1999). It has been well demonstrated that lipid peroxidation is one of the molecular mechanisms involved in OP-induced cytotoxicity (Ranjbar et al., 2002; Akhgari et al., 2003; Abdollahi et al., 2004b). In the present study, the use of tissue homogenates as an experimental model was based on the assumption that OPI may generate free radicals at various sub-cellular sites, such as mitochondria and microsomes, which are functional in tissue homogenates and would provide a representative machinery for the generation of activated species. The validity of using tissue homogenates, as an indicator of whole tissue susceptibility to lipid peroxidation (LPO) has been earlier demonstrated (Kornburst and Mavis, 1980; Wofford and Thomas, 1988).

In vitro studies in cell culture offer a good model system to study the molecular mechanism of xenobiotic-induced cell death (Robertson and Orrenius, 2000). Exposure of isolated human or rodent islets or pancreatic β-cell lines to ROS has been shown to markedly inhibit their function, resulting in cell death. The damaging effects of reactive oxygen species on pancreatic islets have been widely investigated in diabetes mellitus as well as in islet transplantation. Islets of Langerhans are known to exhibit loss of viability following exposure to nitric oxide, ROS and cytokines (Di Matteo et al., 1997). Pancreatic β-cells play a major role in maintaining glucose homeostasis through secretion of insulin in response to changes in extra cellular glucose. ROS may play a central role in β-cell death, leading to the development of type I diabetes (Ho and Bray, 1999; Olejnicka, 1999; Kaneto et al., 2001), and a secondary pathogenic role in the development of type 2 diabetes. Further, when compared with other cell types, pancreatic β-cells are particularly susceptible to destruction caused by ROS and this sensitivity is probably due to islet cells containing low levels and activities of several ROS detoxifying systems.

In this study, we investigated the conditions that result in OPI- induced oxidative stress with an objective to assess its toxicological significance. The study also aimed at selecting the OPI that are capable of inducing significantly increased levels of ROS and or lipid peroxidation and cell death in selected *in vitro* model systems, such as rat pancreatic homogenate and isolated islet cells in culture and in *Caenorhabditis elegans* culture.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Monocrotophos (Technical grade; 95%) [dimethyl (*E*)-1-methyl-2-(methylcarbomoyl) vinyl phosphate] and Quinalphos (Technical grade; 97%) [*O, O*-diethyl O-quinoxalin-2-yl phosphorothioate] were kindly provided by M/s United Phosphorous Ltd., (Mumbai, India). Malathion (Technical grade; 92%) [*S*-1, 2-bis (ethoxycarbonyl) ethyl O, O-dimethyl phosphorodithioate], Ethion (Technical grade; 97%) [*O, O, O', O'*-tetraethyl *S, S'*-methylene bis (phophorodithioate), Dimethoate (Technical grade; 95%) [*O,O*-dimethyl *S*-methylcarbomoylmethyl phosphorodithioate], Chlorpyrifos(Technical grade; 94%) [*O,O*-diethyl *O*-3,5,6 trichloro-2-pyridyl phosphorothioate] and Dichlorvos (Technical grade; 94%) [2, 2-dichlorovinyldimehtyl phosphate] were gifted by M/s Hyderabad Chemical Supplies Ltd., (Hyderabad, India). Thiobarbituric acid (TBA), Dimethyl sulfoxide (DMSO), reduced glutathione (GSH), collagenase type V, Dithizone, 2',7'-Dichlorofluorescin diacetate (DCFH-DA), 2',7'-Dichlorofluorescein (DCF), Soybean trypsin inhibitor (STI), BSA fraction V, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue were purchased from M/s Sigma Chemical Co., (St. Louis, MO, USA). HEPES, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide were procured from M/s Sisco Research Lab, (Mumbai, India). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were purchased from M/s HiMedia (Mumbai, India). Glucometer ("Accu-Check") was procured from M/s Roche diagnostics (GmbH, Mannheim, Germany). All other reagents and chemicals used were of analytical grade.

2.2 Pesticide solution

Stock solutions (1M) of pesticides viz., Monocrotophos (MC); Quinalphos (QP); Malathion (MAL); Ethion (ET); Dimethoate (DM); Chlorpyrifos (CP) and Dichlorvos (DDVP) were prepared in DMSO and further diluted with distilled

water to obtain working solutions for the *in vitro* assays. Stock solutions of DM and DDVP were passed through sterile membrane filters (0.2 µ) before employing in islet studies. Stock solutions of DM (20mg/ml) and DDVP (10mg/ml) prepared in physiological saline were used for intragastric (oral) administration to rats.

2.3 Animals and care

Male Wistar rats, (CFT strain, 8 week old, 180 ± 5 g) obtained from the Central Food Technological Research Institute animal house were used for the studies. They were housed in polypropylene cages (two per cage) at room temperature (25 ± 2º C) with relative humidity of 50- 60% and on a 12h light - darkness cycle. They had free access to commercial pellet diet (M/s Saidurga Feeds and Foods Pvt. Ltd., Bangalore, India) and water *ad libitum*. All animal procedures were conducted in strict conformation with the 'Institute Ethical Committee guidelines' for the care and use of laboratory animals.

The nematode, *Caenorhabditis elegans,* (strain N2, wild type) and OP50 (*E. coli* strain, uracil auxotroph) were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA).

2.4 Preparation of pancreatic homogenate for *in vitro* studies

Rats were sacrificed under diethyl ether anesthesia, and pancreas was excised and blotted. Homogenate of pancreas (20 % w/v) was prepared in phosphate buffer (100 mM, pH 7.4) and 1ml aliquots of the homogenate were used for *in vitro* studies.

2.5 Preparation of islet culture *(Sitasawad et al., 2000)*

Rats were killed following anesthesia by diethyl ether, pancreas was removed aseptically, cut into small pieces and subjected to enzymatic digestion for 10-20 min. in dissociation medium which consisted of Dulbecco's modified minimum essential medium (DMEM) supplemented with Collagenase (1 mg/ml),

Soybean trypsin inhibitor (STI: 2mg/ml) and 2 % BSA. The digest was centrifuged at 1000 rpm for 10 min, pellet washed twice in phosphate buffered saline (pH 7.2) and seeded in culture flasks containing 15ml RPMI 1640 and incubated at 37ºC in CO_2 incubator gassed with 5% CO_2 in air. After 48h of incubation, the islets were separated from exocrine pancreas by hand picking, under an inverted microscope. Islet viability was assessed by trypan blue dye exclusion method and specificity of islets was determined by dithizone staining.

2.6 Worm Cultures

Age synchronous populations of *C. elegans* were obtained by collection and culturing of eggs laid by emergent dauer larvae (Donkin and Williams, 1995). All developmental stages were cultured on 115 mm Petri dishes on K-agar (0.032M KCl, 0.051M NaCl, 0.1M $CaCl_2$, 0.1M $MgSO_4$, 2.5% Bacto-peptone, 0.17% Bacto-agar, and 0.01% cholesterol) (Williams and Dusenbery, 1988) seeded with *Escherichia coli* OP50 to serve as a food source (Brenner, 1974). All exposures were carried out using 3 day old adults.

3.0 EXPERIMENTAL PROCEDURE

3.1 Effect of OPI in rat pancreatic homogenate *in vitro*

3.1.1 Evidence for generation of ROS

Pancreatic homogenate (75µl) was pre-incubated with DCFH-DA (30 µl of 1mM solution in ethanol) for 10 min in a multi-well plate. Each of the OPI (MC, QP, MAL, ET, DM, CP and DDVP) was added to 105µl of the homogenate mixture at a final concentration of 1mM and the total volume of the mixture was made up to 300µl with phosphate buffer. The above mixture was incubated at 37ºC for 60 min in a shaker water bath. After incubation, the mixture was centrifuged at 2000 rpm for 5 min. The DCF formed was measured in the supernatant as described under assay methods.

3.1.2 Evidence for induction of lipid peroxidation

Pancreatic homogenate (300 µl) was incubated with 100mM each of OPI at 37ºC for 60 min in a multiwell plate in a shaker. After incubation, the extent of lipid peroxidation was monitored by measuring the levels of TBARS in homogenate according to the procedure as described under assay methods.

3.1.3 DDVP-induced ROS: concentration-response

Pancreatic homogenate (75µl) was incubated with various concentrations of DDVP (0.25, 0.5, 0.75 and 1mM) and 30 µl of DCFH-DA as described above. The DCF formed in the reaction mixture was measured as described under assay methods.

3.1.4 DDVP-induced ROS: time-course response

Pancreatic homogenate (75µl) was incubated with 1mM DDVP and 30 µl of DCFH-DA at 37ºC and the ROS generated was monitored at different time intervals (15, 30, 45, 60, 75 and 90 min) by measuring the resultant DCF.

3.1.5 DDVP-induced lipid peroxidation: concentration- response

Pancreatic homogenate (75µl) was incubated with various concentrations of DDVP (20, 40, 60, 80 and 100mM) in a multi-well plate at 37ºC for 60 min. After incubation, the extent of lipid peroxidation was monitored by measuring the levels of TBARS in the homogenate.

3.1.6 Acetylcholinesterase inhibition by DDVP

500µl of pancreatic homogenate was incubated with various concentrations of DDVP (50, 100, 150 and 200 µM) at 37ºC for 60min. The activity of AChE in the incubation mixture was determined as described under assay methods.

3.2 Effect of OPI on isolated rat pancreatic islets *in vitro*

3.2.1 Cytotoxicity of DM and DDVP

500 isolated pancreatic islets were incubated in 3ml of RPMI with varying concentrations (0.25, 0.5, 0.75, and 1 µM) of DM or DDVP at 37ºC for 12 h in a CO_2 incubator gassed with 5 % CO_2 and relative humidity of 95%. After incubation, samples were centrifuged at 1000rpm to sediment the islets. The islets were washed 3 times with PBS and finally suspended in a known volume of PBS for assaying reduced glutathione levels while, the supernatant was collected and stored at -20ºC for measurement of LDH activity. Aliquots of islet suspension were used for determination of viability by MTT dye reduction as described under assay methods.

3.2.2 Effect on reduced glutathione levels

The islet suspension was centrifuged at 1000 rpm for 5 min. The pellet was homogenized in 5% TCA in microcentrifuge tubes using a micro pestle followed by centrifugation at 3000 rpm. The resultant supernatant was used for the determination of reduced glutathione as described under assay methods.

3.2.3 Generation of ROS

500 islets were pre-incubated with DCFH-DA, for 30min at 37ºC in a CO_2 incubator gassed with 5 % CO_2 and relative humidity of 95%. Following this, the islets were exposed to varying concentrations of DM or DDVP (0.25- 1.25 µM). After incubation, the reaction mixture was centrifuged at 5000rpm to sediment the islets, which were washed three times with PBS and finally suspended in a known volume of PBS. The islets were homogenized in a microcentrifuge tube using micro pestle and the homogenate was centrifuged at 3000 rpm for 5 min at 4ºC. The supernatant was used for the determination of ROS as described under assay methods .

3.3 Effect of OPI on the nematode, *Caenorhabditis elegans*

3.3.1 Toxicity in C. elegans: 4h-lethality assay

Exposures were performed in 12-well sterile tissue culture plates. Each test consisted of 1 ml exposure volume including one control (K-medium), and 5 different concentrations of DDVP (5, 10, 15, 20 & 25 mM) or DM (5, 10, 15, 20 & 25 mM). Using a dissecting microscope, 9-11 (average ten) worms were transferred into each well with a 20 μl pipette and incubated at 20ºC for 4 h. After 4-h exposure, the wells were observed under a dissecting microscope and the number of live and dead worms was counted in each well. The worms that did not move or respond to gentle probing with a needle were counted as dead. Each test was performed three times on different days. Based on lethality, EC_{50} values were calculated using linear regression analysis of the pesticide concentration versus percentage mortality.

4.0 ASSAY METHODS

4.1 Islet specificity by dithizone staining *(Clark et al., 1994)*

Dithizone (DTZ) stock solution (39 mM) was prepared by dissolving 100 mg of DTZ in 10 ml DMSO, filtered, aliquoted and stored at -15ºC. Staining was carried out by adding 10μl of DTZ stock solution to islets suspended in 1ml of bicarbonate buffer (KRB: pH 7.4) with HEPES (10mM) and incubated at 37ºC for 10 to 15 min. The stained islets were counted under a microscope.

4.2 Cell viability : Trypan blue dye exclusion *(Altman et al., 1993)*

Pancreatic islets were dispersed with PBS and then incubated with trypan blue (final concentration: 0.2 mg/ml) for 1 min. Cells were observed under a microscope, stained and non-stained cells were counted separately, and the viable cell ratio was calculated.

4.3 Cell viability: MTT dye reduction (*Alley et al., 1988)*

Viability of cultured islet cells was also quantified using MTT dye reduction assay. This assay is based on the cleavage of yellow colored MTT to form blue formazan by mitochondrial respiration in viable cells. After treatment, the islets were incubated with MTT (final concentration 0.2 mg/ml) for 4 h at 37ºC in a CO_2 incubator gassed with 5 % CO_2 and relative humidity of 95%. The supernatant was removed, 50 µl of DMSO was added to solubilize formazan and absorbance was measured at 540nm using microplate reader (Elisa Reader, 'Thermo', Electron Corporation, China). The absorbance of cells without pesticide was taken as 100 % viability and cell death was calculated in treated cells.

4.4 Lactate dehydrogenase (LDH) (EC 1.1.1.27) *(Kornberg, 1974)*

10µl of the cell free supernatant was placed in a cuvette and the reaction was started by addition of 0.8 ml NADH (in Tris-HCl. 0.25mM, pH 7.2), 0.15 ml of sodium pyruvate (10.66mM) and 40µl of distilled water. The reaction was monitored over a period of 5 min at 340nm. The results were expressed as nmol NADH oxidized/min/mg protein.

4.5 Reactive oxygen species (ROS) *(Keston and Brandt, 1965)*

100µl of the supernatant was added to 1900µl of phosphate buffer (100mM, pH 7.4) and the intensity of fluorescence was measured at 530nm following excitation at 485nm. The amount of DCF (resulting from the ROS mediated oxidation of DCFH, produced by hydrolytic cleavage of DCFH-DA by cellular esterases) was calculated using the DCF standard graph. Results were expressed as pmoles of DCF/mg protein.

4.6 Lipid peroxidation (*Buege and Aust, 1978*)

250µl of the incubation mixture was added to 2ml of TBA-TCA-HCl (0.374%- 15%- 0.25N) and the tubes were placed in a boiling water bath for 15min. After cooling and centrifugation, the color of the supernatant was read at 535nm. The amount of thiobarbituric acid reactive substances (TBARS) in the supernatant was determined using molar extinction coefficient of $1.56\times10^{-5}M^{-1}$ cm^{-1} and the results were expressed as nmol MDA/min/mg tissue.

4.7 Reduced Glutathione (GSH) *(Benke et al., 1974)*

200µl of the supernatant was added to 4.75ml of sodium phosphate buffer (1.0M, pH 8.0) to which 50µl of DTNB (in phosphate buffer, 0.1M, pH 7.0) was added and mixed. The absorbance of the solution was read at 412nm. Results were expressed as % decrease over control.

4.8 Acetylcholinesterase (AChE) (EC 3.1.1.7) *(Ellman et al., 1961)*

100 µl of pancreatic homogenate was taken in 2.83 ml of phosphate buffer (0.1M, pH 8.0) with 50µl of DTNB solution (10mM) and 20 µl of acetylthiocholine iodide (78mM). The change in absorbance at 412 nm was monitored after the addition of the substrate for 5min. The change in absorbance per minute (ΔA) was calculated. The enzyme activity was expressed as nmoles of substrate hydrolyzed/min/mg protein. Results were expressed as % inhibition of enzyme over control.

4.9 Statistical analysis

All the data are expressed as mean ± S.E. of three separate experiments performed in triplicate using the computer programme Excel software (1999).

5.0 RESULTS

5.1 Effect of OPI on pancreatic homogenate: evidences for induction of oxidative stress

The potential of a few selected OPI (1mM) to induce oxidative stress in rat pancreatic homogenate (exposed for 60 min) was determined in terms of ROS production and the results are presented in **Table 1.1 & Fig. 1.1**. The levels of ROS were markedly elevated in homogenates treated with Ethion followed by DM, DDVP and CP, while other pesticides generated very little ROS at the tested concentration. The order of ROS induction was: ET>DM>DDVP=CP>MC>MAL>QP. Lipid peroxidation which occurs as a consequence of ROS generation was also monitored by quantification of TBARS in the homogenate exposed to higher concentration of the OPI (100mM) for 60 min. Interestingly, significant LPO was elicited only by DDVP as compared to other insecticides (**Table 1.2 & Fig. 1.2**).

From the results presented in **Fig. 1.3**, it is evident that DDVP elicited a concentration-dependent increase in ROS generation. **Fig. 1.4** depicts the time-course of DDVP induced of ROS generation in rat pancreatic homogenate. ROS levels continued to increase as a function of time on exposure to 1mM DDVP. DDVP also elicited a concentration-related increase in MDA levels in pancreatic homogenate (**Fig. 1.5**). Further, DDVP inhibited the activity of rat pancreatic AChE in a concentration-dependent manner (**Fig. 1.6**).

Table 1.1 Extent of reactive oxygen species (ROS) generated in rat pancreatic homogenate exposed to OPI (1mM) for 60 min.

Treatment	ROS (nmol DCF/mg protein)
Control	0.92 ± 0.09
Malathion (MAL)	1.07 ± 0.14
Ethion (ET)	2.39 ± 0.10
Quinalphos (QP)	0.98 ± 0.09
Monocrotophos (MC)	1.24 ± 0.15
Dimethoate (DM)	1.89 ± 0.17
Chlorpyrifos (CP)	1.37 ± 0.16
Dichlorvos (DDVP)	1.41 ± 0.17

Values are mean ± S.E.M. of three determinations each

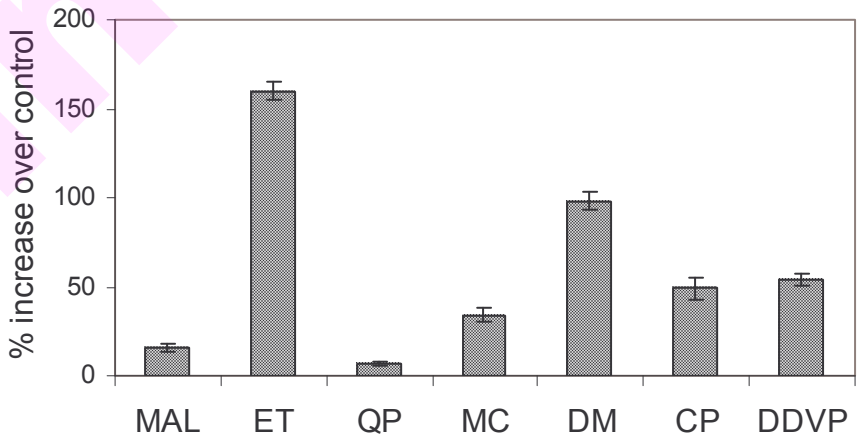


Fig. 1.1 Reactive oxygen species generated in rat pancreatic homogenate exposed to OPI (1 mM) for 60 min.

Values are mean ± S.E.M. of three determinations each

Table 1.2 Extent of lipid peroxidation in rat pancreatic homogenate exposed to OPI (100 mM) for 60 min.

Treatment	nmol MDA/mg protein
Control	7.90 ± 0.39
Dichlorvos (DDVP)	66.64 ± 4.12
Malathion (MAL)	9.07 ± 0.40
Ethion (ET)	10.35 ± 0.55
Quinalphos (QP)	9.85 ± 0.74
Monocrotophos (MC)	8.27 ± 0.63
Dimethoate (DM)	10.89 ± 0.78
Chlorpyrifos (CP)	9.38 ± 0.56

Values are mean ± S.E.M. of three determinations each

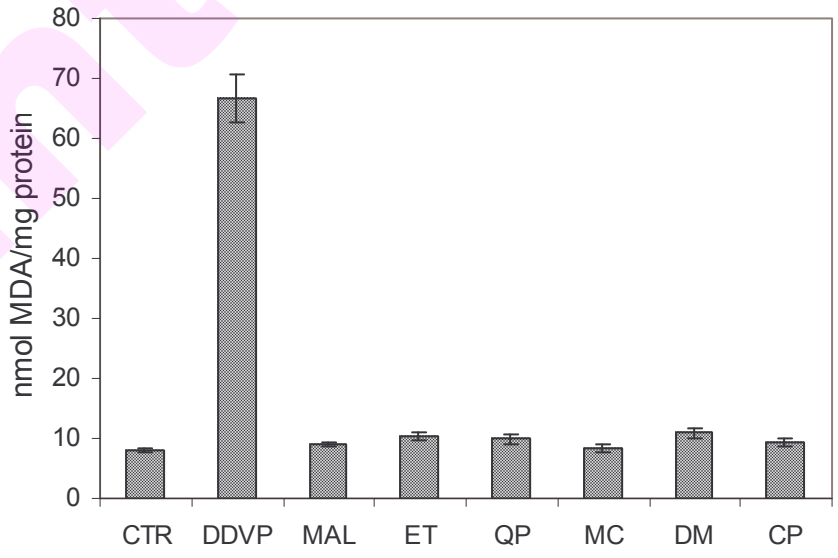


Fig. 1.2 Lipid peroxidation in rat pancreatic homogenate exposed to OPI (100 mM) for 60 min.

Values are mean ± S.E.M. of three determinations each

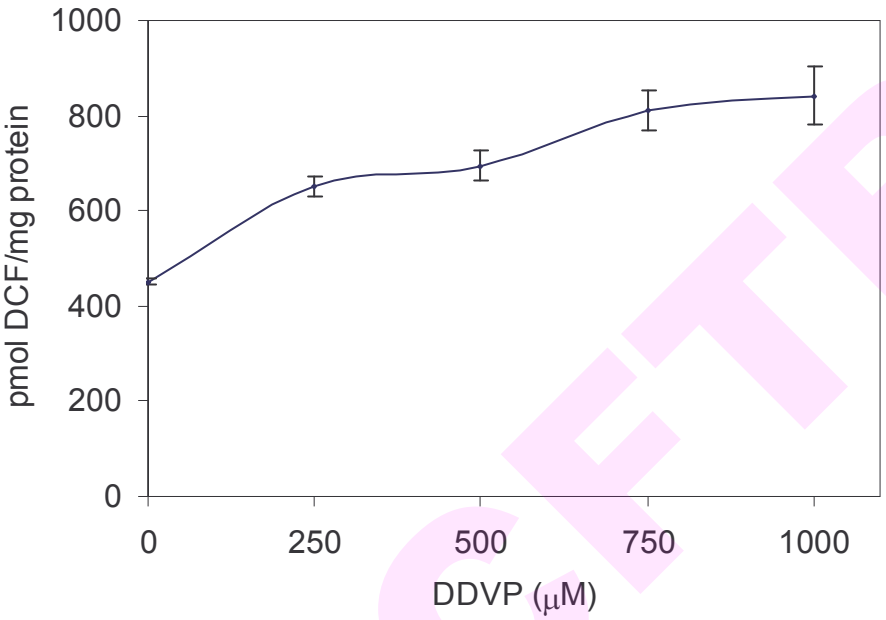


Fig. 1.3 ROS generated in rat pancreatic homogenate exposed to varying concentrations of DDVP for 60 min.

Values are mean ± S.E.M. of three determinations each

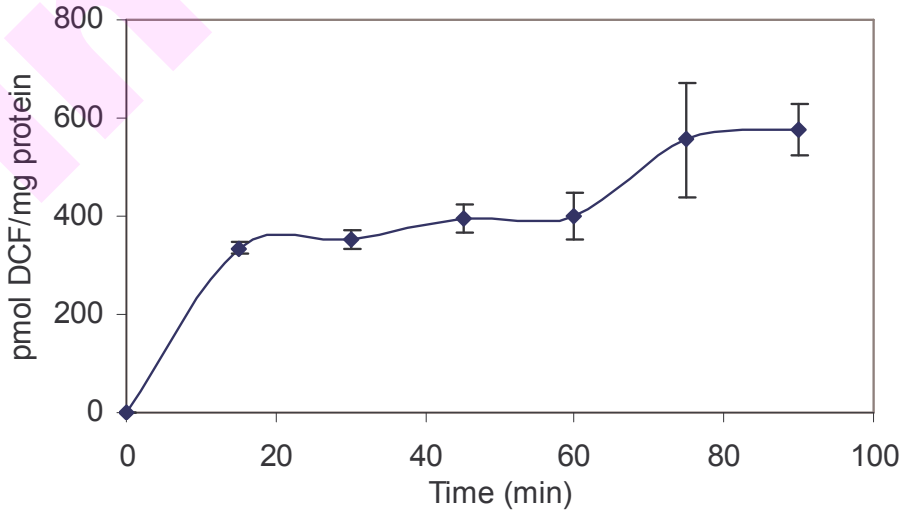


Fig. 1.4 Time-course of ROS generation by DDVP (1mM) in rat pancreatic homogenate

Values are mean ± S.E.M. of three determinations each

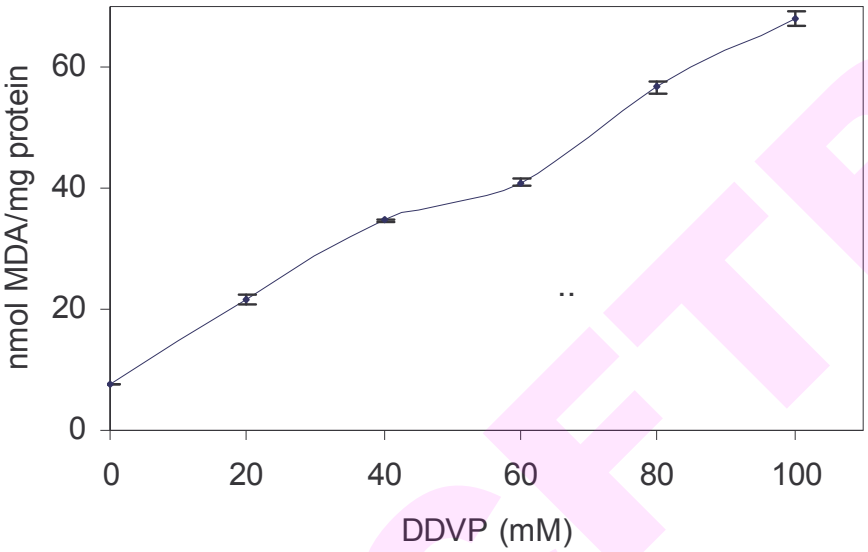


Fig. 1.5 Lipid peroxidation in rat pancreatic homogenate exposed to varying concentrations of DDVP for 60 min.

Values are mean ± S.E.M. of three determinations each

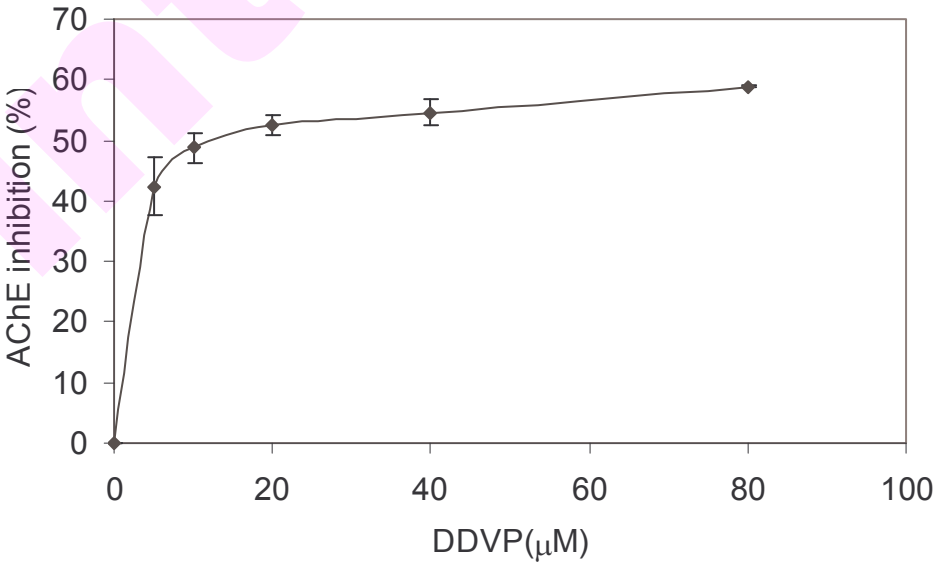


Fig. 1.6 AChE inhibition in rat pancreatic homogenate exposed to varying concentrations of DDVP for 60min.

Values are mean ± S.E.M. of three determinations each

5.2 Effect of DM and DDVP on rat pancreatic islets *in vitro*

Collagenase digestion method followed for the isolation of rat pancreatic islets yielded adequate number of islets for the study (**Fig. 1.7**). Viability of the islets as assessed by trypan blue dye exclusion assay was above 90%. Islet specificity and identity was confirmed by DTZ staining (**Fig. 1.8**). The cytotoxic potential of DM and DDVP were evaluated in pancreatic islet cultures. The islet cell viability was measured in terms of MTT dye reduction and LDH leakage after exposing the islets to varying concentrations of the OPI for 12h. MTT dye reduction on exposure of islets to DDVP and DM are depicted in **Fig. 1.9 A & B** respectively. Both the insecticides induced significant cell death and a concentration-related response. A similar response was also evident in LDH leakage as seen from **Fig. 1.10 A & B**. LDH leakage in unexposed cells was about 0.8 mmol/ min. Employing MTT viability assay, we calculated the IC_{50} values for both DDVP and DM after incubation with varying concentrations of the insecticides for 12h. We obtained IC_{50} values of 1.76 µM for DDVP and 1.6 µM for DM.

ROS levels were quantified in DDVP and DM treated pancreatic islet and **Fig. 1.11 A & B** show the dose-response in ROS production. Even at the lowest concentration (0.25 µM), a marked increase in ROS generation was evident on exposure to DDVP (100%) and DM (60%). Exposure of islets to varying concentrations of DDVP and DM resulted in significant decrease in intracellular GSH content which was evident at 0.25 µM of DDVP (20%) and at 0.75 µM of DM (**Fig. 1.12 A & B**).

5.3 Effect of DM and DDVP on *Caenorhabditis elegans*

The worms showed 0% lethality for the control in three replicates while, there was a concentration-dependent increase in lethality on exposure to both the OPI (data not shown). The LC_{50} values obtained were: DM: 17.52 mM; DDVP: 16.85 mM. Apparently both DM and DDVP were equitoxic to *C.elegans*.

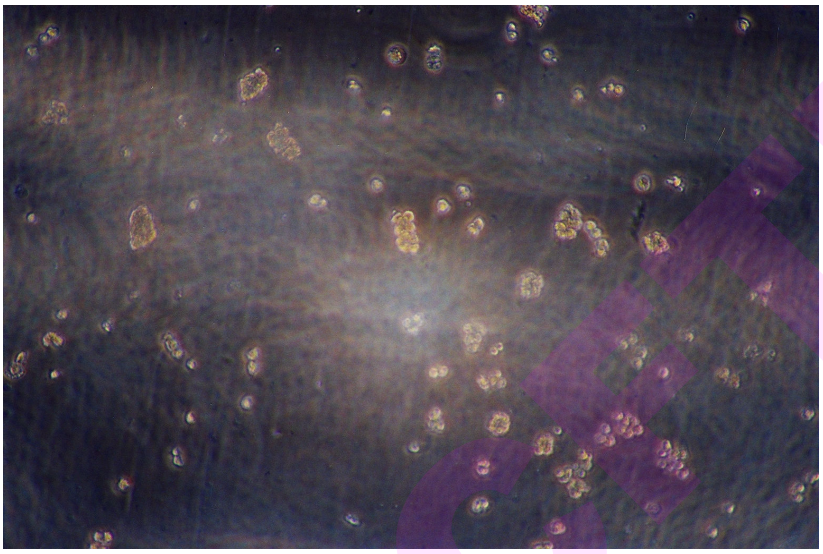


Fig. 1.7 Phase contrast microscopic image of normal isolated rat pancreatic islet cells in culture

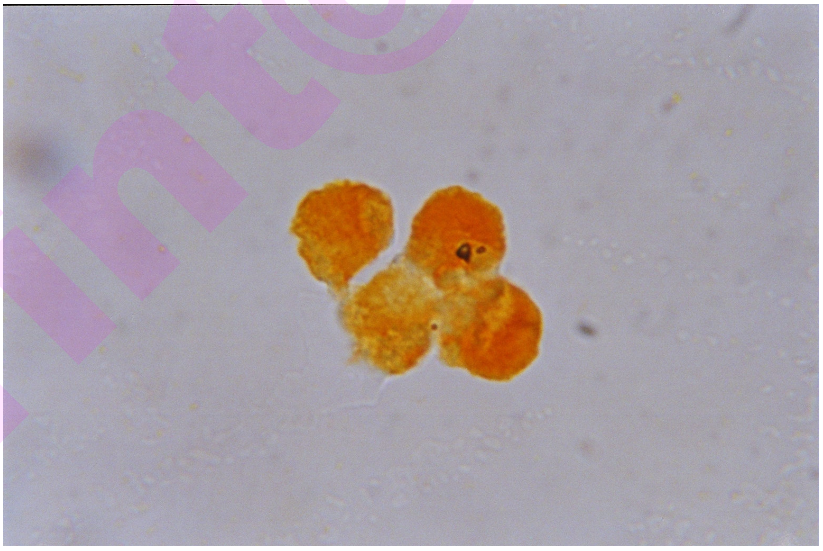


Fig. 1.8 Rat pancreatic islets stained with dithizone

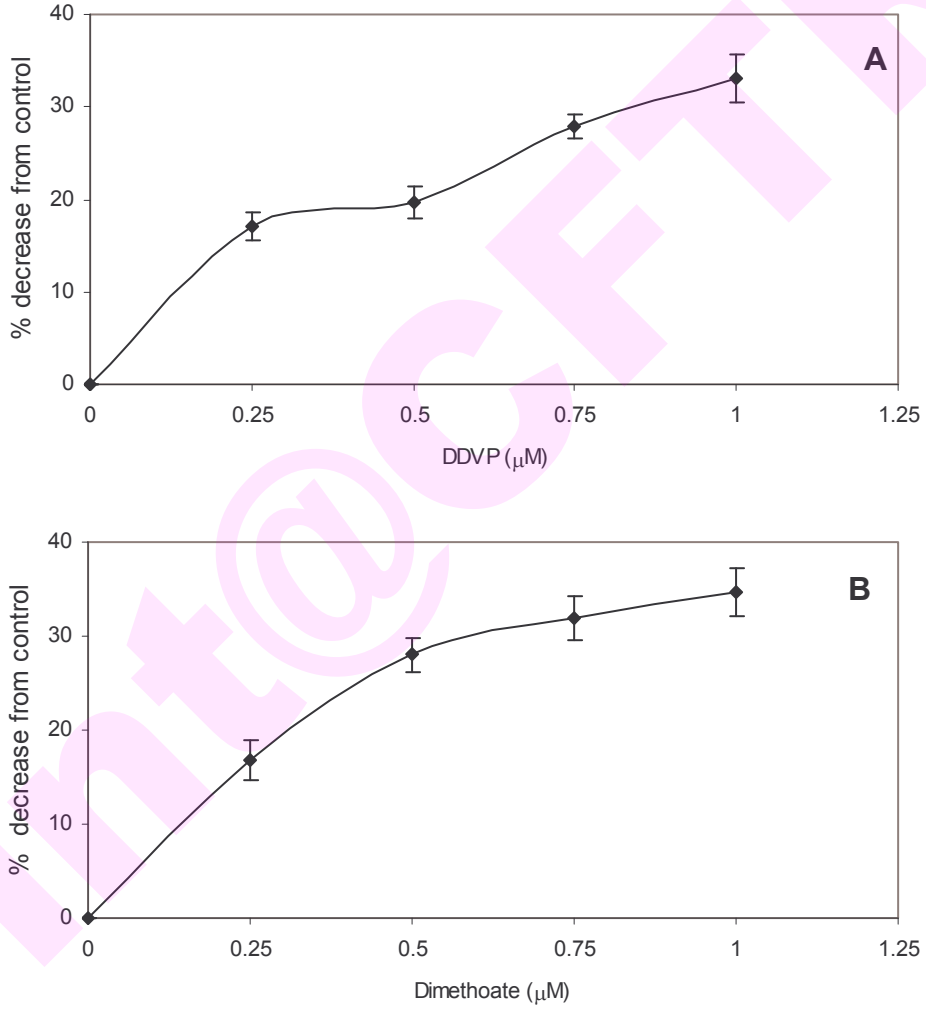


Fig. 1.9 MTT dye reduction in pancreatic islets treated with varying concentrations of (A) DDVP and (B) DM for 12h.

Values are mean ± S.E.M. of three determinations each

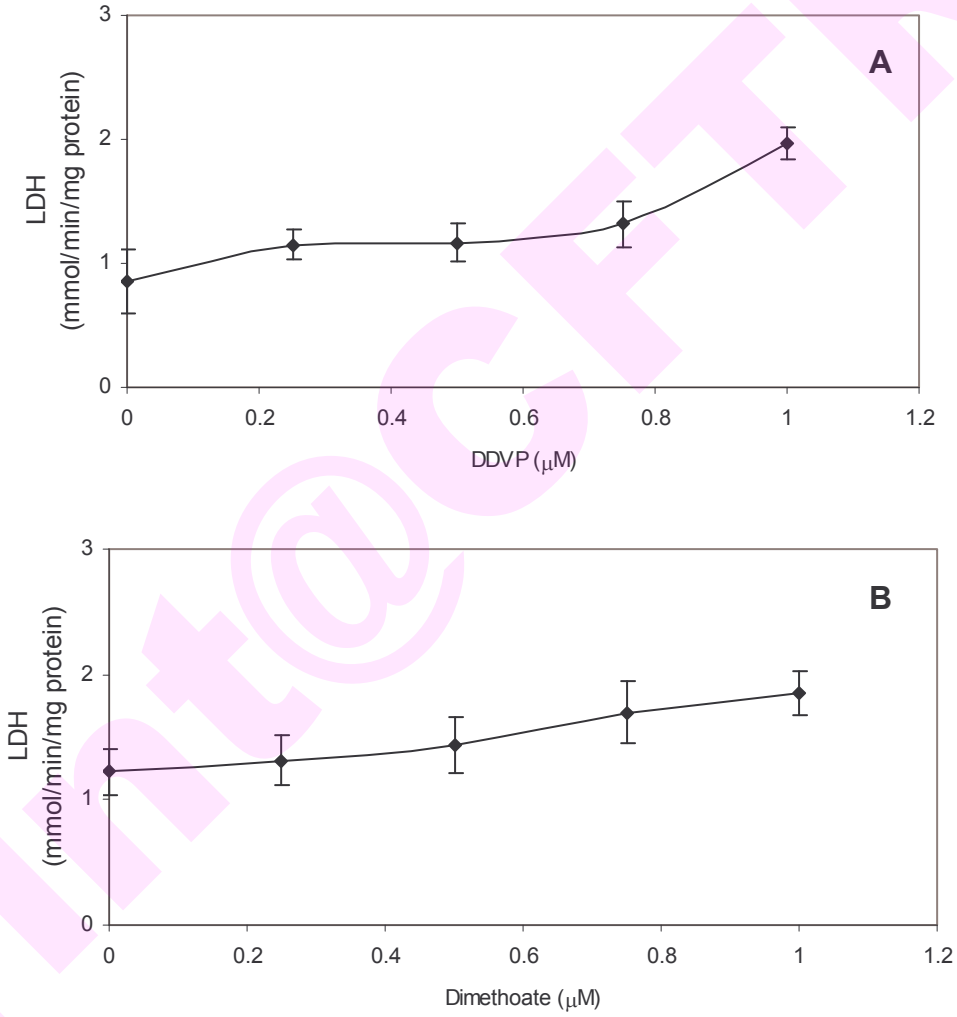


Fig. 1.10 LDH leakage in rat pancreatic islets treated with varying concentrations of (A) DDVP and (B) DM for 12h.

Values are mean ± S.E.M. of three determinations each

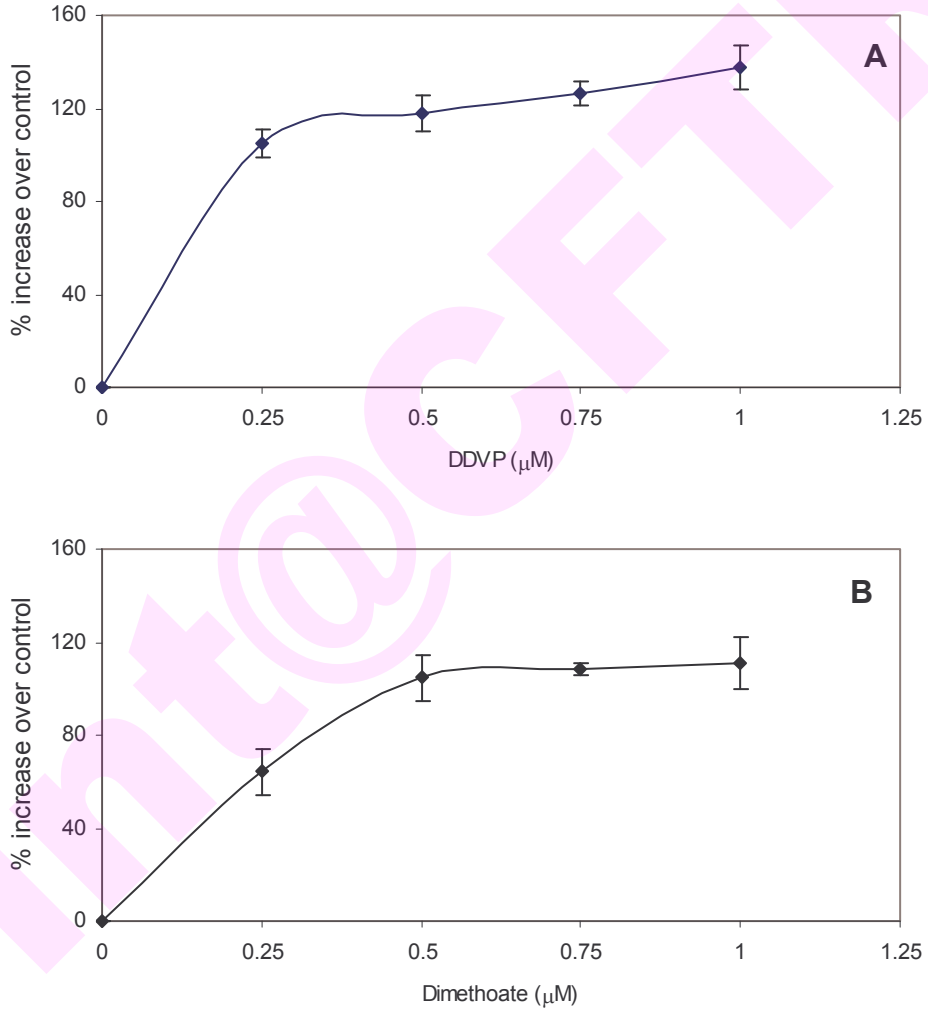


Fig. 1.11 ROS generation in rat pancreatic islets exposed to varying concentrations of (A) DDVP and (B) DM for 12h.

Values are mean ± S.E.M. of three determinations each

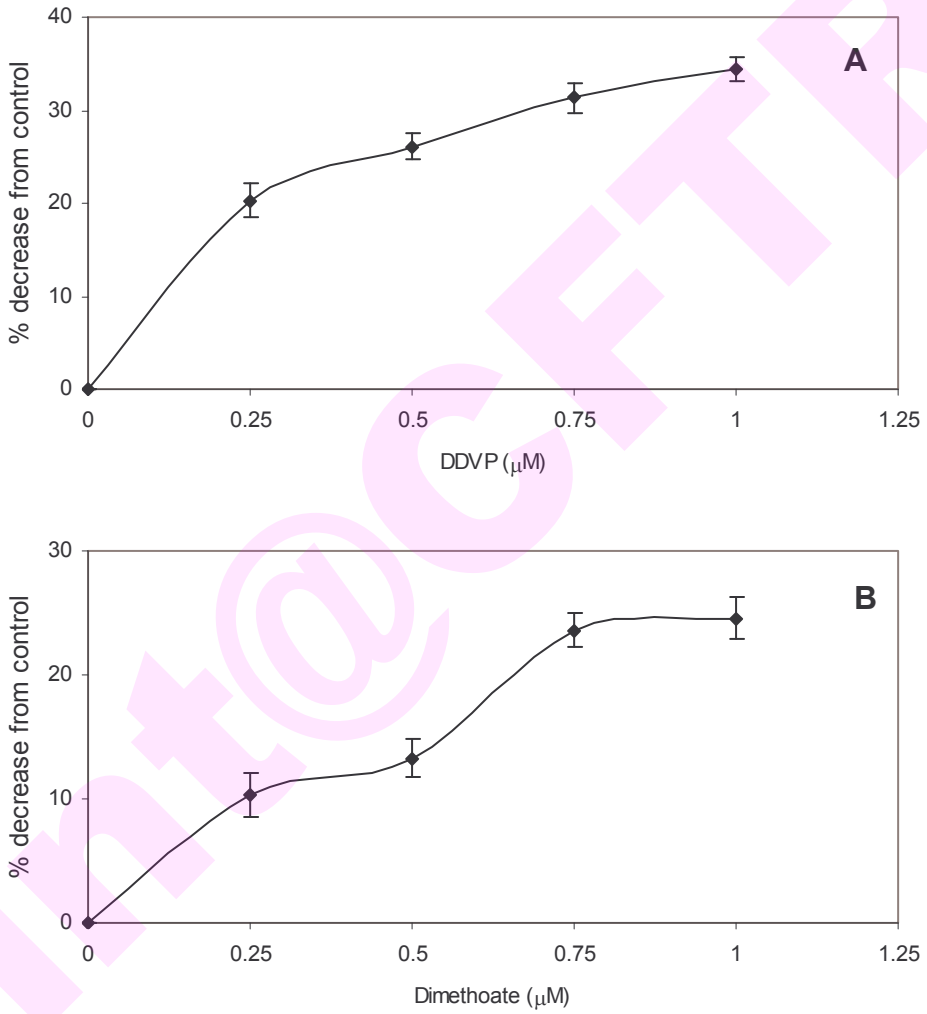


Fig. 1.12 Intracellular GSH content in pancreatic islets exposed to varying concentrations of (A) DDVP and (B) DM for 12h.

Values are mean ± S.E.M. of three determinations each

6.0 DISCUSSION

Generation of free radicals has been implicated in several insecticide toxicities (Bagchi et al., 1995; Yamano and Morita, 1995; Pitrowski et al., 1996; Banerjee et al., 1999). It has been well indicated that the enzymes associated with antioxidant defense mechanism are altered under the influence of OPI and that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced cytotoxicity (Ranjbar et al., 2002, 2005; Akhgari et al., 2003; Abdollahi et al., 2004b). The objectives of this study were to determine the potential of few selected OPI to induce OS in rat pancreatic homogenate *in vitro* in order to elucidate the mechanisms underlying OPI-induced pancreatic dysfunction and altered glucose homeostasis. The validity of using tissue homogenates, as an indicator of whole tissue susceptibility to LPO has been earlier demonstrated (Kornburst and Mavis, 1980; Wofford and Thomas, 1988). In the present study, the use of tissue homogenates as an experimental model was based on the assumption that the OPI may generate free radicals at various sub-cellular sites, such as mitochondria and microsomes which are functional in tissue homogenates and would provide a representative machinery for the generation of activated species.

In the present study, we have made few interesting observations. While the levels of ROS were increased markedly by ethion and dimethoate followed by chlorpyrifos and monocrotophos (1mM), the extent of lipid peroxidation was increased only by DDVP at 100 mM. Induction of LPO must rely on some intermediate rate-limiting mechanisms, which could be unevenly distributed and or active among certain tissues or cells. Further, it cannot be ruled out that components of the antioxidant defense systems could be involved in modulating this process. Further, studies with DDVP revealed that it induced a concentration-related response in terms of ROS generation and LPO induction. OPI such as phosphamidon, trichlorfon and dichlorvos have been reported to induce oxidative stress both *in vivo* and *in vitro* in hepatocytes as shown by inhibition of SOD activity, enhanced MDA production and lactate

dehydrogenase leakage and decreased glutathione peroxidase activity (Julka et al., 1992; Naqvi and Hasan, 1992; Yamano and Morita, 1992). OPI exert their toxicity by inhibiting AChE. In this study, our results demonstrated that DDVP inhibited AChE activity *in vitro* in a concentration-dependent manner and also at concentrations much lower than those inducing LPO.

Cell models have not been extensively deployed to investigate OPI-induced OS mechanisms. Few isolated studies have shown the effect of OPI on rat hepatocytes *in vitro* (Yamano and Morita, 1992). In the present study, we employed isolated rat pancreatic islets for studying the susceptibility to OS by OPI. The damaging effects of ROS on pancreatic islets have been widely investigated in diabetes as well as in islet transplantation (Hara et al., 2006) and the islets of Langerhans have been found to show loss of viability following exposure to ROS. Exposure of isolated islets or pancreatic β-cell lines to ROS reportedly inhibits their function, resulting in cell death. β-cells of pancreatic islets are responsible for control of glucose and maintaining its balance in blood and when compared with other cell types, pancreatic β-cells are particularly more susceptible to destruction caused by ROS. This sensitivity is probably due to islet cells containing low levels and activities of several ROS detoxifying systems. However, they have not been employed often for studying OS induced by pesticides. Wilson and Gaines (1983) studied the effect of the rodenticide, Vacor on cultured rat pancreatic β-cells and demonstrated the utility of this *in vitro* system for studying potential β-cell toxins. The present study revealed that incubation of isolated pancreatic islets with DDVP caused loss of cell viability as evident from MTT dye reduction and LDH leakage into the medium, accompanied by a marked decrease in glutathione and increased ROS production. Similarly, DDVP has been demonstrated to increase MDA production and cause cell death in rat hepatocytes *in vitro* (Yamano and Morita, 1992; Yamano, 1996).

We also studied the toxicity of DDVP and DM in the nematode model, *Caenorhabditis elegans*, as a pre-screen to determine the toxicity of the two

compounds. We found that both the OPI were equitoxic to the nematode in terms of the 4h-$LC_{50.}$ *C.elegans* has been reported to be a good model for studying the toxicity of pesticides (Cole et al., 2004). Studies from our laboratory (unpublished data) has also demonstrated that OPI induce significant oxidative stress in these worms and that OS plays a major role in determining the toxicity of the OPI in *C.elegans.*

In conclusion, the overall results of our study showed that OPI in general, DM and DDVP in particular have the potential to induce OS *in vitro,* as evident by the elevation of the levels of LPO in the *in vitro* models studied and are equally toxic.

7.0 SUMMARY

1. Initially, selected OPI viz., chlorpyrifos (CP), ethion (ET), dimethoate (DM), dichlorvos (DDVP), monocrotophos (MC), malathion (MAL) and quinalphos (QP) were screened for their potential induce oxidative stress in rat pancreatic homogenate *in vitro* .
2. The levels of ROS were markedly elevated in rat pancreatic homogenates following exposure to ET, DM and DDVP and the order of ROS induction was: ET> DM>DDVP=CP>MC>MAL>QP.
3. The extent of lipid peroxidation was significantly increased only on exposure to DDVP.
4. DDVP induced a concentration-related increase in both ROS and LPO in pancreatic homogenate.
5. DDVP also inhibited pancreatic acetylcholinesterase (AChE) *in vitro* in a concentration-dependent manner.
6. DM and DDVP were selected for further mechanistic studies in other model systems: rat pancreatic islet cultures and *Caenorhabditis elegans* cultures.
7. Both DDVP and DM were equally toxic to rat pancreatic islet cells and both elicited a concentration-response as measured by MTT dye reduction and LDH leakage.

8. A concentration-related increase in ROS generation as well as decrease in GSH content was evident in the islets exposed to DM and DDVP.
9. Both the OPI induced significant lethality in *Caenorhabditis elegans*, and were found to be equitoxic on exposure for 4h.

SECTION B

ALTERED GLUCOSE HOMEOSTASIS AND BIOCHEMICAL IMPAIRMENTS IN PANCREAS OF RATS SUBJECTED TO ORGANOPHOSPHOROUS INSECTICIDE INTOXICATION

1.0 INTRODUCTION

Organophosphorous insecticides (OPI) constitute one of the most widely used classes of pesticides being employed for both agricultural and landscape pest control. Use of OPI has increased considerably due to their low toxicity and low persistence in the mammalian system compared to organochlorine pesticides. OPI are primarily recognized for their ability to induce toxicity in mammals through inhibition of acetylcholinesterase (AChE) and subsequent activation of cholinergic receptors (Costa, 2006).

Various complications have been reported in OPI intoxication cases (Hsiao et al., 1996). In fact, OPI are currently responsible for more poisonings than any other single class of pesticides (Sultatos, 1994; Gulr et al., 1996). Hyperglycemia has been widely reported as one of the adverse effects in poisoning by OPI in humans and animals (Shobha and Prakash, 2000; Seifert, 2001; Hagar et al., 2002; Abdollahi et al., 2003; Kalender et al., 2004). Although the precise mechanism/s of OP induced hyperglycemia is not known, it is speculated to be due to inhibition of acetylcholinesterase of central and peripheral synapses that act in the endocrine regulation of glucose metabolism (Matin and Siddiqui, 1982; Kant et al., 1988). Involvement of oxidative stress following acute exposure to OPI has been reported recently (Banerjee et al., 2001) and it has been demonstrated unequivocally that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced cytotoxicity (Ranjbar et al., 2002; Akhgari et al., 2003; Abdollahi et al., 2004b).

Dimethoate (*O*, *O*-dimethyl *S*-*N*-methyl carbomyl methyl phosphorodithioate) (DM) is one of the most important OPI used extensively on

a large number of crops against several pests. The residues of DM and its analog were found in number of foods including cow's milk (Srivastava and Raizada, 1996). While data on acute, subchronic and chronic toxicity of DM in laboratory animals are well documented, its potential to alter glucose homeostasis and impair the endocrine function of pancreas in mammals is less well understood. Interestingly, DM is reported to cause various toxic effects in rat pancreas following chronic exposure (Hagar et al., 2002) as well as pancreatitis in humans following dermal exposure (Panieri et al., 1997). Dichlorvos (2, 2-dichlorovinyldimehtyl phosphate) is another OPI used to control household, public health and stored product insects and like other OP compounds, DDVP is known to inhibit AChE activity. Chronic Dichlorvos exposure has been reported to elicit an increase in the plasma glucose levels in rats (Sarin and Gill, 1999). Our *in vitro* studies (*Section A, Chapter I*) have clearly demonstrated the potential of DM and DDVP to induce oxidative stress. Hence, the present study addressed the potential of dimethoate (DM) and dichlorvos (DDVP) to induce alteration in glucose homeostasis and examined whether oxidative stress plays an important role in pancreatic dysfunction induced by these OPI.

2.0 MATERIALS AND METHODS

As described under *Section A* of this Chapter (*sub sections 2.1 to 2.3*).

3.0 EXPERIMENTAL PROCEDURE

3.1 Effect of Dimethoate (DM) and Dichlorvos (DDVP) on blood glucose levels in rats after acute exposure

Rats were randomly divided into three groups of six rats in each group. Blood was drawn from tail vein of rats, which were fasted over night, and blood glucose was determined using a commercial glucometer. The first group of rats which served as control, received normal saline (500 µl), while the second group of rats received DM at 40 mg /kg b.w. (10 % LD_{50}) and the third group of rats received DDVP at 10 mg/kg b.w. (10% LD_{50}). Blood glucose was monitored

in all the rats at the end of 6, 24, 48 h and on 7th day after treatment. The animals were observed for symptoms and mortality for 7 days and then sacrificed under ether anesthesia.

3.2 Effect of repeated doses of DDVP on biochemical parameters in rats

Rats were randomly divided into five groups of six rats in each group. The first group serving as control received saline while, the other groups of rats were administered DDVP at 10mg/kg b.w. /d for 5 or 10 days or 20mg/kg b.w. /d for 5 or 10 days. Body weights of rats were recorded on the initial and final day and body weight gain was calculated. At the end of the study period, blood glucose was monitored in all the rats and then they were sacrificed under ether anesthesia and pancreas was excised and blotted. The biochemical parameters measured included – blood glucose, AChE and GSH levels in pancreas.

3.3 Preparation of pancreatic homogenate

10 % (w/v) homogenate of pancreas was prepared in phosphate buffer (pH 7.4, 0.2 M), centrifuged at 9000xg at 4ºC for 20 min and the supernatant was used for determination of acetylcholinesterase activity. Reduced glutathione content was determined in supernatant of pancreatic homogenate (20 % w/v in 5 % TCA in 10^{-3} M EDTA), centrifuged at 2,000 rpm for 5 min at room temperature.

4.0 ASSAY METHODS

4.1 Blood glucose

A fresh glucometer biosensor strip was inserted into the slot provided in the glucometer after it was turned on. An aliquot (50 µl) of blood collected from tail vein of rat was applied to the strip as specified by the supplier. The glucose content of the blood displayed on the screen as mg/dl was noted after which the strip was removed and disposed and for every sample a new strip was used. Mean of three such measurements were taken for each rat.

4.2 Acetylcholinesterase activity (AChE) (EC 3.1.1.7) *(Ellman et al., 1961)*

25 µl of pancreatic homogenate was taken in 2.9ml phosphate buffer (0.1M, pH 8.0) with 50 µl of DTNB solution (10mM) and 20 µl of acetylthiocholine iodide (78 mM). The absorbance was read at 412 nm immediately after the addition of the substrate and the absorbance was recorded for 3 min. The change in absorbance per minute (∆A) was calculated and the enzyme activity was expressed as nmoles of substrate hydrolyzed/min/mg protein.

4.3 Reduced Glutathione (GSH) *(Benke et al., 1974)*

200µl of the supernatant was added to 4.75ml of sodium phosphate buffer (1.0M, pH 8.0) to which 50 µl of DTNB (in phosphate buffer, 0.1M, pH 7.0) was added and mixed. The absorbance of the solution was read at 412 nm. Glutathione concentrations were calculated from standard graph obtained with reduced glutathione and the results were expressed as mg GSH/g tissue.

4.4 Protein estimation *(Lowry et al., 1951)*

5 µl of pancreatic homogenate (in phosphate buffer) was added to 495 µl of distilled water and incubated with 2.5ml alkaline copper sulfate solution for 30 min at room temperature. 250 µl of Folin's reagent (1:1 diluted with water) was added and the color was read after 10min at 670nm. Bovine serum albumin (BSA) was used as the standard.

4.5 Statistical analysis

All the results are presented as Mean ± standard error of six determinations each. The data were analyzed employing analysis of variance (ANOVA) using Statistica software (STATSOFT, USA). Duncan multiple regression test (DMRT) for multiple comparisons was performed to determine the significant differences among the groups. *P* values *<0.01* were considered significant.

5.0 RESULTS

5.1 Effect of acute oral dose of Dimethoate (DM) / Dichlorvos (DDVP) on blood glucose levels

Acute oral doses of DM and DDVP failed to induce any distinct clinical signs of toxicity or mortality in rats. Time-course of alterations in blood glucose levels in rats administered acute doses of DM (40 mg / kg b.w) and DDVP (10 mg/ kg b.w) is presented in **Fig. 1.13**. Administration of DM induced significant increase in blood glucose by 6h, and even at the end of 24 h these rats had higher blood glucose levels compared to those of control and DDVP treated rats. After 48 hours, the blood glucose level decreased to the control levels and remained so thereafter. However, DDVP treatment did not cause any significant elevation in blood glucose levels at any of the sampling intervals.

5.2 Effect of repeated oral doses of DDVP on biochemical parameters

Repeated oral doses of DDVP did not elicit any signs and symptoms of toxicity or mortality at both the dosages and both treatment periods. Data on body weight gain of these rats is presented in **Fig. 1.14**. As evident from the figure, DDVP treatment at both doses did not affect the body weight gain while, a marked loss in body weight gain was evident in rats treated at both doses for 10 days.

Data on blood glucose levels, acetylcholinesterase activity and reduced glutathione content in pancreas of rats treated with various dose regimens of DDVP are presented in **Table 1.3 & Fig. 1.15**. Repeated oral administration of DDVP induced a marked increase in blood glucose levels in rats, which was however not increased in a dose-dependent manner. Inhibition of acetylcholinesterase enzyme was marked in rats receiving DDVP at 20 mg/kg b.w/ d for 10 days (40%) followed by that in rats receiving 10 mg/kg b.w /d for 10 days (23 %). Reduced glutathione levels were significantly reduced in rats administered DDVP and rats receiving 20mg/kg b.w/d had lower levels of reduced glutathione compared to that in rats receiving 10 mg/kg b.w./d .

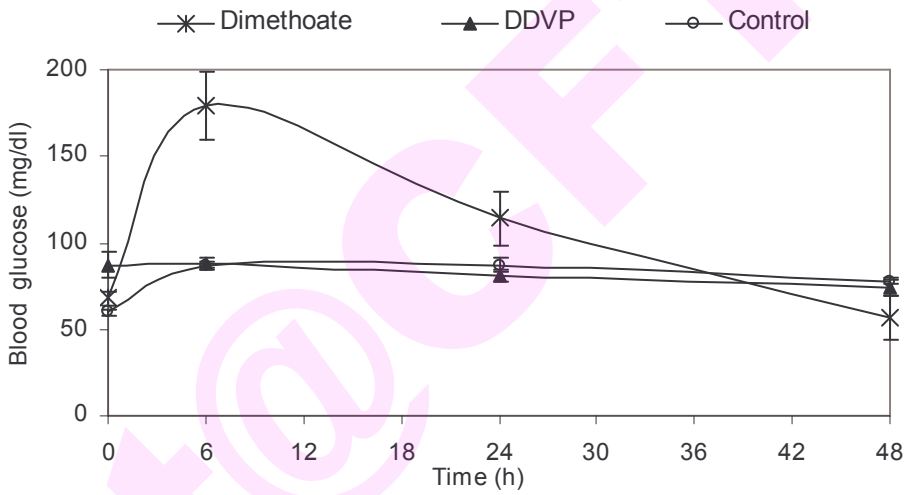


Fig. 1.13 Time-course of blood glucose levels in rats administered a single oral dose of DM (40mg/kg b.w.) and DDVP (10mg/kg b.w.)

Values are mean ± S.E.M. of six rats in each group

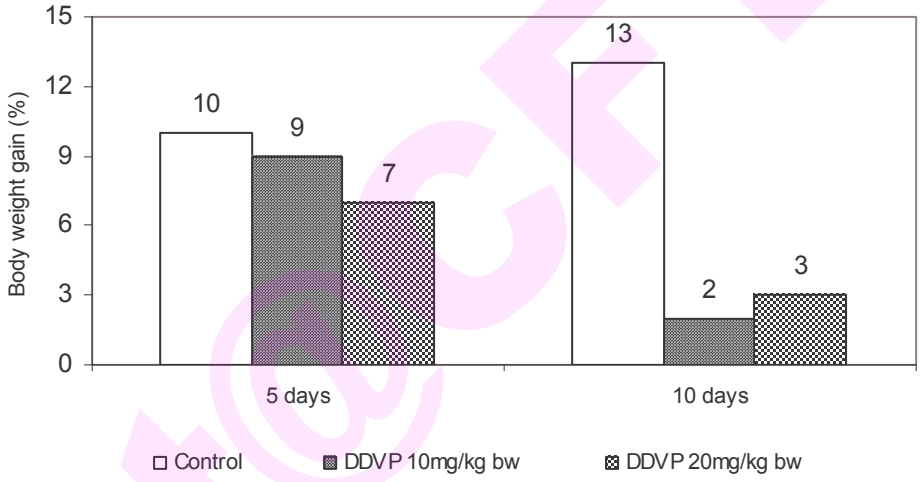


Fig. 1.14 Body weight gain in rats administered oral doses of DDVP (10 or 20 mg/ kg b.w./d for 5 and 10 days)

Values are mean ± S.E.M. of six rats in each group

Table 1.3 Blood glucose, acetylcholinesterase (AChE) and reduced glutathione (GSH) levels in pancreas of rats administered oral doses of DDVP (10 or 20 mg/ kg b.w./d for 5 and 10 days)

	Blood glucose[1]	AChE[2]	GSH[3]
Control	120.14[a] ± 9.06	5.22[a] ± 1.07	1.50[b] ± 0.06
Group I	150.43[c] ± 16.27	4.79[a,b] ± 0.42	1.03[a,b] ± 0.03
Group II	154.11[c] ± 11.36	4.03[b] ± 0.17	1.19[a,b] ± 0.22
Group III	155.51[b] ± 9.20	5.09[a] ± 0.50	1.07[a] ± 0.10
Group IV	150.85[c] ± 5.83	3.09[b] ± 0.65	1.14[a] ± 0.22

[1]mg/dl; [2]nmol/min/mg protein; [3]mg/g tissue
Group I: DDVP-10 mg/ kg b.w/d for 5 d; Group II: DDVP-10 mg/ kg b.w/d for 10 d
Group III: DDVP-20 mg/ kg b.w/d for 5 d; Group IV: DDVP-20 mg/ kg b.w/d for 10d

Values are mean ± S.E.M. of six rats in each group
Means in the same column with different superscript differ significantly (p<0.05)

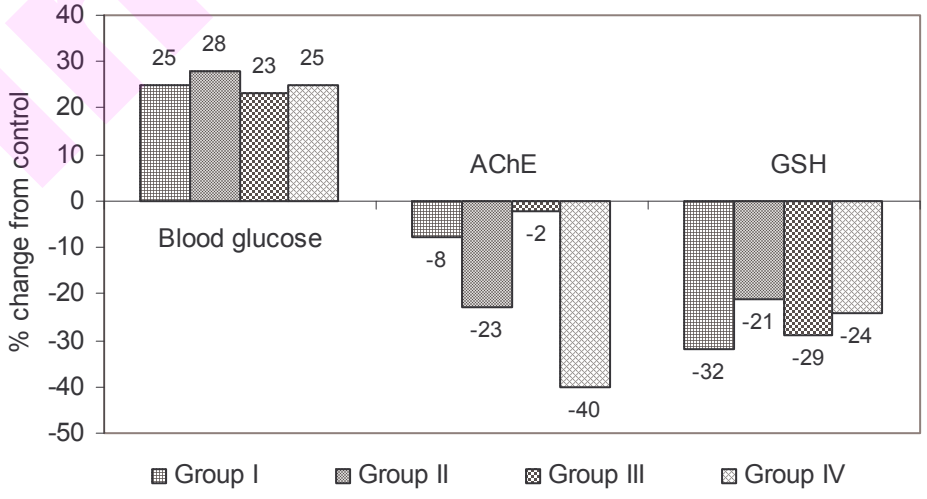


Fig 1.15 Blood glucose, AChE and GSH levels in pancreas of rats administered oral doses of DDVP (10 or 20 mg/ kg b.w./d for 5 and 10 days)

6.0 DISCUSSION

Several observations indicate that OPI affect glucose homeostasis and that blood glucose level is severely increased during organophosphate poisoning in humans (Namba et al., 1971; Hayes et al., 1978; Meller et al., 1981; Teichert-Kuliszenwska et al., 1981; Shobha and Prakash, 2000; Seifert, 2001). The objective of the present investigation was to determine the extent of alterations in blood glucose levels in rats exposed to single / multiple oral doses of DM or DDVP. We observed that while a single oral dose of DM significantly increased the blood glucose levels in rats as early as 6h after dosing, single oral dose of DDVP did not affect the blood glucose level. Hyperglycemic effect of different OPI including diazinon (Matin et al., 1990), dimethoate (Begum and Viayaraghavan, 1999), dichlorvos (Sarin and Gill, 2000), malathion (Mishra and Srivastava, 1983; Abdollahi et al., 2004a; Pournourmohammadi et al., 2005), and malathion (Gowda et al., 1983) following both acute and chronic exposure in animals has been previously reported which strongly supports the present study. Other studies indicate that exposure to an acute dose of malathion causes transient hyperglycemia (Gupta, 1974; Rodriguez et al., 1986; Matin and Husain, 1987c) with glucose levels showing maximum increase at 2 h after dosing, and the levels remained appreciably high up to 6 h and then began decreasing (Gupta, 1974: Rodriguez et al., 1986). However, Abdollahi et al., (2004a) noted significant hyperglycemia only after 18h in rats exposed to sub chronic doses of malathion.

Studies on the effect of DDVP administration during the oral glucose-loading test in rabbits revealed that after 1h of Dichlorvos administration, treated animals presented hyperinsulinemia (Teichert-Kuliszenwska et al., 1981). The cholinergic effects of dichlorvos (Ehrich et al., 1997; Kobayashi et al., 1986) can account for these effects since acetylcholine increase glucose induced insulin secretion by rapid mechanism involving a rise in free cytosolic Ca^{2+} concentration and a marked protein kinase C-mediated increase in

efficiency of Ca^{2+} on exocytosis (Gilon and Henquin, 2001). However, in the present study, we did not measure the insulin levels in rats treated with the OPI.

Hyperglycemia has been known to occur as a consequence of increased accumulation of acetylcholine (ACh) at the nerve endings following AChE inhibition (Anam and Maitra, 1995). ACh, the major parasympathetic neurotransmitter, is released from intra pancreatic nerve endings. The effects of ACh on the β-cells of pancreas are mediated by muscarinic cholinergic receptors (Gilon and Henquin, 2001). Certain studies (Yang et al., 1996; Yang and Dettbarn, 1996) have suggested that AChE inhibitor induced cholinergic hyperactivity initiates accumulation of free radicals leading to LPO, which may be the initiator of AChE inhibitor-induced cell injury. Organophosphates like ethiophate, which inhibit the two cholinesterase isoenzymes in human pancreas (acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), are reported to further increase the sensitivity of pancreas to acetylcholine (Kandlaft et al., 1991; Goodale et al., 1993). Regarding the association of OP-induced hyperglycemia and inhibition of ChE, there is evidence that pralidoxime a reactivator of phosphorylated ChE could alleviate OP-induced hyperglycemia (Seifert, 2001). Furthermore, it has been shown that during the pre-absorptive and absorptive phase of feeding OP, ACh is released by intra pancreatic nerve ending and binds to muscarinic M_3 receptors of β-cells causing a significant increase of glucose–induced insulin secretion (Gilon and Henquin, 2001).

DDVP is an OPI that exerts its neurotoxicity by binding to, and phosphorylating the enzyme AChE in the central and peripheral nervous systems. In the present study, we observed significant AChE inhibition in pancreatic tissue following administration of DDVP, which is indicative of pesticide intoxication. The possible pathogenetic sequel speculated for the pancreatic insult in OP poisoning is excessive cholinergic stimulation of the pancreas and ductal hypertension (Hsiao et al., 1996, Sahin et al., 2002) since

pancreas is a sensitive organ and hence pressure elevation can cause severe tissue damage.

It is known that cells have evolved effective antioxidant defense against ROS. Of these antioxidants, the best characterized endogenous antioxidant is GSH, an abundant and ubiquitous low-molecular weight thiol with putative roles in many cellular processes such as amino acid transport, synthesis of proteins and metabolism of xenobiotics, carcinogens and ROS (Jain et al., 1991). Glutathione (GSH), a tripeptide present in all mammalian cells, participates in many metabolic processes, among which the protection of cells against free radicals and toxic metabolites of endogenous and exogenous origin is one of the most important (Ponsoda et al., 1991). Further, GSH can act as a soluble antioxidant and also as a co-substrate in various cellular enzymic reactions in which it is oxidized to glutathione disulfide (GSSG) (Freeman and Crapo, 1982; Ziegler, 1985; Petkau, 1986). Glutathione is presumed to be an important endogenous defence against the peroxidative destruction of cellular membranes since GSH can act either to detoxify activated oxygen species such as H_2O_2 or reduce lipid peroxides themselves (Freeman et al., 1982). Tissue GSH concentration reflects the potential for detoxification and the levels may decrease due to an increased use of glutathione to detoxify. Low concentration of GSH resulting from increased formation of ROS and the depletion of GSH from mammalian cells have been used as an index of increased oxidative stress (Martensson et al., 1991).

In the present study, multiple doses of DDVP inducing increased blood glucose levels and significantly inhibiting AChE in pancreas also depleted the GSH levels in pancreas to a significant extent. These results indicate that these doses of DDVP induce marked oxidative stress in pancreas which in turn might play a role in the observed alterations in glucose homeostasis in the treated rats.

The results of the present study clearly demonstrate that both DM and DDVP possess the potential to alter blood glucose levels in rats, inhibit AChE and induce OS in pancreas either after single (DM) or multiple doses (DDVP).

7.0 SUMMARY

1. Single oral dose of DM (40 mg/kg b.w) increased the blood glucose levels significantly in rats within 6h after administration and the blood glucose levels remained elevated up to 24h after dosing.
2. While a single dose of DDVP (10 mg/kg b.w) failed to increase blood glucose level to a significant extent, multiple oral doses (10 or 20 mg/kg b.w /d for 5 or 10 days) induced significant increase in blood glucose. However, the response was neither dose nor exposure period related.
3. Other salient alterations comprised of diminution of reduced glutathione levels and marked inhibition of AChE in pancreas of DDVP treated rats.
4. Collectively these data demonstrate the involvement of oxidative stress in the toxicity of DM and DDVP and suggests its role in pancreatic dysfunction and altered glucose homeostasis.

CHAPTER II

SUSCEPTIBILITY OF RAT PANCREAS TO OPI-INDUCED BIOCHEMICAL ALTERATIONS

PREFACE

From our earlier studies (*Chapter I*), it is clearly evident that both dimethoate and dichlorvos possess the potential to induce oxidative stress in rats. Our studies also showed that while a single dose of DM increased blood glucose levels in rats, single dose of DDVP failed to increase the blood glucose levels. However, administration of repeated, sublethal doses of DDVP significantly elevated the blood glucose levels in rats. Hence, in the present study, it was planned to investigate the oxidative stress associated biochemical perturbations in pancreas of rats subjected to repeated oral doses of DM and DDVP. Another important question addressed in the present study was to determine the impact of DDVP pre-treatment on hyperglycemia and associated biochemical alterations induced by streptozotocin, a diabetogenic agent, in order to obtain evidence whether OPI exposure would predispose experimental animals to STZ-induced diabetes.

This Chapter has been presented under two sections.

Section A describes investigations related to the potency of DM and DDVP to impair glucose homeostasis and associated pancreatic dysfunction/s following their repeated oral exposure in rats.

Section B describes the effect of streptozotocin (STZ) in rats pre-treated with repeated oral doses DDVP.

SECTION A

BIOCHEMICAL PERTURBATIONS IN RAT PANCREAS ON EXPOSURE TO DIMETHOATE AND DICHLORVOS *IN VIVO*

1.0 INTRODUCTION

OPI are currently responsible for more poisonings than any other single class of pesticides (Sultatos, 1994; Gulr et al., 1996) and various complications have been reported in OPI intoxication cases (Hsiao et al., 1996). Hyperglycemia has been widely reported as one of the adverse effects in poisoning by OPI in humans and animals (Namba et al., 1971; Ramu and Drexler, 1973; Meller et al., 1981; Shobha and Prakash, 2000; Hagar et al., 2002, Seifert, 2001; Abdollahi et al., 2003). Although the precise mechanism /s of OP-induced hyperglycemia is not known, it is speculated to be due to inhibition of acetylcholinesterase of central and peripheral synapses that act in the endocrine regulation of glucose metabolism (Kant et al., 1988, Matin and Siddiqui, 1982). Acute pancreatitis is also a well known complication of OP poisoning (Dressel et al., 1979; Frick et al., 1987; Hsiao et al., 1996), and epidemiological findings indicate that the incidence of pancreatitis is high in OPI intoxication based on various pathophysiological reports (Gokalp et al., 2005). The precise mechanisms underlying OPI-induced pancreatitis are still undefined, although it is believed to involve obstruction of pancreatic ducts and /or enhanced reactive oxygen species (Dressel et al., 1982, Sevillano et al., 2003, Sultatos, 1994). Involvement of oxidative stress following acute exposure to OPI has been reported recently (Banerjee et al., 2001) and it has been demonstrated unequivocally that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced cytotoxicity (Akhgari et al., 2003; Ranjbar et al., 2002; Abdollahi et al., 2004b).

In view of the above, in the present study, we have focused our attention to understand the potential of repeated oral doses of DM and DDVP to cause alterations in glucose homeostasis and the associated biochemical alterations in pancreas of rats. Further, we have examined the various oxidative impairments in pancreas in terms of lipid peroxidation, generation of ROS, response of antioxidant enzymes and examined its correlation with pancreatic acetylcholinesterase activity.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Thiobarbituric acid (TBA), xanthine oxidase, glutathione reductase (GR), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), 2',7'-Dichlorofluorescein (DCF), *p*-nitrophenyl acetate (PNPA), *p*-nitrophenol (PNP), butyrylthiocholine iodide, streptozotocin and cytochrome 'C' were procured from M/s Sigma Chemical Co., (St. Louis, MO, USA). Ethylenediamine tetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), xanthine, nicotinamide adenine dinucleotide phosphate–reduced (NADPH), nicotinamide adenine dinucleotide–reduced (NADH), nicotinamide adenine dinucleotide phosphate (NADP), trichloroacetic acid (TCA), reduced glutathione (GSH), oxidized glutathione (GSSG), 1-chloro-2,4-dinitrobenzene (CDNB), sucrose, glucose-6-phosphate, glucose-1-phosphate, sodium fluoride, γ-glutamyl p-nitroanilide, glycyl glycine, *N*-ethylmaleimide, *o*-phthalaldehyde, adenosine-5'-monophoshate (AMP), adenosine-tri-phosphate (ATP), L-tyrosine, α-ketoglutaric acid, pyridoxal-5'-phosphate, diethyl dithiocarbamic acid, anthrone, thiourea, glucose-6-phosphate dehydrogenase, β-mercaptoethanol, glucose, sodium pyruvate, 2,6-dichlorophenolindophenol (DCPIP) and acetylthiocholine iodide were procured from M/s Sisco Research Lab, (Mumbai, India). Dimethoate (Technical grade, 97.4 % pure) and Dichlorvos (Technical grade, 94 % pure) were gift from M/s Hyderabad Chemical Supplies Ltd., (Hyderabad, India). Glucometer ("Accu-Check") was procured from M/s Roche

diagnostics (GmbH, Mannheim, Germany). Amylase kit was procured from M/s Span diagnostics (Mumbai, India). All other chemicals used were of analytical grade.

2.2 Animals and care

Adult male rats (CFT-Wistar strain, 12-14 week old, 280 ± 5g) were randomly drawn from the stock colony of our institute animal house facility and were housed individually in polypropylene cages under standard housing conditions (controlled atmosphere with 12:12-hour light/dark cycles, 50% ± 5% humidity, and ambient temperature of 25± 2º C). The rats were acclimatized for 1 week prior to the start of the experiment. They were maintained on commercial pellet diet (M/s Saidurga Feeds and Foods Pvt. Ltd., Bangalore, India) *ad libitum* and had free access to water. All procedures with animals were conducted strictly in accordance with approved guidelines by the 'Institute Animal Ethical Committee', regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. During the experiments, maximum care was taken to minimize animal suffering and in addition, the number of rats used was kept at a minimum.

2.3 Pesticide solution

Stock solution of Dimethoate (DM: 20mg/ml) and Dichlorvos (DDVP:10mg /ml) was prepared in saline and used for intragastric (oral) administration to rats.

2.4 Streptozotocin solution

Streptozotocin solution (STZ: 10mg/ml) was freshly prepared in citrate buffer (0.1M, pH 4.5) and used for intraperitoneal injection.

3.0 EXPERIMENTAL PROCEDURE

3.1 Effect of repeated oral doses of DM

Adult male rats were grouped by randomized design into three groups (n = 6). Rats of the first group (negative control) received saline daily, while rats (non-fasted) of the treatment groups were orally administered daily (0900 to 1100 hours) Dimethoate (DM) at dosages of 20 or 40mg/kg b.w /day (corresponding to 1/20 and 1/10 of LD_{50} value: 400 mg/kg b.w, determined in a preliminary study) for 30 days. Both, control and DM treated rats were subjected to oral glucose tolerance test at the end of 30 days and were subsequently killed. Body weights were recorded weekly, and terminally the rats were sacrificed and blood was collected for separation of serum. Pancreas and other vital organs (viz., liver, kidney and adrenals) were excised and their weights were recorded. The biochemical assays included determination of blood glucose, AChE activity in pancreas, amylase and lipase activity in serum and pancreas, oxidative stress parameters and xenobiotic metabolizing enzymes in pancreas.

3.2 Effect of repeated oral doses of DDVP

Adult male rats were grouped by randomized design into three groups (n = 6). Rats of the first group received saline daily, while rats (non-fasted) of the treatment group were orally administered daily (0900 to 1100 hours) DDVP at 20mg/kg b.w /d (corresponding to 1/5 of LD_{50} value: 100 mg/kg b.w, determined in a preliminary study) for 10 days. Both control and DDVP treated rats were subjected to oral glucose tolerance test at the end of 10 days. Subsequently, the rats were sacrificed and blood was collected for separation of serum. Pancreas and other vital organs (viz., liver, brain and adrenals) were excised, rinsed in ice-cold saline, blotted and stored at 4ºC until use. The biochemical assays included determination of blood glucose, AChE activity in pancreas, amylase, lipase, GGT and LDH activity in serum and pancreas as

biochemical indices of pancreatic damage, oxidative stress parameters and xenobiotic metabolizing enzymes in pancreas, and carbohydrate metabolizing enzyme activities in liver and pancreas, as well as histology of pancreas.

3.3 Oral glucose tolerance test

Oral glucose tolerance test was conducted in control and treated rats 24 h after the last dose of DM or DDVP. Blood samples were collected from tail vein of rats that were fasted overnight to obtain baseline blood glucose levels. Subsequently, rats of both control and treated groups were orally administered a bolus of glucose (3 g / kg b.w.). Blood was collected from tail vein of these rats at intervals of 30 min up to 3 h for estimation of glucose.

3.4 Preparation of tissue homogenates

10 or 20 % (w/v) homogenates of the tissues were prepared in various buffers as per the requirement of a given assay.

3.5 Serum

Blood was drawn by cardiac puncture into tubes and then allowed to clot at room temperature for 20min. It was then kept at 4ºC for 4 h and centrifuged at 3500 rpm for 10min at 4ºC. The supernatant serum was decanted and frozen until use.

4.0 ASSAY METHODS

4.1 Blood glucose

Blood glucose was estimated using glucometer as described earlier (*Section 4.1, Chapter IB*).

4.2 Lipase (EC 3.1.1.3) *(Young et al., 1978)*

Lipase activity was estimated by monitoring the hydrolysis of p-nitrophenyl acetate to p-nitrophenol. The assay involved incubation of 100 µM p-nitrophenyl acetate with 10 µl sample (serum / pancreatic homogenate) in a final volume of 2ml at 37ºC for 10min. The resultant colored product was read at 420nm against water. Enzyme activity was calculated from PNP standard graph and was expressed as nmoles of PNP released/min/mg protein.

4.3 Amylase (EC 3.2.1.1) *(Street and Close, 1956)*

The enzyme activity was measured using a commercial kit. The assay is based on the degradation of starch by the enzyme into reducing dextrins and small oligosaccharides with which iodine solution is reacted to give a blue coloration, which is read at 620 nm. The relative decrease in blue color of test is the measure of enzyme activity. The enzyme activity was expressed as Street-Close units.

4.4 γ-glutamyltranspeptidase (GGT) (EC 2.3.2.2) *(Novogrodsky et al., 1976)*

20 % homogenate of pancreas was prepared in Tris buffer (0.01M, pH 8.0) containing 0.15M NaCl and centrifuged at 10,000g for 10min at 4ºC and the supernatant was used for the assay. Assay solution contained 122 µl of the supernatant, 2.5mM γ-glutamyl p-nitroanilide, 30mM glycyl glycine (adjusted to pH 8.0 with NaOH), 0.05 M Tris-HCl (pH 8.0) and 0.075 M NaCl. The contents were mixed and scanned at 410 nm for 1 min. The enzyme activity was calculated based on molar extinction coefficient: 11.3mM at 410nm. Results were expressed as nmoles of product (p-nitroanline or S-acetophenone-cysteinylglycine) formed/min/mg protein.

4.5 Lactate dehydrogenase (LDH) (EC 1.1.1.27) *(Kornberg, 1974)*

Pancreatic homogenate (10% w/v) was prepared in Tris-HCl buffer (82.4mM, pH 7.2) containing 210mM NaCl, and then centrifuged at 10,000 rpm for 10min at 4ºC. 1µl of the supernatant/serum was placed in a cuvette and the reaction was started by addition of 0.8ml NADH (in Tris-HCl. 0.25mM, pH 7.2), 0.15 ml of sodium pyruvate (10.66 mM) and 49µl of distilled water. The reaction was monitored over a period of 5 min at 340nm. The results were expressed as nmoles of NADH oxidized/ min/ mg protein.

4.6 Acetylcholinesterase (AChE) (EC 3.1.1.7) (*Ellman et al., 1961)*

AChE activity in serum and other tissues viz., brain, liver, adrenal and pancreas was estimated as described earlier (*Section 4.2, Chapter IB*).

4.7 Butyrylcholinesterase (BuChE) (EC 3.1.1.8) (*Ellman et al., 1961)*

100 µl of serum / tissue homogenate was taken in 2.83 ml of phosphate buffer (0.1M, pH 8.0) with 50µl of DTNB solution (10mM) and 20 µl of butyrylthiocholine iodide (1mM). The absorbance was read at 412 nm immediately after addition of the substrate and the absorbance was recorded for 5min. The change in absorbance per minute (∆A) was calculated and the enzyme activity was expressed as nmoles of substrate hydrolyzed/min/mg protein.

4.8 Reactive oxygen species (ROS) *(Keston and Brandt, 1965)*

Tissue homogenate (10%) was incubated with 5µM DCFH-DA in a final volume of 2ml for 45min at room temperature. The intensity of the fluorescence was measured at 530nm following excitation at 485nm. The amount DCF (resulting from the ROS mediated oxidation of DCFH, which is produced by hydrolytic cleavage of DCFH-DA by cellular esterases) was calculated from the DCF standard graph. Results were expressed as pmoles of DCF/min/mg protein.

4.9 Lipid peroxidation (LPO) *(Buege and Aust, 1978)*

10% (w/v) homogenate of tissue was prepared in KCl (1.15%, w/v) and centrifuged at 3500 rpm for 10 min. 250 µl of the supernatant was added to 2ml of TBA-TCA-HCl (0.374%-15%-0. 25N) and the tubes were placed in boiling water bath for 15min. After cooling and centrifugation, the color of the supernatant was read at 535nm. The amount of thiobarbituric acid reactive substances (TBARS) in the supernatant was calculated using the molar extinction coefficient of 1.56X 10^{-5} $M^{-1}cm^{-1}$ and the results were expressed as nmoles of MDA/g tissue.

4.10 Reduced glutathione (GSH) *(Benke et al., 1974)*

Reduced glutathione was estimated in tissue homogenate as described earlier *(Section 4.3, Chapter IB*).

4.11 Oxidized glutathione (GSSG) *(Hissin and Hilf, 1976)*

Tissue homogenate (10% w/v) was prepared in phosphate buffer (0.1M, pH 7.4), centrifuged and 0.5ml of the supernatant was incubated with 200µl of 0.04 M *N*-ethylmaleimide for 30 min at room temperature to interact with GSH present in the tissue. 100 µl aliquot from this mixture was diluted with 1.8 ml of 0.1 N NaOH and incubated with 100 µl of the *o*-phthalaldehyde (OPT) (1mg/ml in alcohol) at room temperature for 15 min. The fluorescence of the mixture was measured at 420nm with the excitation at 350nm. Oxidized glutathione concentrations were calculated from standard graph obtained with GSSG and the results were expressed as pmoles of GSSG/mg tissue.

4.12 Protein carbonyls *(Levine et al., 1990)*

Tissue homogenate (10% w/v) was prepared in 20mM Tris-HCl, 0.14 M NaCl buffer (pH 7.4) and centrifuged at 10,000 rpm for 10 min at 4ºC. 0.1ml of the supernatant was precipitated with an equal volume of 20% TCA and centrifuged. The pellet was resuspended in 1ml of

2,4-dinitrophenylhydrazine solution (10mM, in 2M HCl) and allowed to stand at room temperature for 60min with occasional vortexing. Proteins were then precipitated by adding 0.5ml of 20 % TCA. The resulting mixture was centrifuged and the pellet obtained was washed 3 times with 1ml acetone and 1ml of 2% SDS (in 20mM Tris-HCl, 0.1M NaCl, pH 7.4) was added to dissolve the pellet. The absorbance of the solution was read at 360nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$. Results were expressed as nmoles of carbonyl/mg protein.

4.13 Catalase (CAT) (EC 1.11.1.6) *(Beers and Sizer, 1952)*

Tissue was homogenized (10% w/v) in phosphate buffer (0.1M, pH 7.4), centrifuged at 10,000 rpm for 10 min at 4ºC, and the supernatant was used for the assay. Varying volumes of supernatant along with 25µl of H_2O_2 (3%) was added to 3ml phosphate buffer (50mM, pH 7.4) and the decrease in absorbance due to H_2O_2 degradation was monitored at 240nm for 5min. The enzyme activity was calculated based on molar extinction coefficient at $43.6M^{-1}cm^{-1}$ and the results were expressed as µmoles of H_2O_2 consumed / min/mg protein.

4.14 Superoxide dismutase (SOD) (EC 1.15.1.1) *(Flohe and Otting , 1984)*

Tissue was homogenized (10% w/v) in potassium phosphate buffer (0.1M, pH 7.4) and centrifuged at 10,000 rpm for 10 min 4ºC, and the supernatant was used for the assay. To a semi-micro cuvette, 2.9 ml of solution A (5µl xanthine in 0.01M NaOH + 2µl cytochrome ‘c’ + 50mM phosphate buffer and 0.1mM EDTA, pH7.8) and 0.1ml of solution B (an equal volume of xanthine oxidase and 0.1 mM EDTA) were added. Reaction mixture without enzyme was used as blank. After adding various volumes of supernatant (enzyme source), inhibition of cytochrome ‘c’ reduction was monitored at 560nm for 5min. Results were expressed as units of SOD/mg protein. One unit was defined as the amount of enzyme that decreases the initial rate of cytochrome ‘c’ reduction to 50% of its maximal value for the particular sample being analyzed.

4.15 Glutathione reductase (GR) (EC1.6.4.2) (*Carlberg and Mannervik, 1975)*

The reaction was started by adding 25µl of homogenate to the reaction mixture (final volume 1ml) containing 0.2 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 1mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The enzyme activity was measured indirectly by monitoring the oxidation of NADPH following decrease in OD/min for a minimum of 3 min at 340 nm. One unit enzyme activity was defined as nmoles of NADPH consumed/min/mg protein based on Molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

4.16 Glutathione peroxidase (GPX) (EC 1.11.1.9) (*Flohe and Gunzler, 1984)*

Tissue homogenate (10% w/v) was prepared in phosphate buffer (0.1M, pH 7.0) containing 0.5mM EDTA and centrifuged at 10,000 rpm for 10min at 4°C. 5 or 10µl of supernatant was incubated with 0.575ml phosphate buffer (0.1M, pH 7.0 containing 0.5mM EDTA), 100 µl of glutathione reductase (2.5U/ml), 100µl of GSH (10mM) and 100 µl of NADPH (1% in sodium bicarbonate solution) at 37ºC for 10 min. Finally, 100µl of 12mM t-butylhydroperoxide was added to the above mixture and the oxidation of NADPH over 5min was recorded at 340nm. Results were expressed as nmoles of NADPH oxidized/min/mg protein.

4.17 Glutathione transferase (GST) (EC 2.5.1.18) *(Jakoby and Habig, 1980)*

To 0.5ml of potassium phosphate buffer (0.2M, pH 7.2) were added 0.1ml of GSH (10mM), 0.01ml of 1-chloro-2,4-dinitro benzene (CDNB, 0.1M) and 390µl of distilled water and mixed. 1-2.5 µl of the tissue homogenate was then added to the above mixture and the formation of the adduct of CDNB (S-2,4-dinitrophenyl glutathione) was monitored by measuring net increase in absorbance at 340nm. The enzyme activity was calculated based on absorption coefficient of 9 mM^{-1} cm^{-1}. Results were expressed as µmoles of adduct formed/min/mg protein.

4.18 NADPH-Cytochrome P_{450} reductase (EC 1.6.2.4) (*Omura and Takesue, 1970)*

The reaction mixture containing 0.3M potassium phosphate buffer (pH 7.5), 0.2mM potassium ferricyanide and tissue homogenate in a final volume of 1 ml was incubated with 0.1mM NADPH at 25ºC. The enzyme activity was calculated using extinction coefficient 6.22$mM^{-1}cm^{-1}$. One unit of enzyme activity was defined as that causing the oxidation of one mole of NADPH / min. The enzyme activity was expressed as µmoles of NADPH utilized/min/mg protein.

4.19 NADH-Cytochrome b_5 reductase (EC 1.6.2.2) *(Mihara and Sato, 1972)*

NADH-Cytochrome b5 reductase was assayed by measuring the rate of reduction of potassium ferricyanide. The reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.5), 1 mM potassium ferricyanide and tissue homogenate in a final volume of 1ml was incubated at 25ºC with 0.1 mM NADH. The enzyme activity was calculated using extinction coefficient of 1.02 mM^{-1} cm^{-1}. One unit of enzyme activity was defined as that causing the reduction of one mole of ferricyanide/min. The enzyme activity was expressed as µmoles of NADH utilized/min/mg protein.

4.20 DT-diaphorase (DT-D) (EC 1.6.99.3) *(Ernestr et al., 1962)*

This method involves measurement of reduction, at 550nm using NADH as the electron donor and 2, 6-dicholorophenolindophenol as the electron acceptor. The reaction was started by adding 50 µl of the homogenate to the reaction mixture containing 2.73ml of phosphate buffer (50mM, pH 7.6), 50 µl of 0.3 mM NADH, 50 µl of 0.4mM DCPIP, 100 µl of 33µM cytochrome 'c' and 20 µl 0.07% bovine serum albumin. The enzyme activity was calculated using the extinction coefficient 21$mM^{-1}cm^{-1}$ and expressed as µmoles of NADH utilized/ min /mg protein.

4.21 NADPH Diaphorase (NADPH-D) (EC 1.6.99.1) *(Huennekens et al., 1957)*

This method is based on reduction of methylene blue to leukomethylene blue during the transfer of hydrogen from NADPH to NADPH-diaphorase. The reaction mixture containing 830µl of phosphate buffer (50mM, pH 7.6), 100 µl of NADPH (2mM) and 20 µl sample was in incubated at 37^{o}C for 10 min. To this 50µl of methylene blue (0.8mM) was added. Enzyme activity was assayed by following the decrease in absorbance of NADPH at 340nm. The enzyme activity was calculated using extinction coefficient 6.22mM^{-1}cm^{-1} and expressed as nmoles of NADPH utilized/ min/ mg protein.

4.22 Glycogen (*Nicholas, 1956*)

Liver was homogenized (5 % w/v) in TCA (5%) for 3 min at 4ºC. The homogenate was spun and to an aliquot of the supernatant, 5 volumes of cold 95% EtOH was added and left overnight to precipitate glycogen. After removal of EtOH, the glycogen precipitate was dissolved in 2 ml of distilled water. Blank and standard were prepared with distilled water and glucose solution (0.5 mg/ml) respectively. 10 ml of Anthrone reagent was added to all the tubes and the tubes were placed in a boiling water bath for 15 min. After cooling, the absorbance was measured at 620 nm against the blank. Glycogen content was measured using glucose standard and the results were expressed as mg of glycogen /g tissue.

4.23 Glycogen phosphorylase (EC 2.4.1.1) *(Niemeyer et al., 1961)*

The enzyme was assayed by measuring the release of orthophosphate from glucose-1-phosphate in the presence of glycogen and AMP. A stock solution containing 25 mM glucose-1-phosphate, 75 mM NaF, 2.5 mM AMP, and 1% glycogen was prepared in 67 mM citrate buffer, pH 6.0. 1.2 ml of the above solution was incubated for 2 min at 30ºC, further 0.2 ml of liver homogenate was added and immediately 0.5 ml of the assay mixture was withdrawn into 10% TCA. After incubation for 5 min at 37ºC, an aliquot (0.5 ml)

of the assay mixture was transferred into 10% TCA. The TCA extracts were spun and the iP in an aliquot of supernatant was determined. Results were expressed as nmoles of Pi released/ min / mg protein.

4.24 Inorganic phosphate *(Fiske and Subbarow, 1925)*

300 µl of the supernatant was added to 3ml of solution containing 2% ammonium molybdate, 6 N H_2SO_4, and 10% ascorbic acid mixed in 1:2:3:4 ratio. The resultant mixture was incubated at 37ºC for 1h and read aginst blank at 820nm. The amount of inorganic phosphate liberated was calculated using potassium dihydrogen phosphate as the standard.

4.25 Glucose-6-phosphatase (G-6-P) (EC 3.1.3.9) *(Hers, 1959)*

0.1 ml of 1 % liver homogenate prepared in 250 mM sucrose, containing 1 mM EDTA (pH 7.0), was incubated with 50 mM glucose-6-phosphate at 37ºC for 30 min. The reaction was stopped by addition of 1ml 10 % TCA, and the phosphate released by the enzymatic activity was measured as described earlier. Results were expressed as nmoles of Pi released /min /mg protein.

4.26 Glucokinase (GK) (EC 2.7.1.2) *(Crane and Sols, 1955)*

The assay mixture in a volume of 2 ml, consisted of 0.2 IU of G-6-PDH, 0.25 mM NADP, 5 mM ATP, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 25 µl of liver homogenate and either 0.05 mM glucose or 100 mM glucose. The rate of reduction of NADP by G-6-PDH, which is directly proportional to formation of G-6-P, was monitored at 340 nm. Activity of glucokinase was obtained by subtracting the rate of reduction of NADP with 0.5 mM glucose from that with 100 mM glucose and the results were expressed as µmol of NADP utilized/min/mg protein.

4.27 Glutamate dehydrogenase (GDH) (EC 1.4.1.3) *(Bryla et al., 1994)*

A solution containing final concentration of NADPH (100µM), α-ketoglutarate (5 mM), ammonia (50 mM), EDTA (0.1 mM), and bovine serum albumin (1mg/ml) in 0.1 M phosphate buffer (pH 7.6) was incubated in a 1cm path length quartz cuvette at 25ºC and the reaction was started by adding homogenate (10 µl). The decrease in absorbance at 340 nm was monitored for 1 min. One unit of activity was defined as that amount of enzyme required to oxidize 1 µmol of NADH per minute at 30ºC and the results were expressed as mmoles of NADPH utilized/min/mg protein.

4.28 Protein estimation *(Lowry et al., 1951)*

Protein content of tissue homogenate and serum was estimated as described earlier (*Section 4.4, Chapter I B*).

4.29 Histopathology

Pancreas was excised, and blotted. A portion of the organ was fixed in 10% neutral buffered formalin, embedded in paraffin, processed by standard histological techniques (*Lillie and Fullmer, 1976*), stained with hematoxylin /eosin and examined by light microscopy.

4.30 Statistical analysis

Mean and standard error values were determined for all the parameters and the results were expressed as mean ± S.E from six rats in each group. The results were processed by the Programme: Microsoft Office Excel 2002. Paired Students t-test was applied to the results obtained in the two groups of rats: control and DDVP treated. In all cases, P values lower than 0.05 were considered to be statistically significant. Tukey test for multiple comparisons was performed on data of DM toxicity study to determine the significant differences among the groups and '*p*' values < 0.01 were considered significant.

5.0 RESULTS

5.1 Biochemical perturbations induced in rat pancreas by repeated oral doses of DM

5.1.1 Growth and organ weights

Repeated oral doses of DM failed to induce any distinct clinical signs of toxicity or mortality. Data on the body weights along with the relative organ weights are presented in **Table 2.1**. While the weight gain in rats of control group at the end of 30 days was 17.9%, rats treated with the higher dose of DM showed only 7.6% weight gain. There was a marked increase (32%) in pancreatic weight in rats administered higher dose of DM (170.73mg ± 6.74 vs. 129.05mg ± 6.43). However, only marginal increases in liver and kidney weights were observed in rats administered with higher dose of DM.

5.1.2 Oral glucose tolerance

Data on blood glucose levels monitored in control and DM treated rats following glucose overload over a 3h period is presented in **Fig.2.1**. Fasting blood glucose levels in rats of all the three groups were similar. There was no significant difference in blood glucose levels measured at 30 min. However DM treated rats showed higher blood glucose level (135 mg/dl) at 60 min and beyond. The glucose levels in control rats returned to normal level (90 mg/dl) at the end of 3h suggesting normal glucose tolerance while on the other hand DM treated rats showed higher levels of blood glucose (125 and 129 mg/dl) at the end of 3 h indicating altered glucose tolerance.

5.1.3 Blood glucose, acetylcholinesterase and reduced glutathione

Data on blood glucose levels, pancreatic AChE activity and reduced glutathione levels in control and DM treated rats are presented in **Table 2.2 & Fig. 2.2**. The blood glucose levels in DM treated rats were elevated in a dose-dependent manner and the percent increase in glucose levels was 15 and 51 % at the low and high dose respectively. The activity of AChE in pancreatic tissue

was markedly reduced (40 and 90 %) among DM treated rats. While the decrease in the reduced GSH was marginal in the lower dose (11%), it was 18 % at the higher dose.

5.1.4 Pancreatic damage

The activity of enzymes viz., amylase and lipase in serum and pancreatic tissue following DM treatment is presented in **Fig. 2.3 & Fig. 2.4**. There was a significant increase in serum lipase activity (20 and 38 %) in DM treated rats compared to the activity in control rats (5.79 ± 0.26 nmol PNP/mg protein/min). However, pancreatic tissue lipase activity was significantly decreased in rats administered with DM (18 and 63%) with respect to control (37.1nmol PNP/mg protein/min). While serum amylase activity was increased (by 2-3 folds) in DM treated rats, pancreatic amylase activity was marginally decreased.

5.1.5 Oxidative stress in pancreas

ROS and TBARS levels determined in the pancreatic tissue are presented in **Fig. 2.5**. There was a dose-related elevation in ROS levels among treated rats. While the increase at the lower dose was 66%, a dramatic (150%) increase was evident at the higher dose. Concomitantly, a dose-related increase in TBARS levels was observed in DM treated rats. There was 2.5 and 3.7 fold increase in TBARS level at lower and higher doses of DM respectively.

5.1.6 Activities of antioxidant enzymes and phase II enzymes in pancreas

Data on the activities of various antioxidant enzymes in pancreas following DM treatment is presented in **Table 2.3 & Fig. 2.6**. In general, activities of SOD, CAT, GR and GST were significantly elevated in DM treated rats compared to the control rats. At the higher dose, activities of SOD, CAT, GR and GST were increased by 112%, 64%, 45% and 100% over the control. However, the GPX activity was diminished (50%) at the higher dose.

5.1.7 Activities of phase II enzymes in pancreas

Data on the activities of phase II enzymes such as NADPH-diaphorase and DT-diaphorase are presented in the **Table 2.4 & Fig. 2.7**. There was a 27% increase in the activity of NADPH-diaphorase and a 16% increase in the activity of DT-diaphorase in the rats treated with higher dose of DM.

Table 2.1 Body weight and relative organ weights of rats administered repeated oral doses of DM for 30 days

	Control	DM (mg/kg b.w/d)	
	0	20	40
Initial body weight (g)	282.67 ± 9.04	287.28 ± 7.41	287.00 ± 5.51
Final body weight (g)	333.33 ± 9.27	327.67 ± 4.23	309.00 ± 7.03[a,b]
Pancreas (mg/100g b.w.)	129.05 ± 6.43	144.25 ± 7.96[a]	170.73 ± 6.74[a,b]
Liver (g/100g b.w.)	3.08 ± 0.21	3.25 ± 0.32	3.56 ± 0.51
Kidney (g/100g b.w.)	0.75 ± 0.10	0.74 ± 0.08	0.82 ± 0.06
Adrenals (mg/100g b.w.)	15.26 ± 1.28	15.41 ± 1.97	14.89 ± 2.17

Values are mean ± SEM (n=6);
[a] Comparison of control and other groups;
[b] Comparison of DM: 20mg /kg b.w/d treated group with DM: 40 mg/kg b.w/d treated group

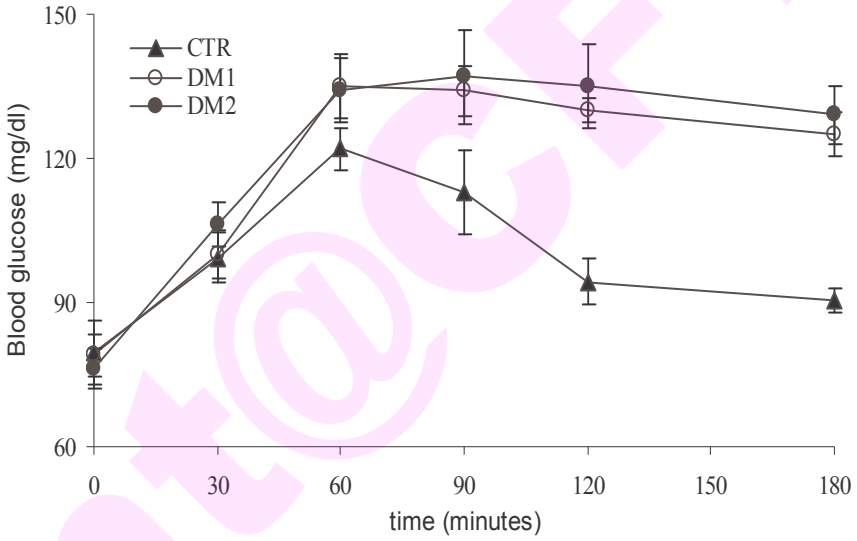


Fig. 2.1 Oral glucose tolerance at the end of 30 days in control (CTR) and DM treated rats (DM1: 20 mg/kg b.w/d; DM2: 40 mg/kg b.w/d)

Values are mean ± SEM (n=6)

Table 2.2 Blood glucose, acetylcholinesterase (AChE) and reduced glutathione (GSH) levels in pancreas of rats administered oral doses of DM for 30 days

	Control	DM (mg/kg b.w.)	
	0	20	40
Initial blood glucose[1]	85.33 ± 3.85	87.34 ± 5.23	85.00 ± 5.30
Final blood glucose[1]	91.33 ± 2.41	105.28 ± 3.57[a]	138.67 ± 5.70[a,b]
AChE[2]	4.96 ± 1.47	2.94 ± 1.75	0.43 ± 0.21[a,b]
GSH[3]	1.11 ± 0.02	0.99 ± 0.05[a]	0.91 ± 0.07[a,b]

[1]mg/dl; [2] nmoles substrate hydrolyzed /min/mg protein; [3]mg/g tissue
Values are mean ± SEM (n=6);
[a] Comparison of control and other groups;
[b] Comparison of DM (20mg /kg b.w.) group with DM (40 mg/kg b.w.) group

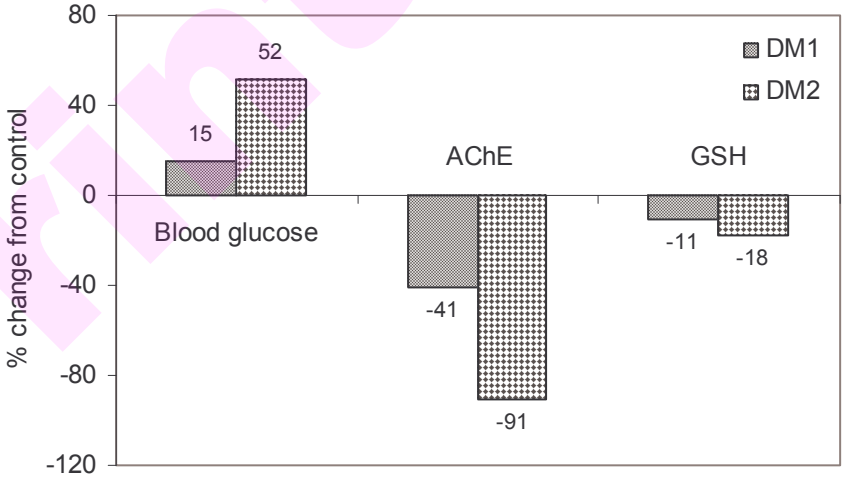


Fig. 2.2 Blood glucose, AChE and GSH levels in pancreas of rats administered oral doses of DM (DM1: 20 mg /kg b.w/d; DM2: 40mg/kg b.w/d) for 30 days

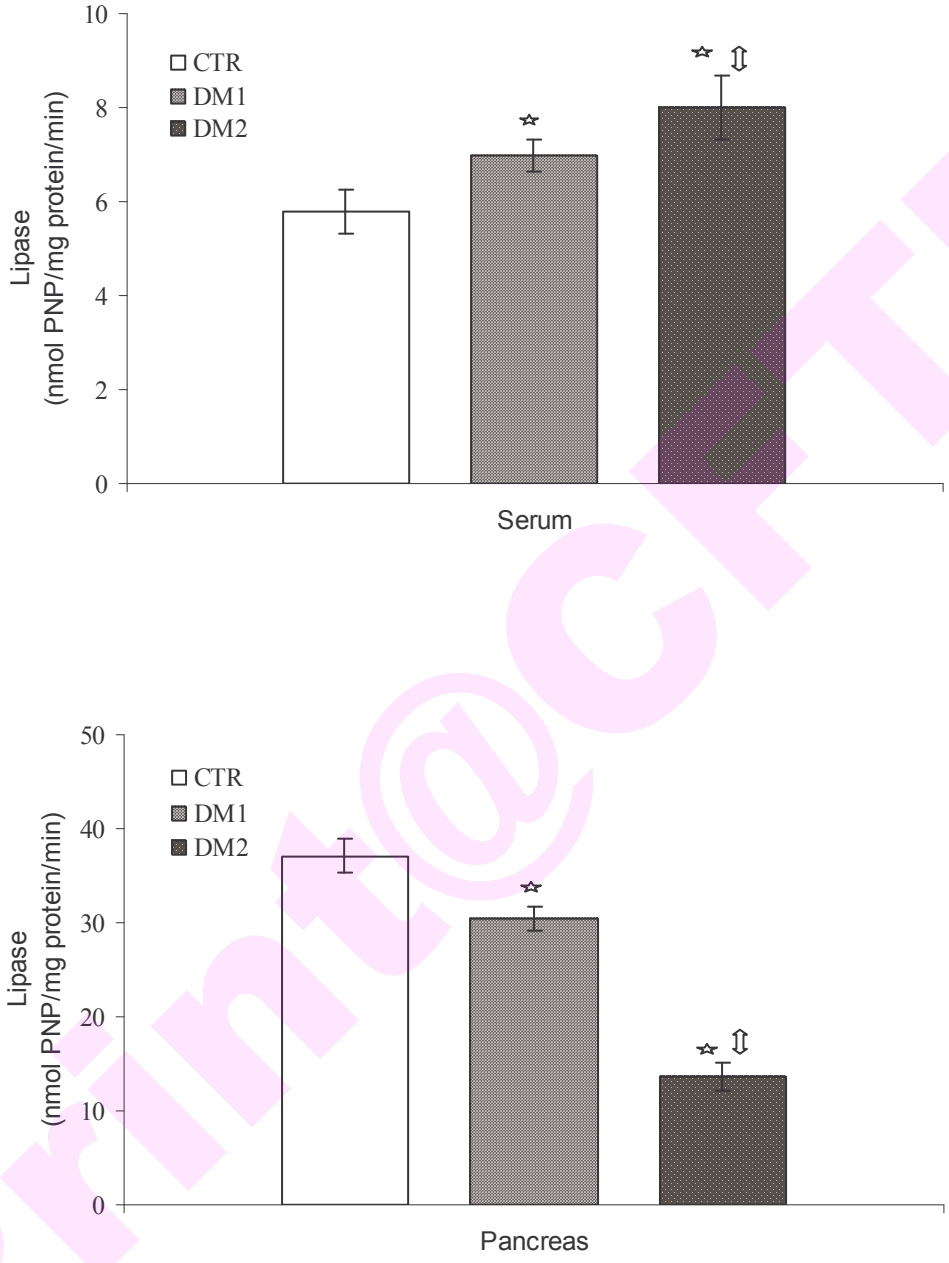


Fig 2.3 Activities of lipase in serum and pancreas of control (CTR) and DM treated rats (DM1: 20 mg/kg b.w/d; DM2: 40 mg/kg b.w/d)

Values are mean ± SEM (n=6)
* Comparison of control and other groups ($P < 0.01$),
⇳ Comparison of DM1 with DM2 ($P < 0.01$)

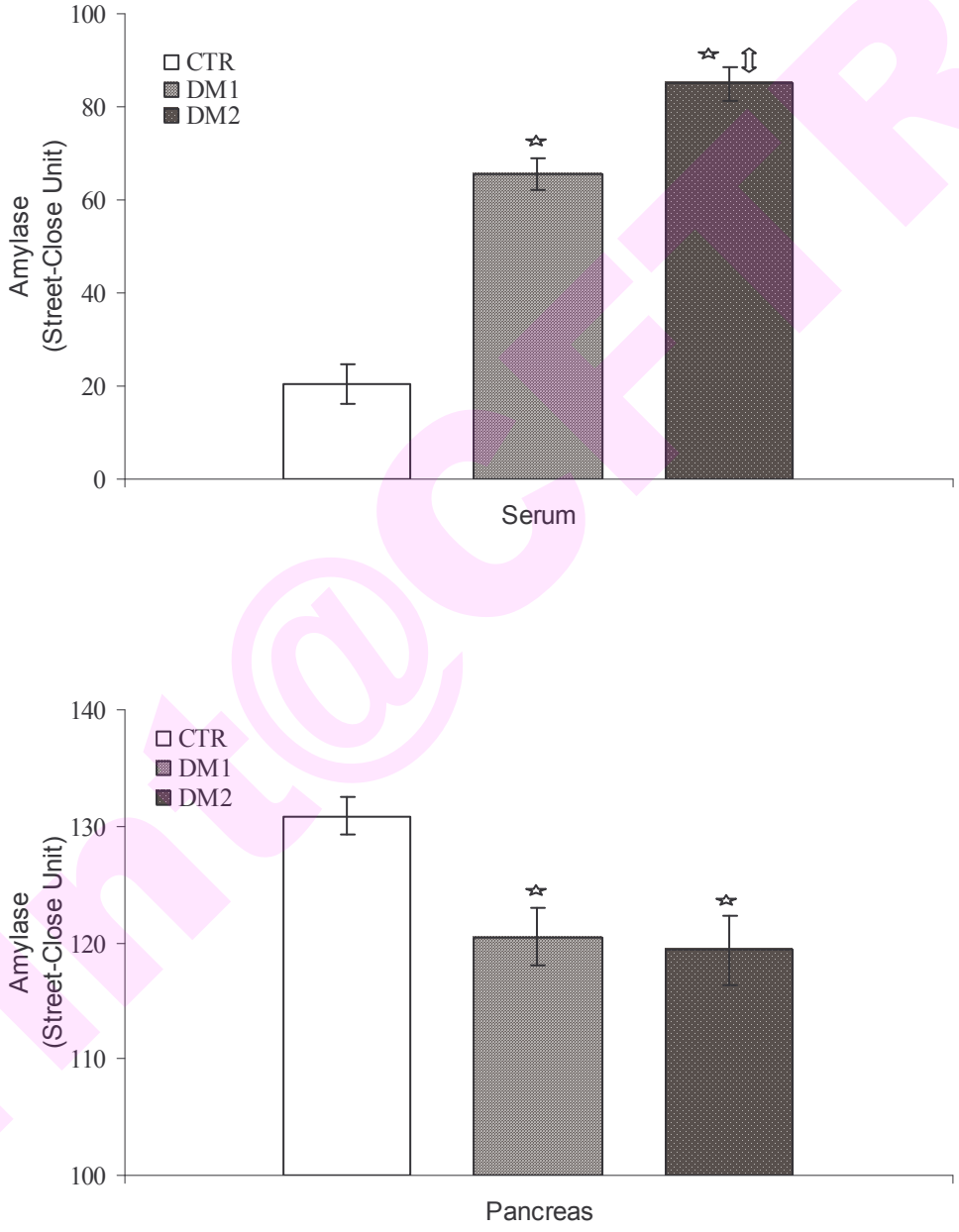


Fig. 2.4 Activities of amylase in serum and pancreas of control (CTR) and DM treated rats (DM1: 20 mg/kg b.w/d; DM2: 40 mg/kg b.w/d)

Values are mean ± SEM (n=6)
* Comparison of control and other groups ($P < 0.01$),
⇳ Comparison of DM1 with DM2 ($P < 0.01$)

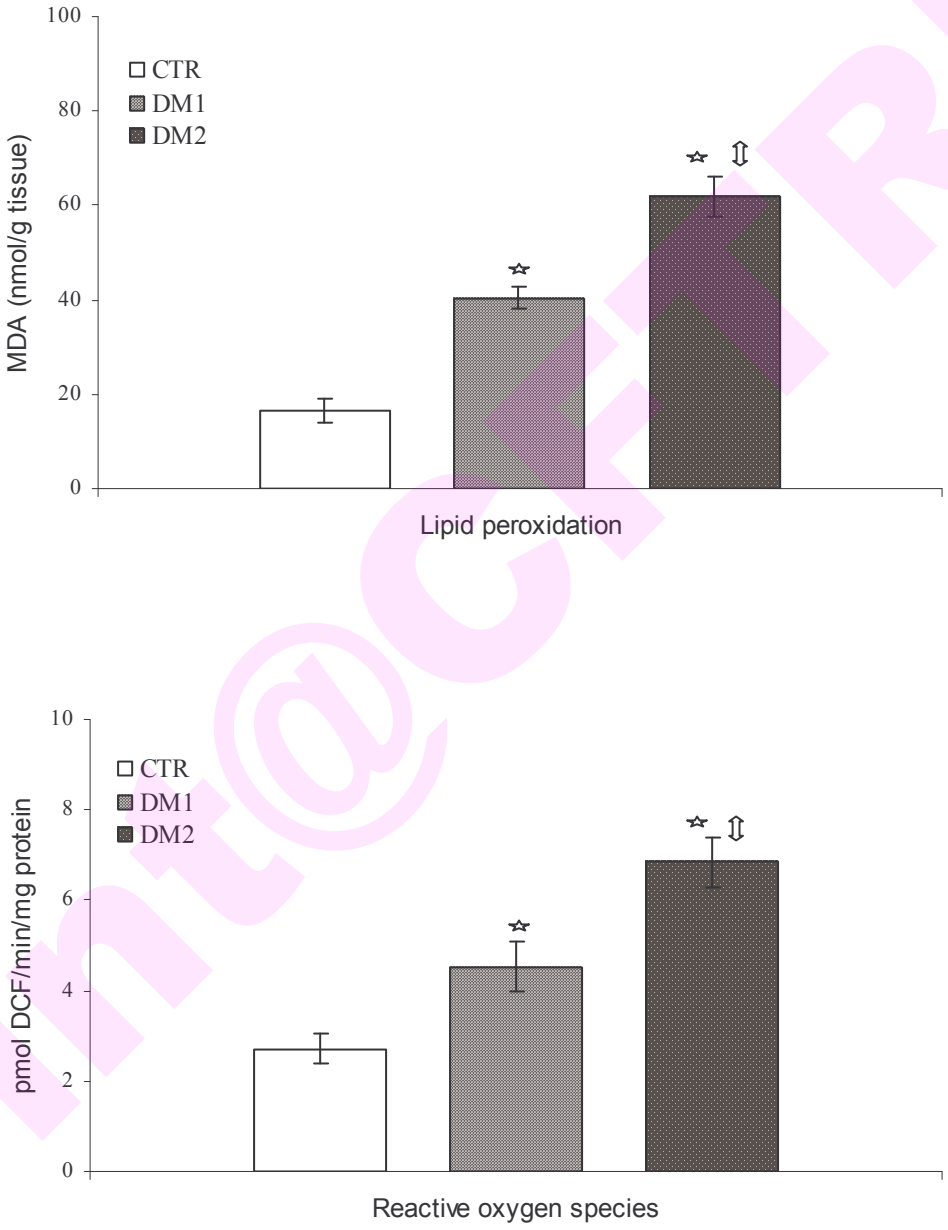


Fig. 2.5 ROS levels and extent of LPO in pancreas of control (CTR) and DM treated rats (DM1: 20 mg/kg b.w/d; DM2: 40 mg/kg b.w/d)

Values are mean ± SEM (n=6);
* Comparison of control and other groups ($P < 0.01$),
⇳ Comparison of DM1 with DM2 ($P < 0.01$)

Table 2.3 Activities of antioxidant enzymes in pancreas of rats administered oral doses of DM for 30 days

Group	Enzyme Activity				
	SOD[1]	CAT[2]	GPX[3]	GR[3]	GST[4]
CTR	26.42 ± 2.2	9.38 ± 0.31	27.18 ± 5.24	17.50 ± 1.60	0.03 ± 0.004
DM1	42.72 ± 0.38[a]	10.24 ± 0.32	25.23 ± 3.89	19.72 ± 2.03	0.04 ± 0.003[a]
DM2	56.23 ± 1.18[a,b]	15.44 ± 0.51[a,b]	13.85 ± 2.20[a.b]	25.30 ± 1.30[a,b]	0.06 ± 0.003[a,b]

[1]units/mg protein; [2]µmol/min/mg protein; [3]nmol/ min/ mg protein; [4]µmol/ min / mg protein
Values are mean ± SEM (n=6)
[a] Comparison of control (CTR) and other groups;
[b] Comparison of DM1 (DM: 20mg /kg b.w/d) group with DM2 (DM: 40 mg/kg b.w/d) group

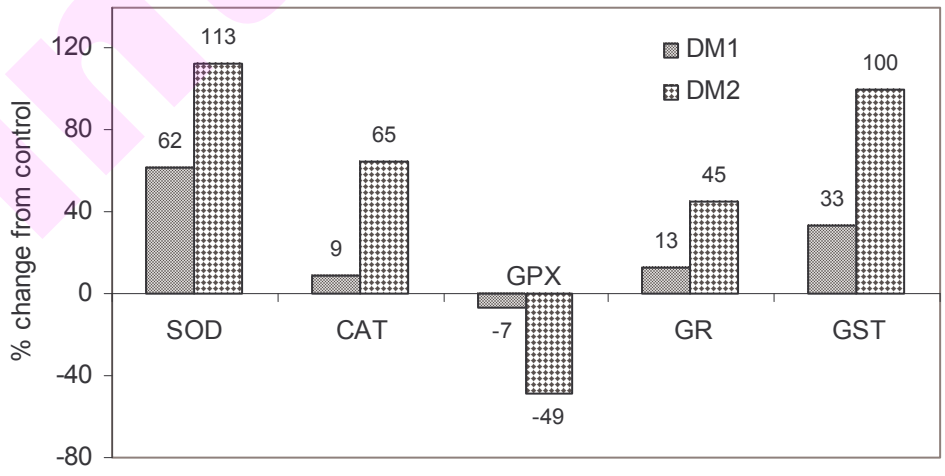


Fig. 2.6 Activities of antioxidant enzymes in pancreas of rats administered oral doses of DM (DM1: 20 mg /kg b.w/d; DM2: 40mg/kg b.w/d) for 30 days

Table 2.4 Activities of Phase II enzymes in pancreas of rats administered oral doses of DM for 30 days

Group	Enzyme Activity	
	NADPH-D[1]	DT-Diaphorase[2]
CTR	8.78 ± 0.27	0.62 ± 0.05
DM1	9.12 ± 0.37	0.60 ± 0.02
DM2	11.17 ± 0.42[a,b]	0.72 ± 0.02[a,b]

[1]nmoles of NADPH utilized/min /mg protein; [2]µmoles of NADH utilized/min/mg protein
Values are mean ± SEM (n=6)
[a] Comparison of control (CTR) and other groups
[b] Comparison of DM1 (DM: 20mg /kg b.w/d) group with DM2 (DM: 40 mg/kg b.w/d) group

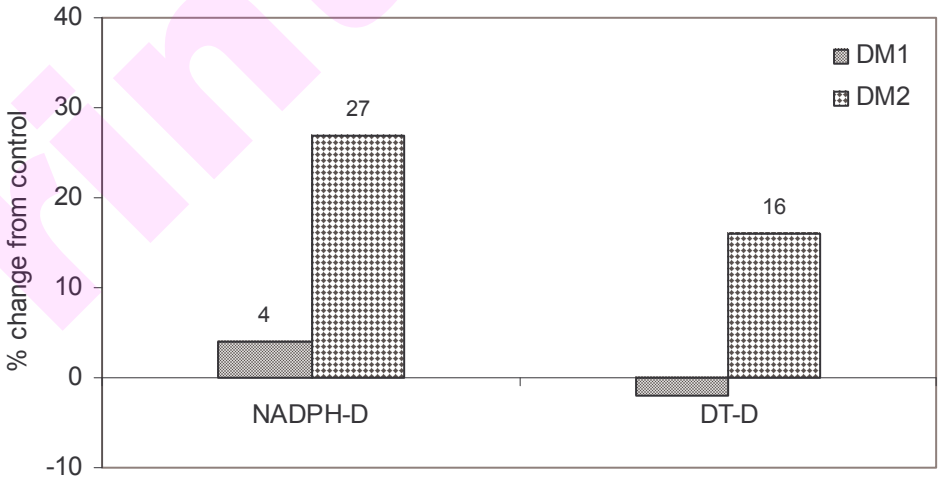


Fig. 2.7 Activities of Phase II enzymes in pancreas of rats administered oral doses of DM (DM1: 20 mg /kg b.w/d; DM2: 40mg/kg b.w/d) for 30 days

5.2 Biochemical perturbations induced in rat pancreas by repeated oral doses of DDVP

5.2.1 Body and organ weights

Repeated oral doses of DDVP failed to induce any distinctive clinical signs and symptoms of toxicity or mortality. There was no significant change in the body weights among control and DDVP treated rats. The relative weights of the vital organs of DDVP treated rats were in the same range as that in control rats (data not shown).

5.2.2 Oral glucose tolerance

The blood glucose levels monitored in control and DDVP treated rats following glucose overload over 3h period is presented in **Fig.2.8**. Fasting blood glucose levels in DDVP treated rats were significantly higher (108 mg/dl) compared to that of control rats (75 mg/dl). DDVP treated rats showed higher blood glucose level at all the measured time points. Blood glucose levels in control rats returned to normal level (99 mg/dl) at the end of 3h suggesting normal glucose tolerance. On the other hand, DDVP treated rats showed higher levels of glucose (133 mg/dl) at the end of 3h indicating altered blood glucose tolerance.

5.2.3 Blood glucose

Data on the blood glucose levels in control and DDVP treated rats is presented in **Table 2.5.** Blood glucose level in DDVP treated rats was elevated significantly (75%) at the end of 10 days.

5.2.4 Pancreatic damage

The activity of enzymes viz., lipase and amylase in serum and pancreas of rats following DDVP intoxication are presented in **Table 2.6 & Fig. 2.9.** There was a 35 % increase in serum lipase activity and 12 % increase in serum amylase activity in rats administered DDVP. However, pancreatic lipase activity

was significantly reduced (26%), while pancreatic amylase activity was markedly elevated (47%) in rats administered DDVP. While GGT activity in serum was markedly higher (86%), serum LDH activity was elevated by 45% indicating the generalized tissue damage due to DDVP administration **(Table 2.7 & Fig. 2.10)**. Similarly, pancreatic GGT activity was elevated by 58% while pancreatic LDH activity was reduced significantly (31%) in DDVP treated rats.

5.2.5 ChE activity in tissues

Acetylcholinesterase enzyme activity in various vital organs of rats administered DDVP is presented in **Table 2.8 & Fig. 2.11** and BuChE activity is presented in **Table 2.9 & Fig. 2.12.** AChE activity was reduced in all the vital tissues to varying extent due to DDVP administration while both AChE and BuChE were equally and significantly reduced (30%) in pancreas. Adrenal AChE and BuChE activity were decreased by 27% while liver AChE was not affected and BuChE (34%) was reduced. Serum pseudocholinesterase activity in rats administered DDVP were 35 % and 42% lower than the control values.

5.2.6 Oxidative damage in pancreas

The levels of ROS and LPO in tissues of rats of various groups are presented in **Table 2.10 & Fig. 2.13**. ROS generation was significantly increased in all the vital organs such as pancreas (51%), adrenals (61%), liver (22%) and brain (37%). A significant increase in the levels of TBARS was also evident in pancreas (38%), adrenals (64%), liver (23%) and brain (50%).

5.2.7 Non-enzymic antioxidant levels in tissues

The levels of reduced glutathione were marginally decreased in adrenals (13%), liver (15%) and brain (16%) while, the levels were increased marginally in pancreas. Oxidized glutathione levels were decreased in pancreas (20%), adrenals (30%) and liver (31%) but increased in brain (70 %) of rats administered DDVP. Protein carbonyl levels in all the organs were increased

significantly with pancreas showing 28% increase over control **(Table 2.11 & Fig. 2.14).**

5.2.8 Antioxidant enzymes in tissues

Activities of various antioxidant enzymes in tissues of rats subjected to repeated oral doses of DDVP are presented in **Table 2.12 & 2.13; Fig. 2.15 & 2.16.** The enzymes activities were elevated in tissues of rats treated with DDVP except in brain where the activities of GPX and GST were decreased. In pancreas, increase in the enzyme activities over control were as follows: catalase (44%), SOD (76%), GR (30%), GPX (68%) and GST (27%); in adrenals: catalase (105%) and SOD (28%); in liver: GR (50%) and GPX (50%). In brain, the activities of SOD and GR were significantly increased while, the activity of GPX was significantly reduced compared to that in control rats.

5.2.9 Phase I enzymes in liver and pancreas

Activities of Phase I enzymes in pancreas and liver of rats administered DDVP along with control values are presented in **Table 2.14 & Fig. 2.17**. In liver, cyrochromeB5 reductase activity was elevated marginally (9%) in rats administered DDVP, and there was marginal decrease (13 %) in cytochromeP450 reductase activity. Similarly in pancreas, the activity of cytochromeB5 reductase was increased marginally (12%) while activity of cytochrome P450 reductase enzyme was decreased (22%) in treated rats.

5.2.10 Phase II enzymes in liver and pancreas

DT-diaphorase and NADPH-diaphorase activity were reduced by 16 and 18% respectively in liver of DDVP administered rats while, in pancreas, activity of DT-diaphorase was increased significantly (41%), and the activity of NADPH-diaphorase was decreased (33 %) **(Table 2.15 & Fig. 2.18).**

5.2.11 Glycogen and carbohydrate metabolizing enzymes in liver

Liver glycogen and carbohydrate metabolizing enzymes in rats subjected to DDVP intoxication are presented in **Table 2.16 & Fig. 2.19**. Liver glycogen was reduced significantly (40%) due to DDVP administration. Activities of glycogen phosphorylase and glucose-6-phosphatase enzymes were significantly increased (by 100 % and 87% respectively) while a 50 % reduction in the activity of liver glucokinase was evident in liver of DDVP treated rats.

5.2.12 Glucokinase and Glutamate dehydrogenase activities in pancreas

Pancreatic glucokinase and glutamate dehydrogenase enzyme activities in control and DDVP administered rats are presented in **Table 2.17 & Fig. 2.20**. Both the enzyme activities were reduced significantly (by 52% and 37%) in pancreas of DDVP treated rats.

5.2.13 Histopathology of pancreas

The islets in the pancreas of control rats **(Fig. 2.21 A and C)** appeared to be mostly intact while, most of the islet tissue in pancreas of DDVP-treated rats **(Fig. 2.21 B and D)** was atrophied. Pancreatic histology of DDVP administered rats revealed packed cells with mixed inflammatory cells and areas of hemorrhage, suggestive of acute inflammatory lesion.

Table 2.5 Blood glucose levels in rats administered oral doses of DDVP (20 mg/ kg b.w/d for 10 d)

	Blood glucose (mg/dl)	
	Initial	Final
Control	95.33 ± 3.85	100.14 ± 9.06
DDVP	87.34 ± 5.23	150.85[a] ± 5.83

Values are mean ± S.E.M. of six rats in each group
Values significantly different from controls by Student's *t* Test are indicated:
[a] P≤ 0.01

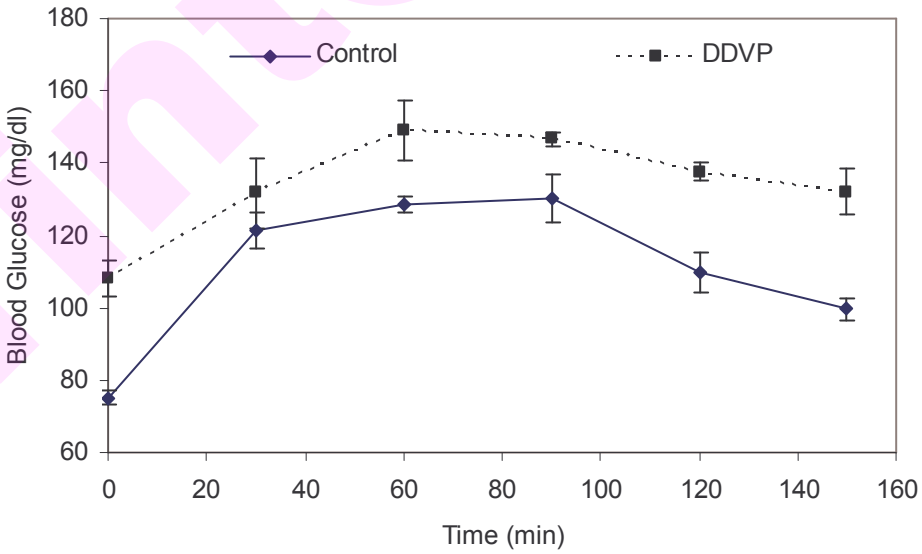


Fig. 2.8 Oral glucose tolerance at the end of 10 days in control (CTR) and DDVP (20 mg/kg b.w./d) treated rats

Values are mean ± SEM (n=6)

Table 2.6 Activities of lipase and amylase in serum and pancreas of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	Lipase[1]		Amylase[2]	
	Serum	Pancreas	Serum	Pancreas
Control	35.13 ± 2.92	397.14 ± 35.38	10.24 ± 2.47	75.79 ± 6.61
DDVP	47.32 ± 2.77[a]	292.54 ± 15.04[a]	11.47 ± 7.85[a]	111.70 ± 13.95[b]

[1]nmol PNP /min/ mg protein; [2]Street Close Unit
Values are mean ± SEM (n=6) ; Values significantly different from controls by Student's *t* Test are indicated: [a] P≤ 0.05; [b] P≤ 0.01

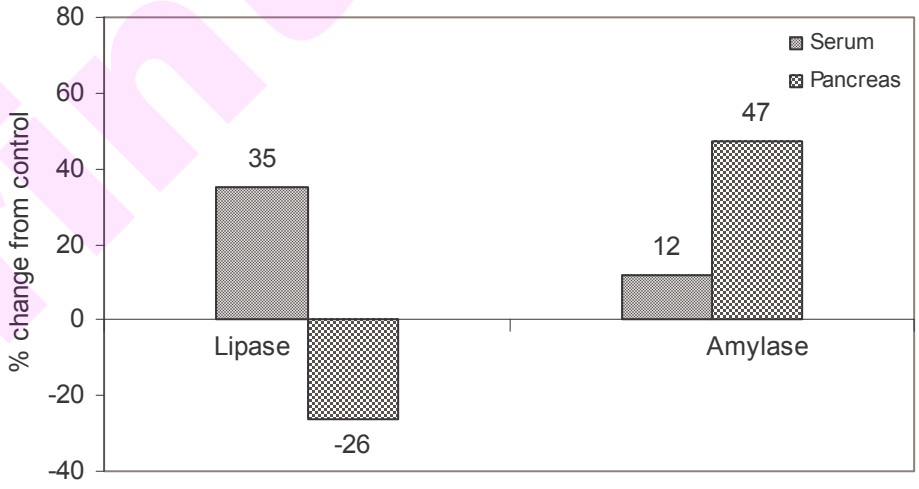


Fig. 2.9 Activities of lipase and amylase enzymes in serum and pancreas of control and DDVP (20 mg/kg b.w/d for 10 d) treated rats

Table 2.7 Activities of γ-glutamyl transpeptidase (GGT) and lactate dehydrogenase (LDH) in serum and pancreas of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	GGT[1]		LDH[2]	
	Serum	Pancreas	Serum	Pancreas
Control	49.49 ± 7.07	938.89 ± 146.91	24.00 ± 2.92	165.00 ± 10.58
DDVP	91.91 ±14.14[a]	1480.46 ± 98.08[a]	34.83 ±0.90[b]	113.66 ± 1.85[b]

[1]IU/L; [2]µmol /min/mg protein
Values are mean ± SEM (n=6); Values significantly different from controls by Student's *t* Test are indicated: [a] P≤ 0.05; [b] P≤ 0.01

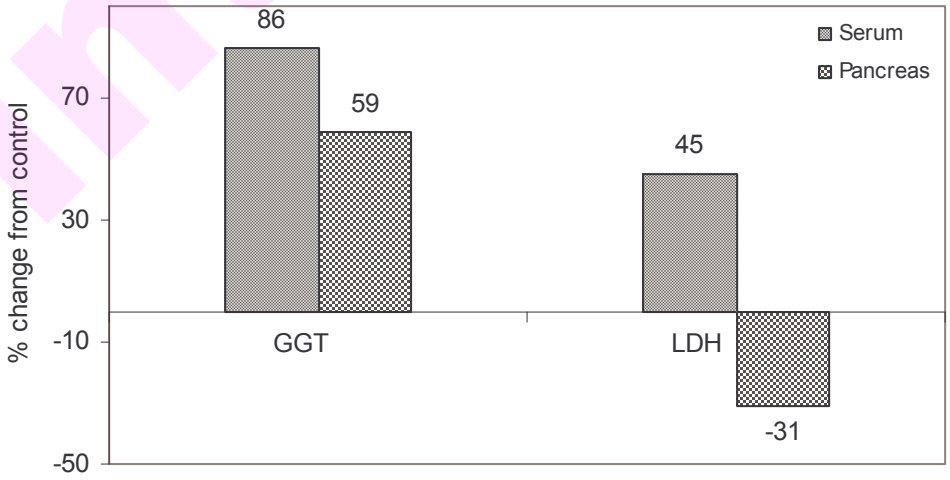


Fig. 2.10 Activities of GGT and LDH in serum and pancreas of control and DDVP (20 mg/kg b.w/d for 10 d) treated rats

Table 2.8 Acetylcholinesterase (AChE) activity in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	AChE (nmol substrate hydrolyzed /min/mg protein)				
	Pancreas	Adrenals	Liver	Brain	Serum
Control	9.77 ± 1.17	12.39 ± 0.55	7.07 ± 0.27	64.19 ± 9.39	8.95 ± 0.19
DDVP	6.80 ± 0.47[a]	8.92 ± 0.68[c]	6.58 ± 0.28	42.71 ± 0.82[a]	5.85 ± 0.49[a]

Values are mean ± SEM (n=6)
Values significantly different from control by Student's *t* Test are indicated:
[a] P≤ 0.05; [c] P≤ 0.001

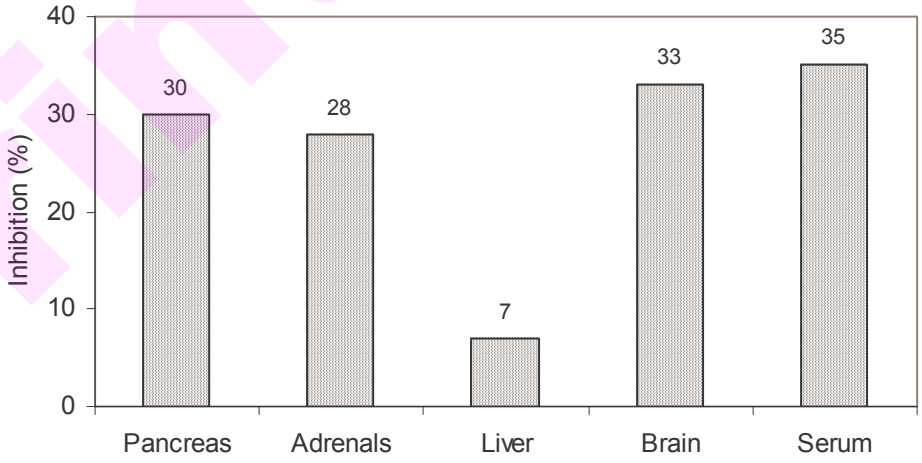


Fig. 2.11 AChE activity in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.9 Butyrylcholinesterase (BuChE) activity in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	BuChE (nmol substrate hydrolyzed /min/mg protein)				
	Adrenal	Pancreas	Liver	Brain	Serum
Control	5.01 ± 0.09	5.25 ± 0.33	3.99 ± 0.37	4.78 ± 0.19	0.72 ± 0.04
DDVP	3.66 ± 0.20[a]	3.82 ± 0.12[a]	2.65 ± 0.38	3.31 ± 0.13[c]	0.42 ± 0.03[b]

Values are mean ± SEM (n=6)
Values significantly different from controls by Student's *t* Test are indicated: [a] P≤ 0.05; [b] P≤ 0.01; [c] P≤ 0.001

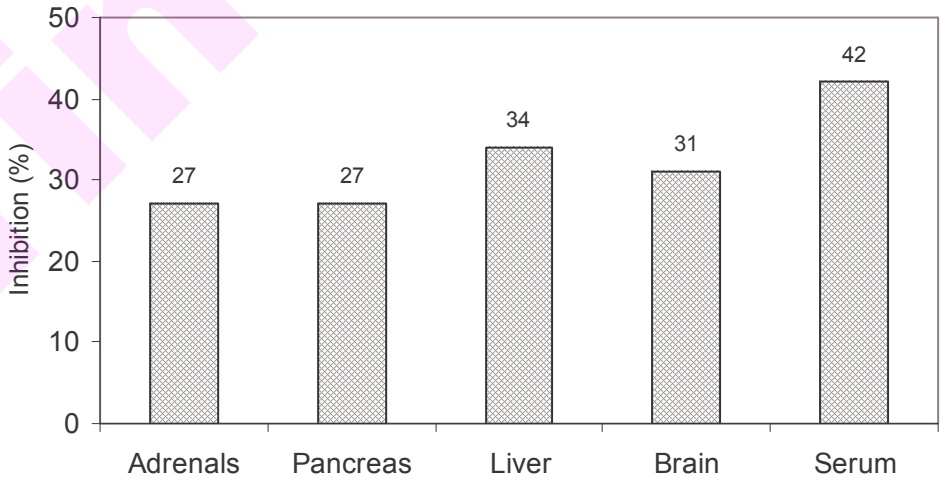


Fig. 2.12 BuChE activity in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.10 ROS and TBARS levels in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	ROS[1]		TBARS[2]	
	Control	DDVP	Control	DDVP
Pancreas	57.50 ± 0.05	86.63 ± 5.72[a]	213.90 ± 11.03	294.26 ± 4.08[a]
Adrenals	99.76 ± 8.15	160.51 ± 5.70[b]	1028.03 ± 63.11	1689.36 ± 279.10[a]
Liver	81.46 ± 3.62	99.66 ± 4.39[c]	189.67 ± 21.57	232.78 ± 17.25[a]
Brain	220.17 ± 8.03	302.53 ± 22.98[a]	292.51 ± 28.42	437.46 ± 51.59[a]

[1]pmol DCF/min/mg protein; [2]nmol MDA/g tissue
Values are mean ± SEM (n=6)
Values significantly different from controls by Student's *t* Test are indicated:
[a] P≤ 0.05; [b] P≤ 0.01; [c] P≤ 0.001

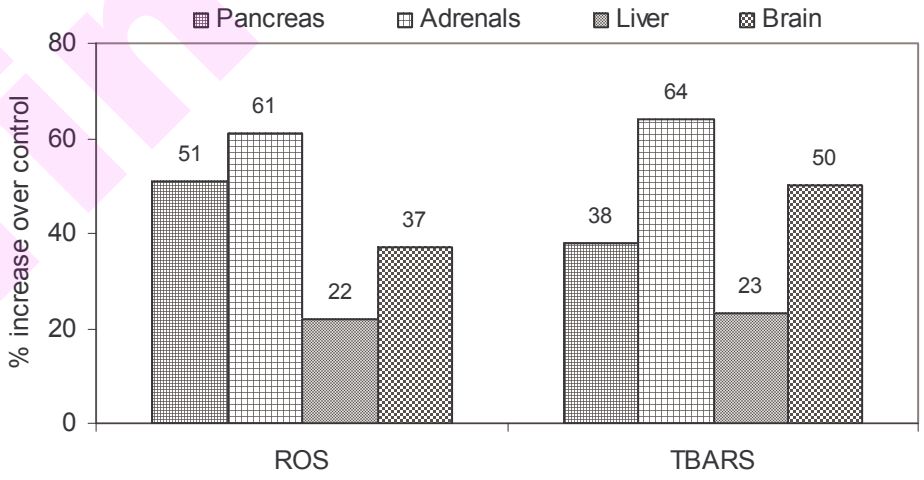


Fig 2.13 ROS and TBARS levels in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.11 GSH, GSSG and protein carbonyl (PC) levels in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	GSH[1]		GSSG[2]		PC[3]	
	Control	DDVP	Control	DDVP	Control	DDVP
Pancreas	1.01 ± 0.01	1.12 ± 0.01[b]	716.71 ± 52.20	574.93 ± 6.60[a]	5.68 ± 0.64	7.29 ± 0.08[a]
Adrenals	1.38 ± 0.07	1.20 ± 0.01	9267.81 ± 589.77	6486.05 ± 156.07[a]	30.77 ± 2.16	52.97 ± 10.05
Liver	1.55 ± 0.04	1.33 ± 0.06[a]	2180.7 ± 308.36	1503.95 ± 204.92	3.86 ± 0.31	5.09 ± 0.09[a]
Brain	1.78 ± 0.11	1.50 ± 0.05[a]	1993.88 ± 343.22	3465.95 ± 271.35[b]	1.35 ± 0.11	1.67 ± 0.72[b]

[1]mg/g tissue; [2]pmol/mg tissue; [3]nmol/mg protein
Values are mean ± SEM (n=6); Values significantly different from controls by Student's *t* Test are indicated: [a] P≤ 0.05; [b] P≤ 0.01

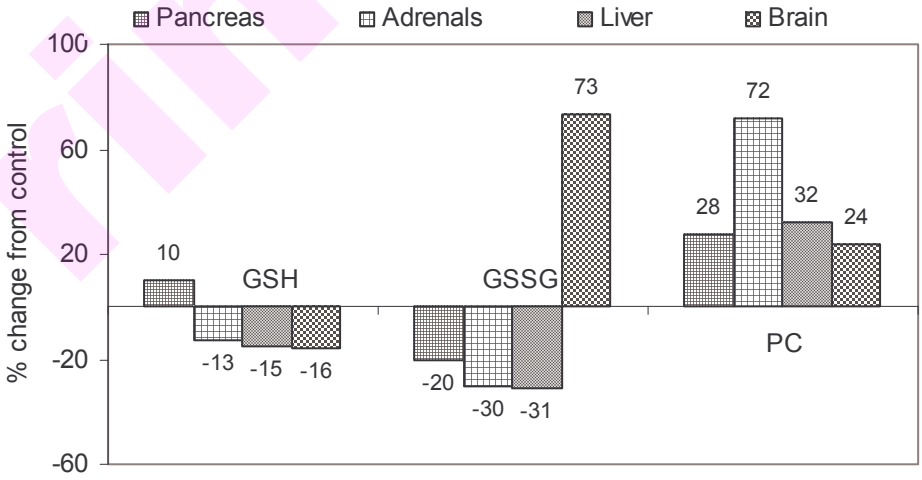


Fig 2.14 GSH, GSSG and PC levels in tissues of rats administered oral dose of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.12 Activities of catalase (CAT) and superoxide dismutase (SOD) in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	CAT[1]		SOD[2]	
	Control	DDVP	Control	DDVP
Pancreas	2.76 ± 0.26	3.97 ± 0.05[a]	13.69 ± 2.06	24.03 ± 3.97[a]
Adrenals	15.47 ± 0.88	31.78 ± 7.94[b]	58.71 ± 1.88	75.09 ± 2.09[b]
Liver	522.56 ± 23.49	661.60 ± 92.33	57.58 ± 4.13	80.33 ± 12.48
Brain	0.32 ± 0.017	0.40 ± 0.048	52.67 ± 4.38	68.86 ± 6.51[a]

[1]µmol/min/mg protein; [2]units/mg protein
Values are mean ± SEM (n=6); Values significantly different from controls by Student's *t* Test are indicated: [a] P≤ 0.05; [b] P≤ 0.01

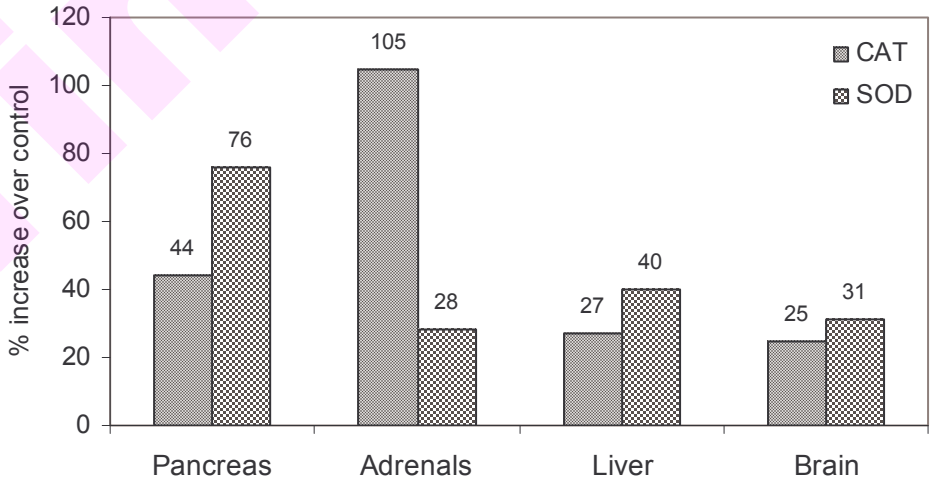


Fig. 2.15 Activities of CAT and SOD in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.13 Activities of glutathione-dependent antioxidant enzymes in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	GR[1]		GPX[1]		GST[2]	
	Control	DDVP	Control	DDVP	Control	DDVP
Pancreas	23.59 ± 1.06	30.66 ± 1.33[a]	32.15 ± 1.99	54.58 ± 5.63[b]	0.059 ± 0.01	0.075 ± 0.01[b]
Adrenals	30.24 ± 1.91	33.38 ± 1.24	179.53 ± 29.47	209.01 ± 19.32	0.47 ± 0.06	0.72 ± 0.08 [b]
Liver	58.48 ± 1.99	87.71 ± 3.66[b]	132.70 ± 10.9	198.31 ± 18.08[b]	1.65 ± 0.09	2.04 ± 0.14 [a]
Brain	22.09 ± 0.64	27.25 ± 1.68[b]	80.39 ± 9.28	46.00 ± 6.76[b]	0.14 ± 0.02	0.10 ± 0.01[a]

[1]nmol/min/mg protein; [2]µmol/min/mg protein
Values are mean ± SEM (n=6)
Values significantly different from controls by Student's *t* Test are indicated: [a] P≤ 0.05; [b] P≤ 0.01

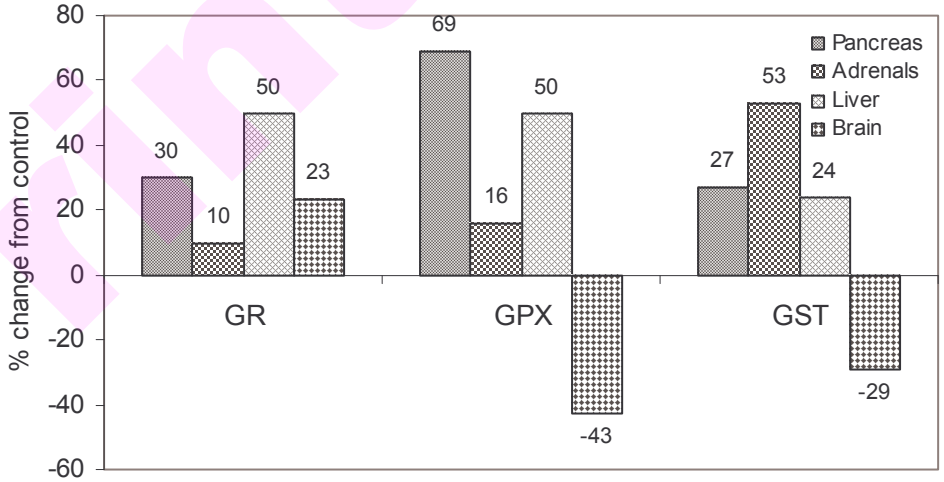


Fig. 2.16 Activities of glutathione-dependent antioxidant enzymes in tissues of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.14 Activities of Phase I enzymes in pancreas and liver of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	µmol/min/mg protein			
	Cytochrome B_5 Reductase		Cytochrome P_{450} Reductase	
	Control	DDVP	Control	DDVP
Pancreas	500.33 ± 30.44	560.00 ± 36.47	11.80 ± 0.70	9.24 ± 0.63[a]
Liver	1508.33 ± 59.06	1649.00 ± 7.57	163.78 ± 5.58	142.67 ± 5.27[a]

Values are mean ± SEM (n=6)
Values significantly different from controls by Student's *t* Test are indicated:[a] P≤ 0.05

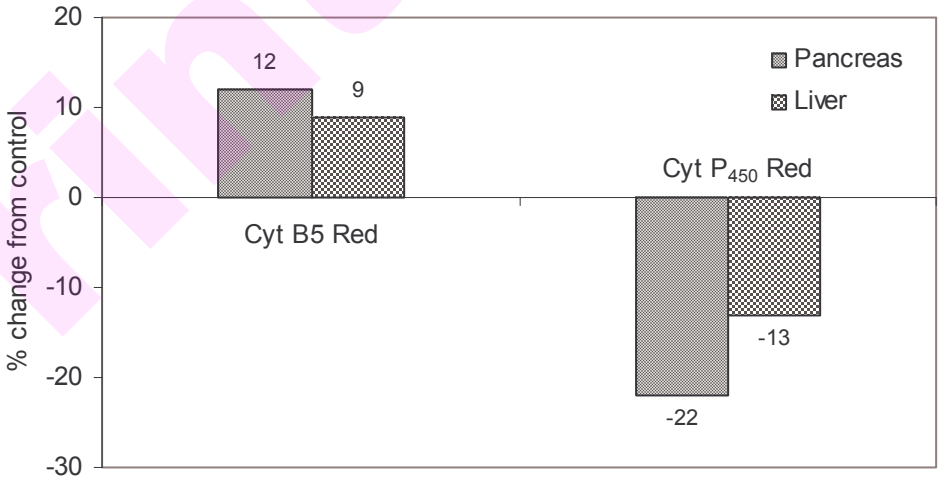


Fig. 2.17 Activities of Phase I enzymes in pancreas and liver of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.15 Activities of Phase II enzymes in pancreas and liver of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	DT-Diaphorase[1]		NADPH-Diaphorase[2]	
	Control	DDVP	Control	DDVP
Pancreas	9.34 ± 0.99	13.16 ± 0.93[a]	13.51 ± 1.87	9.07 ± 0.35[a]
Liver	115.14 ± 2.54	96.46 ± 5.99[a]	128.89 ± 9.42	106.19 ± 5.25[a]

[1]µmol NADH utilized/min/mg protein; [2] nmol NADPH utilized/min/mg protein
Values are mean ± SEM (n=6)
Values significantly different from controls by Student's *t* Test are indicated:[a] P≤ 0.05

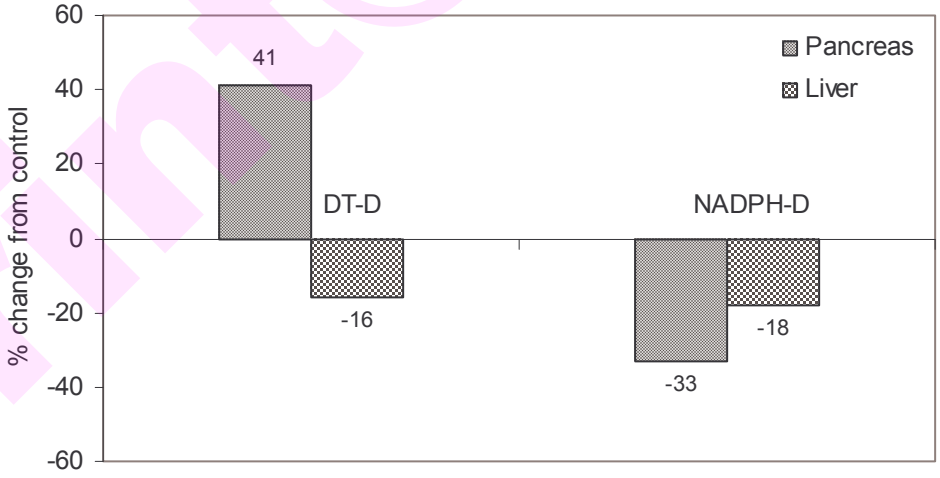


Fig. 2.18 Activities of Phase II enzymes in pancreas and liver of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.16 Glycogen content and carbohydrate metabolizing enzyme activities in liver of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	Glycogen*	Glycogen phosphorylase** (GP)	Glucose-6-phosphatase** (G-6-P)	Glucokinase#
Control	63.42 ± 2.21	10.56 ± 0.54	872.00 ± 130.00	71.90 ± 4.75
DDVP	37.95 ± 7.94[a]	20.09 ± 3.15[b]	1631.00 ± 283.00[a]	35.64 ± 6.10[a]

* mg /g tissue; ** nmol iP/min/mg protein; # µmol/min/mg protein
Values are mean ± SEM (n=6)
Values significantly different from controls by Student's *t* Test are indicated: [a] P≤ 0.05; [b] P≤ 0.01

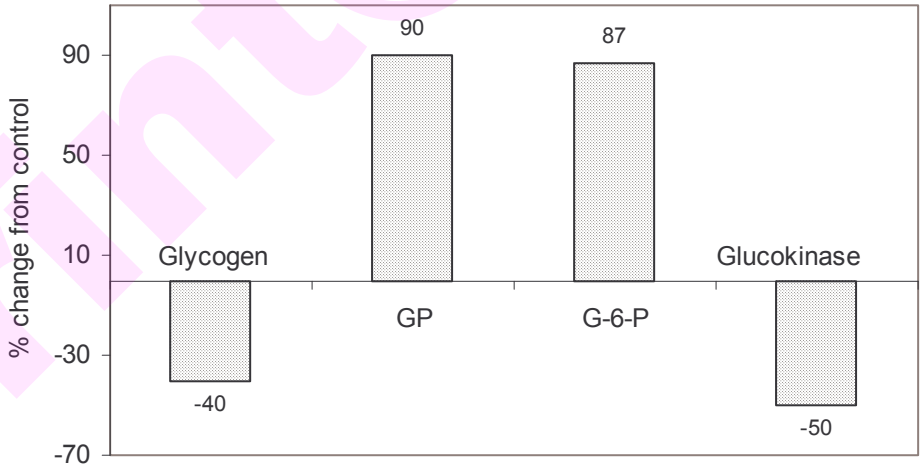


Fig. 2.19 Glycogen content and carbohydrate metabolizing enzyme activities in liver of rats administered oral dose of DDVP (20 mg/kg b.w/d for 10 d)

Table 2.17 Glucokinase and glutamate dehydrogenase enzyme activity in pancreas of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

	Glucokinase*	Glutamate dehydrogenase**
Control	46.61 ± 5.13	27.98 ± 3.35
DDVP	21.98 ± 3.97[c]	17.53 ± 1.29[a]

* µmol/min/mg protein; ** mmol/min/mg protein
Values are mean ± SEM (n=6)
Values significantly different from controls by Student's *t* Test are indicated:
[a] P≤ 0.05; [c] P≤ 0.001

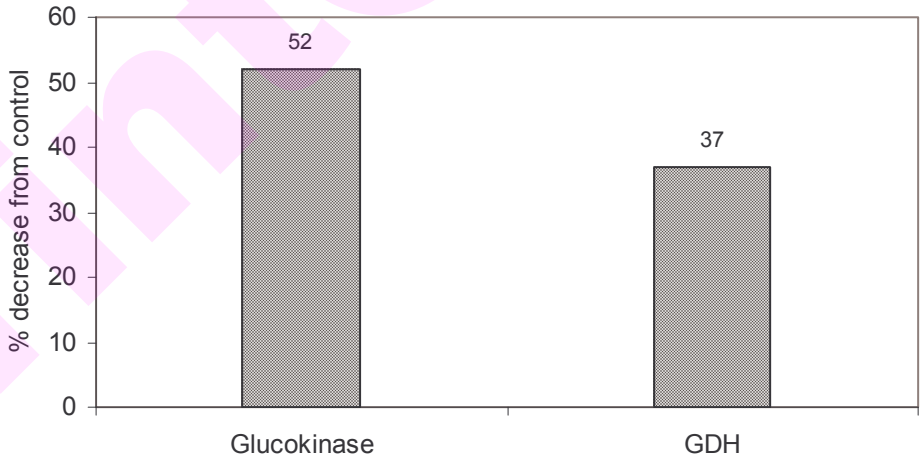


Fig 2.20 Glucokinase and glutamate dehydrogenase activity in pancreas of rats administered oral doses of DDVP (20 mg/kg b.w/d for 10 d)

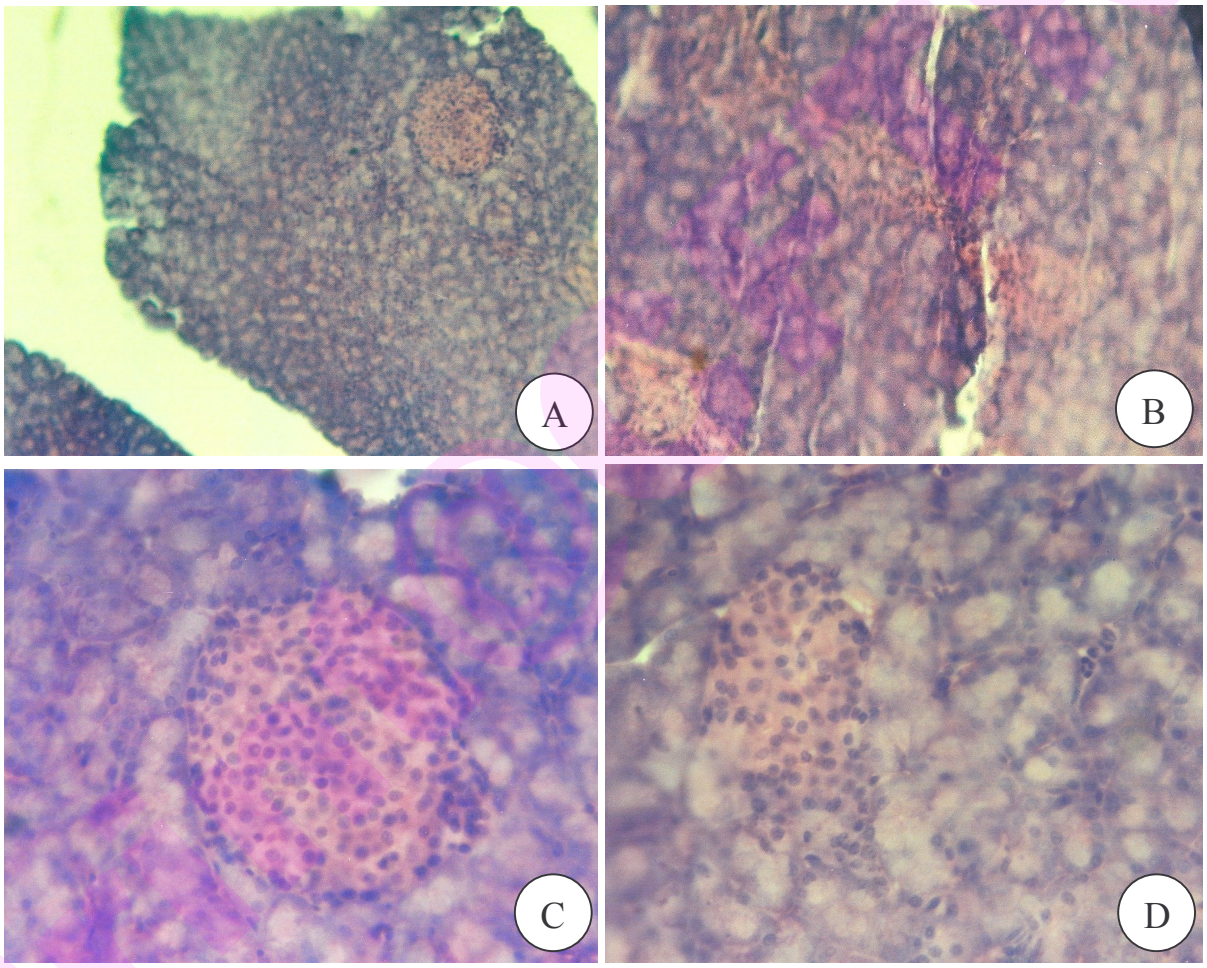


Fig 2.21. Hematoxylin-eosin stained sections of pancreas of - control rats showing normal islets with clusters of purple stained β-cells (A & C) ; DDVP-treated (20 mg/kg b.w/d for 10 d) rats showing irregular morphology of islets with degenerative changes and infiltration (B & D); (A & B: 10X; C & D: 40X)

6.0 DISCUSSION

The mechanisms involved in blood glucose alterations following OPI exposure have been under investigation in recent years since hyperglycemia is one of the prominent side effects of OPI poisoning in humans (Namba et al., 1971; Hayes et al., 1978; Meller et al., 1981). The ability of OPI to cause hyperglycemia has also been demonstrated in laboratory animals (Matin and Siddiqui, 1982; Fletcher et al., 1988). OPI are known to impair both, endocrinal and biochemical functions of pancreas (Sikk et al., 1985; Hsiao et al., 1996). Accordingly, the present study addressed the potential of repeated doses of dimethoate (DM) and dichlorvos (DDVP) to induce perturbations in glucose homeostasis and also examined whether oxidative stress plays a role in these OPI-induced pancreatic dysfunctions.

Our results showed that oral administration of DM at both the dosages for one month induced significant elevation in blood glucose levels in rats. Further, we observed significant alteration in oral glucose tolerance among DM-treated rats. These data are in agreement with the earlier reports, which have shown elevated blood glucose levels after administration of DM (Hagar et al., 2002). Further, we observed increased blood glucose levels in rats administered repeated oral doses of DDVP for 10 days accompanied with altered glucose tolerance at the end of the treatment period. Earlier, hyperglycemic effect has been reported in experimental animals following both acute and chronic OPI exposures (Begum and Vijayaraghavan, 1999; Sarin and Gill, 1999; Abdollahi et al., 2004a; Pournourmohammadi et al., 2005).

Hyperglycemia has been suggested to occur as a consequence of increased accumulation of acetylcholine (ACh) at the nerve endings in pancreas subsequent to AChE inhibition (Gallo and Lawryk, 1991). In the present study, we observed significant dose-dependent AChE inhibition in pancreatic tissue following administration of DM, which is indicative of OPI intoxication. Administration of DDVP for 10 days resulted in inhibition of AChE in both

pancreas and brain to an equal extent. The possible pathogenetic sequel speculated for the pancreatic insult in OP poisoning is excessive cholinergic stimulation of the pancreas and ductal hypertension (Hsiao et al., 1996, Sahin et al., 2002). Pancreas is a sensitive organ and hence pressure elevation can cause severe tissue damage.

In the present study, repeated oral doses of DDVP significantly inhibited BuChE in pancreas. It is known that butyrylcholinesterase enzyme is more abundant in pancreatic tissue (Sahin et al., 2002; Harputluoglu et al., 2003). Although the real substrate(s) is still unknown, BuChE can hydrolyze hydrophobic and hydrophilic carboxylic or phosphoric acid ester containing compounds. Butyrylcholinesterase (pseudocholinesterase or nonspecific cholinesterase) has no known physiological function but it can be considered as an endogenous scavenger of anticholinesterase compounds. BuChE detoxifies them before they reach to AChE at physiologically important target sites (Cokugras, 2003). Our results indicate the possible extent of protection offered by BuChE against inhibition of AChE in pancreas.

Liver plays a major role in blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose as glycogen and the release of glucose via glycogenolysis and gluconeogenesis (Hers, 1990; Nordlie et al., 1999; Abdollahi et al., 2003). Liver glycogenolysis and gluconeogenesis might be stimulated, at least to some extent, by the detoxification mechanisms, essential for metabolism or degradation and elimination of the pesticides from the body (Begum and Vijayaraghavan, 1999). Acute intraperitoneal treatment of rats with diazinon has been reported to result in depletion of brain glycogen and increase the activities of glycogen phosphorylase (GP) (Matin and Husain, 1987a, b). Treatment of rats with acute diazinon also resulted in hyperglycemia, depletion of glycogen from brain and peripheral tissues accompanied by increased activity of GP in the brain and liver, and increased activity of the hepatic gluconeogenic enzymes, phophoenolpyruvate carboxykinase (PEPCK)

(Matin et al., 1990). Sublethal single dose of dimethoate an OP insecticide has been shown to increase the hepatic and muscle GP activity of Indian catfish (*Clarias batrachus;* Begum and Vijayaraghavan 1995, 1999). In another study, fish exposed to sublethal doses of malathion showed muscle glycogenolysis with concomitant hyperglycemia (Mishra and Srivastava, 1983).

Chronic exposure to Dichlorvos is also reported to cause a significant depletion in brain glycogen content accompanied with an increase in the activity of GP (Sarin and Gill, 2000). Dichlorvos exposure has also been reported to decrease liver glycogen content and affect glucose homeostasis (Omkar and Shukla, 1985; Verma et al., 1983). Single dose of DDVP (50% LD_{50}) has been found to increase the activity of glycogen phosphorylase, while decreasing UDP-glucose pyrophosphorylase activity (Teichert-Kuliszenwska et al., 1981). Recent study of Abdollahi et al., (2004a) reported stimulatory effect of liver GP in rats exposed to Malathion. One possible explanation for stimulated GP and increased glycogenolysis could be the release of catecholamines from adrenal medulla. Therefore one mechanism suggested for increased activities of liver GP in response to OPI exposure might be the induction of metabolic processes to meet the augmented stress conditions, which could finally result in hyperglycemia and diabetes.

Hepatic glucokinase plays an important role in glycogen synthesis (Ferre et al., 1996). Increased glucokinase activity has a potent enhancing effect on glycogen synthesis (Seoane et al., 1996; O'Doherty et al., 1996). In contrast, impaired hepatic glycogen synthesis is observed in glucokinase deficiency (Postic et al., 1999). Stimulatory effect of malathion on activities of GK and GDH was reported by Panahi et al., (2006). In fact, it has been proposed that the glycolysis and gluconeogenesis are stimulated to counteract increased oxidative stress conditions caused by OPI (Begum and Vijayaraghavan, 1999; Abdollahi et al., 2004b). In the present study, DDVP treatment induced a reduction in the activity of both hepatic and pancreatic

glucokinase. Pancreatic beta cells also play a major role in maintaining glucose homeostasis through secretion of insulin in response to changes in the extracellular glucose. Glucose –stimulated insulin secretion is regulated by the rate of glucose metabolism within beta cells (Megalsson and Matschinsky, 1986; Newgard and McGarry, 1995) and a key event in this process is the phosphorylation of glucose by glucokinase (GK). GK, also referred as hexokinase IV, is a member of the mammalian hexokinase (HK) family that catalyses the initial step in glucose metabolism by the most metabolic pathways (e.g. glyocolysis, pentose phosphate cycle and glycogen synthesis) (Wilson, 1995). In beta cells, glucokinase plays a key role in regulating insulin secretion in response to glucose (Meglasson and Matschinsky, 1986; Matshinsky, 1996).

In the present study, DM administration significantly increased the activity of lipase in serum. This finding is suggestive of the potential of DM to cause acute pancreatitis, since the increase in lipase activity by two-fold is specific for the diagnosis for pancreatitis. Increased serum lipase activity has also been reported after administration of methidathion, an OPI (Mollaoglu et al., 2003). Several studies have demonstrated acute pancreatitis after oral exposure to various OPI (Dressel et al., 1979, Hsiao et al., 1996, Moore and James, 1988). Our results corroborate with earlier reports of acute pancreatitis in humans after accidental cutaneous exposure to DM (Marsh et al., 1988). Another biochemical marker, often employed to evaluate pancreatitis, is the increased amylase activity. DM and DDVP treatment elevated serum amylase activity supporting the evidence of pancreatic damage. Similar increase in amylase activity has been reported in dogs after diazinon administration (Dressel et al., 1982).

Our histopathological evidences confirmed the pancreatic damage induced by DDVP. The degeneration of the acini observed in the section of pancreas of DDVP treated rats might be considered as a toxic inflammatory process inducing pancreatitis. Chronic dimethoate administration has been

shown to induce degenerative changes in pancreatic islets (Hagar et al., 2002), while several other reports have also suggested that acute pancreatitis may follow oral ingestion of OPI such as mevinphos, malathion, parathion and diazinon (Hsiao et al., 1996; Moore and James, 1989; Dressel et al., 1979).

Another major question addressed in the present investigation was whether the OPI-induced pancreatic dysfunction is mediated through oxidative stress. Active oxygen metabolism plays a vital role in the normal functioning of the pancreas. Several key enzymes in reactive oxygen species (ROS) defense are usually low in pancreatic islets compared to other tissues suggesting that islet cells are uniquely susceptible to oxidative stress-induced damage (Grankvist et al., 1981, Kakkar et al., 1998). Further, the low levels of antioxidant enzyme gene expression may account for exquisite sensitivity of β-cell to ROS and free radical induced damage leading to β-cell death and type-1 diabetes (Ho and Bray, 1999).Oxidative stress has been implicated to be an important component in the toxicity of several OPI (Yamano and Morita, 1993; Bagchi et al., 1995; Yamano and Morita, 1995; Yang and Dettbarn, 1996). In the present study, we obtained a dose-dependent increase in levels of ROS and TBARS in pancreas due to both DM and DDVP treatment. These findings suggest that exposures to both DM and DDVP induce significant oxidative stress in the pancreas thereby leading to its dysfunction.

We also observed increased activities of serum and pancreatic GGT and LDH in DDVP treated rats. γ-glutamyltranspeptidase, a key enzyme in GSH metabolism provides high intracellular levels of GSH required for conjugation by GST (Stark et al., 2002). In the current investigation, the observed increase in the activity of GGT both in serum and pancreas may be due to the tissue response to cope up with the oxidative stress. The biological significance of GGT-dependent LPO *in vivo* might be multifold. It is conceivable that the prooxidant effects of GGT activity are normally balanced by its established role in favouring the cellular uptake of precursors for GSH re-synthesis, thus

allowing the reconstitution of cellular antioxidant defence. We also observed increased activity of LDH in serum and pancreas of rats administered DDVP. LDH is a pivotal cellular metabolic enzyme between the glycolytic pathway and carboxylic acid cycle (Lehninger, 1984). Lactate dehydrogenase is an oligomeric enzyme and cytoplasmic marker enzyme, which is well known indicator of damage by xenobiotic compounds (Reddy and Lokesh, 1996).

Pesticides that induce free radical generation may also oxidatively modify cellular proteins. Stadtman (1992) reported that some amino acid residues are oxidized to carbonyl derivatives and consequently, the carbonyl content of proteins is being employed as a measure of protein damage. In our study, DDVP significantly elevated the levels of PC in pancreas. This correlated with the pattern of OS response seen in pancreas.

Depletion of GSH, the most abundant cellular non-protein thiol, is associated with oxidative stress and cytotoxicity of pro-oxidant xenobiotic. Glutathione is presumed to be an important endogenous defence against the peroxidative destruction of cellular membranes. GSH can act either to detoxify activated oxygen species such as H_2O_2 or to reduce lipid peroxides themselves (Freeman et al., 1982). Tissue GSH concentration reflects the potential for detoxification and the levels may decrease due to an increased use of glutathione to detoxify. Enhanced status of lipid peroxidation and concomitant depletion of GSH pools in pancreas provides further evidence for the occurrence of the oxidative stress in DM treated rats. Interestingly, we did not observe any alteration in GSH levels in pancreas of DDVP treated rats. This could probably due to the shorter treatment regime or suggestive of lesser oxidative stress in pancreas.

Cytosolic free radicals are removed either non-enzymatically or by antioxidant enzymes such as SOD and GPX, which oxidize GSH to GSSG. GSSG is then reduced back to GSH by GR through oxidation of NADPH to NADP+, which is recycled by the pentose phosphate pathway. Furthermore,

glutathione S-transferase (GST) catalyzes the conjugation of GSH to OPI, leading to its detoxification and elimination (Ziegler, 1985). The level of GSSG was significantly reduced in pancreas of DDVP treated rats.

The results of the present study revealed that levels of all the antioxidant components namely, SOD, CAT, GPX and GR, which take care of ROS generated *in vivo* were enhanced by DM and DDVP. Thus, the superoxide generated is accounted for by the enhanced SOD and converted to H_2O_2, which in turn is converted to H_2O by catalase or GPX. The GSSG generated thereby is again reduced by GR, thus replenishing the depleted pool of GSH. This may be the reason that the level of GSH remained unchanged following DDVP treatment. Our results showed increased activity of SOD and CAT in the pancreas of DM as well as DDVP treated rats. Superoxide dismutase is a ubiquitous chain breaking antioxidant and is found in all aerobic organisms. It is a metalloprotein widely distributed in all cells and plays a protective role against ROS-induced oxidative damage. The increased activity of SOD in DM and DDVP treated rats probably indicates an activation of the compensatory mechanism through the effects of DM on progenitor cells. Pancreas also has certain amount of CAT activity and the elevated activity of CAT in DM and DDVP treated rats may be due to an adaptive response to the generated free radicals (Koner et al., 1997). These findings suggest that a state of oxidative stress is induced in pancreas of DM and DDVP treated rats.

We also observed a decrease in the activity of GPX and increase in the activity of GR in pancreas of DM treated rats while, pancreas of DDVP treated rats showed increase in activity of both GPX and GR. Knowing that GR is the enzyme responsible for providing reduced GSH from its oxidized for (GSSG) and that GGT activity is important for the re-synthesis of GSH inside the cell, it is not surprising, therefore, to detect an increase in GR activity as a compensatory mechanism for replenishing the GSH concentration inside the cell. GPX converts H_2O_2 or other lipid peroxides to water or hydroxy lipids, and

during this process GSH is converted to GSSG. To recycle GSSG, the cell utilizes the enzyme NADPH-dependent GSH reductase, the NADPH being supplied to the reaction by glucose-6-phosphate dehydrogenase (Bachowski et al., 1997).

Many environmental chemicals that are not chemically reactive are metabolically activated by phase I and phase II xenobiotic-metabolizing enzymes in order to exert their effects. In normal cells, the reactive intermediates formed by phase I reactions are conjugated with glucuronides, sulfate or glutathione, facilitating their excretion. The conjugation reactions are catalyzed by phase II biotransformation enzymes including glutathione-s-transferase, DT-diaphorase, sulfotransferases and UDP-glucuronyl transferases (Shweita and Timisany, 2003). While Phase I enzymes increase the toxicity of a chemical, Phase II enzymes serve to detoxify the electrophilic metabolites. Imbalances in phase I and Phase II metabolizing enzymes has been reported in various disease conditions (Pelkonen et al., 1999; Subapriya et al., 2005; Williams and Phillips, 2000).

Cytochrome P450, a phase I enzyme catalyses the oxidation of lipophilic chemicals via C- or N-hydroxylation to electrophilic ultimate carcinogens. The catalytic efficiency of cytochrome P450 is enhanced by the ubiquitous electron transport protein cytochrome b5. The reactive metabolites generated by phase I enzymes interact with DNA, including mutations that can initiate oncogene transformation (Modugno et al., 2003; Williams and Philips, 2000). In the present study, DDVP treatment resulted in decreased activity of cytochrome P450 reductase. Pancreatitis represents a manifestation of uncoordinated detoxification reactions between pancreatic cytochrome P450 monooxygenases and phase II conjugating enzymes, resulting in the irreversible consumption of glutathione in the acinar cell (Walling, 1998).

Activities of the GSH-dependent phase II detoxifying enzymes in pancreas, GR and GST were also significantly increased in DM and DDVP-

treated rats. Among the phase II enzymes, GSTs are a family of enzymes that catalyzes the conjugation of reactive chemicals with GSH and play a major role in protecting cells. After generating conjugated GSH, these are subsequently eliminated via a GSH conjugated-recognizing transporter (Nakamura et al., 2000). The inducers of GST were suggested to induce isoforms of GST, which specifically detoxify products of free radical damage (Fiander and Schneider, 2000). The increase in GST activity in the pancreatic tissue among DM and DDVP treated rats indicate the role played by this system in detoxification of these OPI.

Interestingly, we found increase in activity of DT-diaphorase in pancreas of both DM and DDVP treated rats. DT-diaphorase and NADPH-diaphorase are generally induced in coordination with GST and GGT that are involved in protecting cells against toxins and carcinogens. While DT-diaphorase, a flavoprotein facilitates the 2-electron reduction of quinones, NADPH diaphorase catalyzes hydrogen transfer from NADPH (Begleiter et al., 1997; Huennekens et al., 1957). Phase II enzymes, such as GST, and DT-diaphorase, are considered to be a major mechanism of protection against chemical stress (Gerhauser et al., 1997). They are a class of widely distributed enzymes that detoxify toxins either by destroying their reactive centers or by conjugating them with endogenous ligands, facilitating their excretion. DT-diaphorase is a flavoprotein that catalyses the two electron reduction of quinines to hydroquinones and nitrogen oxide. This reaction prevents the formation of semiquinones by one electron reduction and in turn the generation of free radicals from the autooxidation of semiquinone. Reduction of quinones and nitrogen oxide might also make them available for conjugation with UDP-glucuronic acid, facilitating their excretion (Begleiter et al., 1997; Talalay et al., 1995).

In conclusion, our results indicate that both DM and DDVP have the propensity to significantly alter glucose homeostasis and the associated

oxidative impairments observed in pancreas may wholly or in part contribute towards the development of pancreatitis in adult rats. Our studies also demonstrate the impairment in carbohydrate metabolism in rats due to OPI exposures.

7.0 SUMMARY

1. Administration of multiple oral doses of DM (20 and 40 mg/kg b.w/d for 30 d) caused significant elevation (15 and 51%) in blood glucose levels in rats.
2. DM treated rats subjected to oral glucose tolerance test at the end of 30 d showed significantly higher blood glucose levels (135 mg/dl) at 60 min and beyond which remained high (125 and 129 mg/dl) up to 3 h suggesting altered glucose tolerance.
3. A marked increase (32%) in pancreatic weight was evident in DM treated rats only at the higher dose.
4. DM treated rats showed significant elevation in serum lipase (20 and 38%) and amylase (2-3 folds) activities which were accompanied with marginal decrease in lipase (18 and 63%) and significant decrease in amylase activity in pancreas.
5. Dose-related elevation in ROS (66% and 150%) and lipid peroxide levels were accompanied by marginal decrease in the reduced GSH levels in DM treated rats.
6. At the high dose, increased activities of antioxidant enzymes such as SOD, CAT, GR and GST and altered activities of xenobiotic metabolizing enzymes viz., NADPH-diaphorase (27% increase) and DT-diaphorase were evident.
7. Marked reduction (40 and 91 %) in the activity of AChE was also demonstrable in pancreas of DM treated rats.
8. Administration of repeated oral doses of DDVP (20 mg/kg b.w /d for 10 d) induced significantly higher blood glucose levels in rats.
9. Following glucose overload, DDVP treated rats showed higher glucose levels (149mg/dl) at 60 min and beyond indicating altered glucose tolerance.

10. DDVP also induced increases in serum lipase (35 %), serum amylase (12%) and pancreatic amylase (47%) activities, while it significantly reduced pancreatic lipase activity (26%).
11. Serum lactate dehydrogenase and pancreatic γ-glutamyltranspeptidase activities were elevated (45 and 59%) while, pancreatic and adrenal AChE activity was significantly reduced (by 31%) in DDVP treated rats.
12. Enhanced ROS levels and lipid peroxidation were accompanied by depleted glutathione levels in pancreas and other vital organs in DDVP treated rats, concomitant with elevated activities of pancreatic antioxidant enzymes such as catalase (44%), SOD (76%), GR (30%), GPX (68%) and GST (27%).
13. However, varying degrees of increase in the activities of DT-diaphorase (41%) and cytochrome B_5 reductase (12%) and decrease in the activities of NADPH-diaphorase (33%) and cytochrome P_{450} reductase (22%) were evident in pancreas of DDVP treated rats.
14. Carbohydrate metabolism was markedly affected in rats administered DDVP as evidenced by significantly reduced liver glycogen (40%), increased activities of glycogen phosphorylase and glucose-6-phosphatase enzymes and marked reduction (50%) in liver glucokinase activity.
15. Pancreatic glucokinase and glutamate dehydrogenase enzyme activities in DDVP treated rats were also reduced significantly (by 52% and 37%).
16. Further, histology of pancreas of DDVP administered rats revealed atrophied islets, packed cells with mixed inflammatory cells and areas of hemorrhage, suggestive of acute inflammatory lesion.

SECTION B

IMPACT OF DDVP PRE-TREATMENT ON HYPERGLYCEMIA AND BIOCHEMICAL ALTERATIONS IN STREPTOZOTOCIN TREATED RATS

1.0 INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin. Although the etiology of this disease is not well defined, viral infection, autoimmune disease, and environmental factors have been implicated (Kataoka et al., 1983; Paik et al., 1982; Sandler et al., 2000; Shewade et al., 2001). Further, increased oxidative stress has been a widely accepted participant in the development and progression of diabetes and its complications (Ceriello, 2000; Baynes and Thorpe, 1999; Baynes, 1991) since diabetes is usually accompanied by increased production of free radicals (Baynes and Thorpe, 1999; Baynes, 1991; Chang et al., 1993) or impaired antioxidant defenses (Halliwell and Gutteridge,1990; Saxena et al., 1993).

Diabetes is rapidly emerging as a major health problem in the world (Zimmet et al., 2001; Onkamo et al., 1999) and its prevalence is rising due to the westernized life style, which includes excessive energy intake and physical inactivity (Zimmet et al., 2001). However, several drugs (Ferner, 1992) and environmental factors also contribute towards the development of diabetes (Ferner, 1992; Yoon et al., 1987; Department of Veterans Affairs, 2001). The increasing use of organophosphate pesticides with their adverse effects on glucose metabolism may probably be an additional factor in the speedy expansion of diabetes in the world.

Little information is currently available regarding the toxicity of environmental chemicals in the presence of diabetes. It is also not well known if exposure to certain pesticides will worsen the complications of diabetes.

Retrospective studies have only begun to examine the interaction of environmental agents and diabetes. It has been reported that the metabolic rate and inactivation of drugs are altered in diabetes, especially in type I (Dixon et al., 1961; Thummel and Schenkman, 1990). Based on these findings, diabetic patients may be more susceptible to pesticide toxicity due to changes in the degree of their action, detoxification or elimination or vice versa.

The purpose of the present study was to investigate the effect of DDVP on the expression of diabetic condition in rats using sub-diabetic dose of streptozotocin.

2.0 MATERIALS AND METHODS

Presented under 2.1 to 2.4 of *Section A* of this Chapter.

3.0 EXPERIMENTAL PROCEDURE

3.1 Animal treatment and experimental protocol

Growing adult male rats were grouped by randomized design into two groups (n = 12). Rats of the first group (control) received saline daily for 10 d, while rats (non-fasted) of the second group were orally administered daily (0900 to 1100 hours) DDVP at 20mg/kg b.w/d (corresponding to 1/5 of LD_{50} value: 100 mg/kg b.w, determined in a preliminary study) for 10 d. After 10 days, rats of the control group were further divided into two sub groups of six animals each ; the first sub group served as control (‘untreated control’), while the second sub group of rats was intraperitoneally injected streptozotocin (STZ, 25 mg/kg b.w.) (‘STZ’). The group of rats administered with DDVP was also divided into two sub groups; the first sub group of rats served as DDVP control (‘DDVP’), while the second sub group of rats was injected with streptozotocin (i.p, 25mg/kg b.w.) (‘DDVP+STZ’). Following streptozotocin injection, the rats were given glucose water (5% w/v) for 48 h to prevent initial STZ-induced hypoglycemic mortality. Seven days after streptozotocin administration, all the

rats were sacrificed under mild diethyl ether anesthesia. Samples of blood were collected by cardiac puncture for serum, and pancreas were excised and stored at 4ºC until use. The biochemical assays included determination of blood glucose, liver glycogen, amylase and lipase activity and oxidative stress parameters in pancreas.

3.2 Preparation of tissue homogenates

10 or 20 % (w/v) homogenates of the tissues were prepared in various buffers as per the requirement of a given assay.

3.3 Serum

Blood was drawn by cardiac puncture into tubes and processed separately for obtaining serum as described earlier (*Section 3.5, Chapter II A*).

4.0 ASSAY METHODS

4.1 Blood glucose estimation

Blood glucose was estimated using glucometer as described earlier *(Section 4.1, Chapter I A).* Results were expressed as mg glucose/ dl blood. Mean of three such measurements are taken for every rat.

4.2 Glycogen *(Nicholas et al., 1956)*

Glycogen in liver was estimated as described earlier *(Section 4.22, Chapter II A).*

4.3 Reactive oxygen species (ROS) *(Keston and Brandt, 1965)*

ROS in tissue was estimated by DCFH oxidation method as described earlier *(Section 4.8, Chapter II A)*.

4.4 Lipid peroxidation *(Buege and Aust, 1978)*

Lipid peroxidation in pancreas was estimated by measuring the thiobarbituric acid reactive substances as described earlier *(Section 4.9, Chapter II A).*

4.5 GSH (reduced glutathione) *(Benke et al., 1974)*

Reduced glutathione in pancreas was estimated as described earlier *(Section 4.3, Chapter I A).*

4.6 Lipase (EC 3.1.1.3) *(Young et al., 1978)*

Lipase activity was estimated by monitoring the hydrolysis of p-nitrophenyl acetate to p-nitrophenol as described earlier *(Section 4.2, Chapter II A).*

4.7 Amylase (EC 3.2.1.1) *(Street and Close, 1956)*

The enzyme activity was measured using a commercial kit as described earlier *(Section 4.3, Chapter II A)*.

4.8 Protein estimation *(Lowry et al., 1951)*

Protein content of tissue homogenate and serum was estimated as described earlier *(Section 4.4, Chapter I B).*

4.9 Statistical analysis

Mean and standard error values were determined for all the parameters and the results were expressed as mean ± S.E from six rats in each group. The data were analyzed employing analysis of variance (ANOVA) using Statistica software (STATSOFT, USA). Duncan's multiple regression test for multiple comparisons was performed to determine the significant differences among the groups. '*p*' values < 0.01 were considered significant.

5.0 RESULTS

5.1 Body and organ weights

Rats administered a single dose of STZ, which were pre-treated with DDVP for 10 days showed no clinical signs of toxicity or mortality. Terminally, there were no significant alterations either in body weights among various treatment groups. The relative weights of vital organs were similar in rats of all four groups (data not shown).

5.2 Blood glucose and liver glycogen levels

Data on blood glucose and liver glycogen levels in rats treated with repeated doses of DDVP and further subjected to a single dose of STZ are given in **Table 2.18**. DDVP treated rats showed higher (22%) levels of blood glucose compared to normal control rats. As expected, rats injected with STZ alone also showed elevated (37%) level of blood glucose **(Fig. 2.22)**. However, blood glucose levels of DDVP pretreated rats administered STZ showed relatively higher (66%) blood glucose level compared to all the groups.

Liver glycogen levels were significantly lower in rats administered either DDVP (18%) or STZ (19%) alone while, rats administered DDVP followed by STZ revealed further lower levels of glycogen (46 %).

5.3 Oxidative stress in pancreas

Data on ROS, LPO and GSH levels among rats treated with repeated doses of DDVP and further subjected to a single dose of STZ are presented in **Table 2.19 & Fig. 2.23**. ROS levels were significantly elevated in STZ (40%) and DDVP (55%) groups compared to 'untreated control' group. However, ROS levels were markedly higher (81.23 ± 6.52 pmole DCF/min/mg protein) in 'DDVP+STZ' group of rats. Pancreas of rats administered with either DDVP or STZ alone showed marginally higher levels of the lipid peroxides compared to that in 'untreated controls' while, the levels of lipid peroxides generated in

pancreas of 'DDVP+STZ' rats showed significant increase (110%) compared to all other groups. Pancreatic reduced glutathione level in 'DDVP+STZ' rats was significantly lower (37%) while, rats administered with DDVP or STZ alone also had significantly lower levels of GSH, although to a lesser extent.

5.4 Pancreatic damage markers

Activities of pancreas specific enzymes such as lipase and amylase in serum and pancreas of rats of the various groups are shown in **Table 2.20.** STZ treatment alone did not alter serum and pancreatic lipase amylase activities. Both, serum and pancreatic lipase and amylase activities were significantly elevated in rats administered DDVP alone. Rats of 'DDVP+STZ' group showed significant increase in activities of both lipase and amylase in serum (30% and 83 % respectively) while in these rats the pancreatic lipase activity was decreased significantly (20 %) and pancreatic amylase activity was increased (35%).

Table 2.18 Blood glucose and liver glycogen levels in rats pretreated with oral doses of DDVP (20 mg/kg b.w/d for 10 d) followed by a single dose of STZ (i.p , 25 mg/kg b.w)

Group	Blood glucose[1]	Liver glycogen[2]
Untreated control	113.53[a] ± 2.31	41.55[c] ± 2.01
DDVP	138.37[b] ± 4.17	34.20[b] ± 1.42
STZ	155.03[c] ± 5.09	33.34[b] ± 2.23
DDVP+STZ	188.99[d] ± 4.44	22.62[a] ± 3.52

[1]mg/dl; [2]mg/g tissue
Values are mean ± SEM (n=6); Mean in the same column with different superscript differ significantly (*p<0.05*)

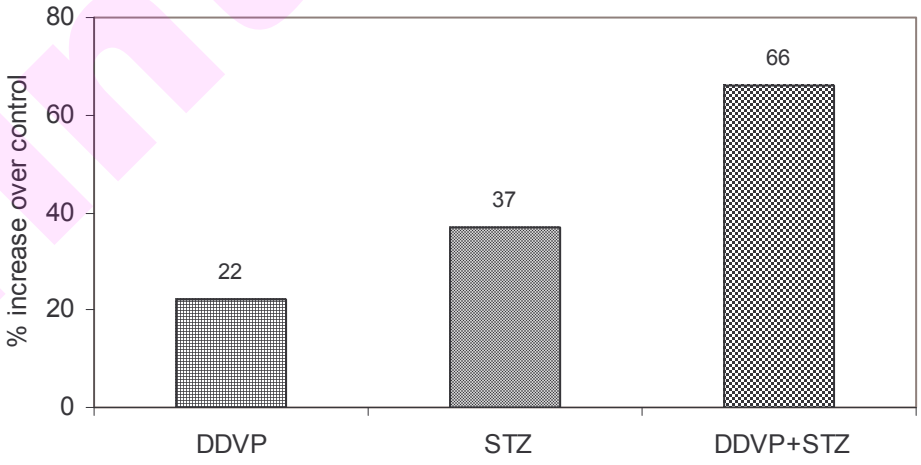


Fig. 2.22 Blood glucose levels in rats pretreated with oral doses of DDVP (20 mg/kg b.w/d for 10 d) followed by a single dose of STZ (i.p , 25 mg/kg b.w)

Table 2.19 ROS, TBARS and GSH levels in pancreas of rats pretreated with oral doses of DDVP (20 mg/kg b.w/d for 10 d) followed by a single dose of STZ (i.p , 25 mg/kg b.w)

Group	ROS[1]	TBARS[2]	GSH[3]
Untreated control	38.76[a] ± 4.04	242.76[a] ± 19.18	1.07[c] ± 0.03
DDVP	60.18[b] ± 4.59	294.94[a] ± 10.65	0.83[b] ± 0.01
STZ	54.27[b] ± 2.89	283.63[a] ± 7.27	0.78[b] ± 0.02
DDVP+STZ	81.23[c] ± 6.52	389.38[b] ± 38.47	0.67[a] ± 0.02

[1]pmol DCF/min/mg protein; [2]nmol/g tissue; [3]mg/g tissue
Values are mean ± SEM (n=6)
Mean in the same column with different superscript differ significantly (*p<0.05*)

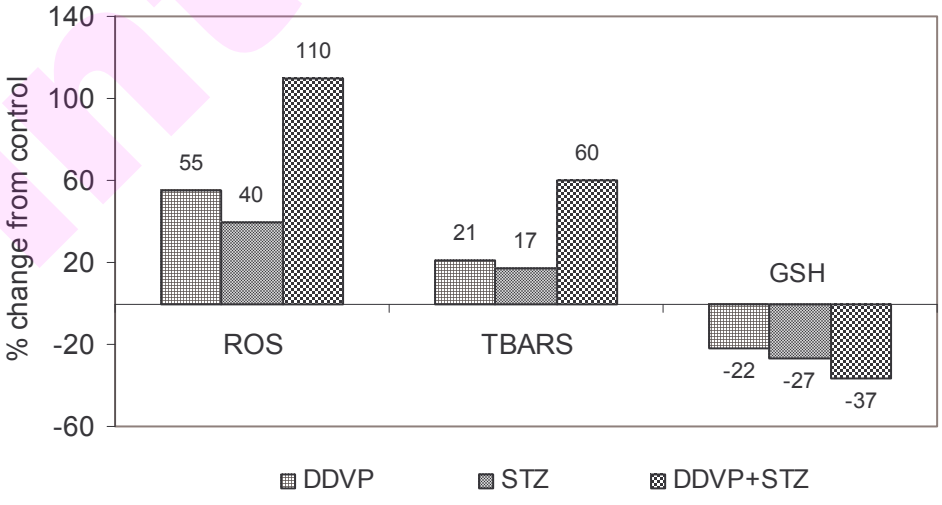


Fig. 2.23 ROS, TBARS and GSH levels in pancreas of rats pretreated with oral doses of DDVP (20 mg/kg b.w/d for 10 d) followed by a single dose of STZ (i.p , 25 mg/kg b.w)

Table 2.20 Activities of lipase and amylase in serum and pancreas of rats pretreated with oral doses of DDVP (20 mg/kg b.w/d for 10 d) followed by a single dose of STZ (i.p , 25 mg/kg b.w)

Group	Lipase (nmol PNP/mg protein/min)		Amylase (Street-Close Unit)	
	Serum	Pancreas	Serum	Pancreas
Untreated control	52.73^{a} ± 3.51	257.77^{b} ± 13.79	37.11^{a} ± 1.61	188.39^{a} ± 52.60
DDVP	70.96^{b} ± 3.36	178.76^{a} ± 10.06	68.58^{b} ± 3.94	261.46^{b} ± 64.08
STZ	51.43^{a} ± 4.56	255.22^{b} ± 17.37	38.10^{a} ± 4.00	167.95^{a} ± 11.69
DDVP+STZ	68.10^{b} ± 3.28	206.69^{a} ± 6.18	68.01^{b} ± 3.01	253.47^{b} ± 13.25

Values are mean ± SEM (n=6)
Mean in the same column with different superscript differ significantly ($p<0.05$)

6.0 DISCUSSION

The primary objective of the present study was to examine whether pretreatment with DDVP renders rats more susceptible to a single dose of STZ, a diabetogen. Increase in blood glucose and LPO levels with reduction in GSH content were the salient features observed in DDVP as well as STZ-treated rats. Our results clearly showed that a pre-existing disrupted glucose homeostasis and pancreatic damage caused by DDVP increased the STZ-mediated hyperglycemia relative to control rats. Although DDVP and STZ are structurally and mechanistically different, our results indicate the possibility of a common mechanism of potentiation of pancreatic injury in rats.

Streptozotocin is frequently used to induce diabetes mellitus in experimental animals through its toxic effects on pancreatic β-cells (Yamagishi et al., 2001; Stefek et al., 2002; Kalender et al., 2002). Streptozotocin (STZ) is an antiobiotic derived from *Streptomycetes achromogenes*. It has the property of selective destruction of β-cells after injection, while the exocrine part remains unchanged (Richter et al., 1971). STZ enters the β-cell via a glucose transporter (GLUT2) and promotes alkylation of DNA. The potent alkylating property of STZ is the principle reason for its toxicity (Szkudelski, 2001). Because of its highly specific β-cell toxicity and its subsequent diabetogenic effects, STZ has been used to study β-cell damage in a wide variety of *in vivo* models (Bolaffi et al., 1987). The cytotoxic action of STZ is also associated with the generation of reactive oxygen species causing oxidative damage (Szkudelski, 2001). Acute STZ injection has also been used to study cellular or tissue oxidative damage because it generates ROS and reduces antioxidant enzyme activities especially in pancreatic tissues (Coskum et al., 2005). In fact, STZ can stimulate H_2O_2 generation in islet cells (Friesen et al., 2004) where the activity of antioxidant enzymes such as SOD, CAT and GPX is relatively low when compared to other tissues (Tiedge et al., 1997). On exposure to STZ, most islets are impacted to

death and remaining islet cells almost exhibit a significant decrease in the activity of these enzymes (Srinivasan et al., 2003).

Generally, an acute intraperitoneal dose of 40–60 mg/kg b.w is employed to induce significant hyperglycemia in rats (Szkudelski, 2001). In our earlier studies (Singh et al., 2005a), we have induced significant hyperglycemia in adult rats with an i.p dosage of 52 mg/kg b.w. However for the present study, we employed a lower dose of 25 mg/kg b.w ('sub-diabetogenic dose') in order to examine if pretreatment with DDVP renders these rats more susceptible to hyperglycemia.

In the present study, STZ treatment at a dosage well below that needed to induce diabetes in rats was found to elevate blood glucose levels by 36% above control after 7 days of treatment. This is in contrast to the report of Mythili et al., (2004) who did not observe increased blood glucose after 7 days of treatment of rats with STZ at 30 mg/kg b.w. DDVP treated rats injected with STZ showed 30% more of blood glucose levels than that of STZ treated rats. Concomitantly, the liver glycogen was also further decreased in DDVP+STZ rats, suggesting a severely impaired carbohydrate metabolism in these rats.

Diabetes manifested by experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system (Baynes and Thorpe, 1999). Increased oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus, are thought to be the etiology of diabetic complications (Baynes, 1991). Oxidative stress thus plays an important role in chronic complications of diabetes and is postulated to be associated with increased lipid peroxidation (Kakkar et al., 1995).

We observed that LPO induced by STZ was associated with a decreased non-enzymatic (GSH) antioxidant content. GSH protects tissue from damage caused by diabetes, which impairs the antioxidant system. Depletion of tissue

GSH is one of the primary factors that permit lipid peroxidation (Konukoglu et al., 1998). It has been proposed that antioxidants that maintain the concentration of GSH may restore the cellular defense mechanisms, block lipid peroxidation and thus protect the tissue against oxidative damage (Chugh et al., 1999). GSH is an important substrate for GPX and GR. The reduction in GSH due to STZ was to a similar extent to that induced by DDVP treatment. Further depletion in pancreatic GSH content in DDVP+STZ rats clearly suggests an increased oxidative stress.

We did not observe any alteration in the levels of pancreatic marker enzymes due to STZ treatment. This is justified due to the fact that STZ does not affect the acinar cells of the pancreas (Richter et al., 1971). However, 'DDVP+STZ' rats showed altered pancreatic marker enzymes due to the impact of DDVP treatment alone.

Impaired glucose tolerance is a category that permits classification of individuals whose glucose tolerance is above the conventional normal range but lower than the level considered diagnostic of diabetes. Such persons do have a high risk of developing diabetes mellitus. Our earlier results (*Section A, Chapter II*) with DDVP had clearly demonstrated impaired glucose tolerance in rats treated at the dosage employed for the present study. Hence, based on the results of the present study, it could be hypothesized that the rats pre-treated with DDVP could be at a risk of developing diabetes if exposed to even a sub-diabetogenic dosage of a diabetogen.

7.0 SUMMARY

1. Blood glucose levels in rats treated with DDVP or STZ alone were only 22% and 37% respectively higher compared to control rats. Interestingly, blood glucose levels were further elevated (66%) among rats administered STZ pretreated with DDVP.
2. Liver glycogen levels were significantly lower in rats administered with DDVP (18%) or STZ (19%) alone while rats pretreated with DDVP followed by STZ injection had further lower levels of glycogen (46 %) compared to that of the control rats.
3. Markedly higher ROS and lipid peroxide were evident in pancreas of 'DDVP+STZ' rats.
4. Reduced glutathione levels in pancreas were significantly lower among STZ+DDVP rats compared to those administered either DDVP or STZ alone.
5. Rats administered with DDVP+STZ showed significant increases in lipase and amylase activities in serum (30% and 83 % respectively), while pancreatic tissue lipase activity was decreased significantly.
6. These studies provide new evidence for the potential of OPI to damage rat pancreas and thereby exacerbate condition leading to diabetes.

CHAPTER III

AMELIORATION OF OPI-INDUCED PANCREATIC DAMAGE BY ANTIOXIDANT PHYTOCHEMICALS

PREFACE

Our previous studies clearly demonstrated that the OPI – dimethoate and dichlorvos induce oxidative stress in pancreatic tissue and that OS in pancreas plays a role towards its dysfunction and the ensuing alterations in glucose homeostasis in rats. Results of our studies also point towards the fact that exposure to OPI may contribute towards induction or exacerbation of diabetes. There are many reports on the protective effects of antioxidants in the management of diabetes as well as OPI poisoning. Therefore, it seems reasonable to believe that antioxidants can play an important role in the improvement of hyperglycemia and pancreatic dysfunction induced by OPI. Hence, the aim of studies in this chapter was to: (i) identify a novel antioxidant phytochemical and (ii) to establish its efficacy to ameliorate the toxic effects of DM and DDVP in experimental animals.

This Chapter is presented under two sections.

Section A includes studies on the comparative antioxidant activities of ethanolic extracts of three plant novel derived food-processing wastes such as potato peel (PPE), tamarind seed coat (TSCE) and cashew nut skin (CSE) in selected *in vitro* antioxidant assay models. Included are also investigations on the antioxidant efficacy of CSE and standard antioxidants (epicatechin and β-carotene) against oxidative stress induced by DM and DDVP in rat pancreatic homogenate and rat pancreatic islets, *in vitro*.

Section B includes studies describing the protection offered by oral supplementation of CSE in rats subjected to repeated oral doses of DM.

SECTION A

ANTIOXIDANT ACTIVITIES OF SELECTED PHYTOCHEMICALS *IN VITRO*

1.0 INTRODUCTION

Currently, there is considerable emphasis on identifying the potential of natural plant products present in food consumed by humans as chemopreventive agents. Several plant products exert antioxidative effects and some of them are widely used in food in different parts of the world. Recently, a growing interest in the substitution of synthetic antioxidants by natural ones has promoted research on the screening of plant materials, especially from inexpensive or residual sources from agricultural/ food processing industries for evaluating newer antioxidants (Moure et al., 2001; Makris et al., 2007).

Antioxidant activity have been detected in seed processing wastes such as hulls from peanut (Yen et al., 1993), mung bean (Duh et al., 1997) and buck wheat (Watanabe et al., 1997), red and black seed coat of *Phaseolus vulgaris* (Muanza et al., 1998), sesame seed coat (Chang et al., 2002), peanut testa (Lou et al., 2004) and almond skins (Chen et al., 2005). Industrial residues have also served as attractive sources of antioxidants, such as potato peel waste (Rodriguez de Sotillo et al., 1994a, b), grape pomace peels (Bonilla et al., 1999; Makris et al., 2007) citrus peels (Bocco et al., 1998). Studies related to the value assessment of food processing wastes are mainly based on the content and profile of phenolics as well as their *in vitro* antioxidant potency. In the present study, we selected three food processing by-products viz., potato peel, tamarind seed coat and cashew nut skin whose antioxidant activities in water extract or extract in other solvents have been demonstrated. Recent comprehensive studies from our laboratory demonstrated the antioxidant activities of potato peel extract (Singh and Rajini, 2004; Singh et al., 2005b). The antioxidant activity of tamarind seed coat was earlier reported by Tsuda et al., (1994). The

antioxidant potential of cashew nut skin is hitherto unknown although it is reported to be rich in polyphenols (Mathew and Parpia, 1970).

In the present study, we have compared the antioxidant activities of ethanolic extracts derived from potato peel, tamarind seed coat and cashew skin. It is evident from our own findings and that of others that active principles of these extracts are widely different and hence, their antioxidant potential is also expected to be significantly different. We employed various *in vitro* model systems to assess their antioxidant activity and also quantified the total polyphenol content in order to select the most potent antioxidant extract for our *in vivo* amelioration studies. Studies were carried out to evaluate the efficacy of the selected extract and its antioxidant-active component to offer protection against oxidative damage induced by dimethoate and dichlorvos in rat pancreatic homogenate and isolated rat pancreatic islet culture, *in vitro*.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), diphenyl-β-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), gallic acid, bovine serum albumin (BSA), β-carotene, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ethylenediaminetetraacetic acid (EDTA), reduced glutathione (GSH), dimethyl sulfoxide (DMSO), collagenase type V, 2',7'-Dichlorofluorescein diacetate (DCFH-DA), 2',7'-Dichlorofluorescin (DCF), Dithizone, Soybean trypsin inhibitor (STI), BSA fraction V, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), trypan blue, epicatechin, β-carotene and Tween-20 were purchased from M/s Sigma Chemicals Co. (St. Louis, MO, USA). Silica gel thin layer chromatography plates (TLC pre-coated plates) were procured from, M/s Merck KgaA (Darmstadt, Germany). Trichloroacetic acid (TCA), linoleic acid,

tris hydroxymethyl aminomethane, adenosine dinucleotide phosphate (ADP), ferrous sulphate, nicotinamide adenine dinucleotide phosphate-reduced (NADPH), nicotinamide adenine dinucleotide-reduced (NADH), ammonium molybdate, Folin's reagent, glucose, Ferrozine, deoxyribose (DR), HEPES, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), and ferric chloride were purchased from M/s Sisco Research Laboratories (Mumbai, India). Dulbecco's minimum essential medium (DMEM) and RPMI 1640 were purchased from M/s HiMedia (Mumbai, India). Technical grade Dimethoate (97%) and Dichlorvos (94%) were gifted by M/s Hyderabad Chemical Supplies Ltd., (Hyderabad, India). All other chemicals used were of analytical grade.

2.2 Samples

Food processing wastes viz., potato peel, tamarind seed coat and cashew nut skin were selected for the study. Fresh potato peels obtained from a local potato chips making unit, were washed three times with tap water and then dried at 70ºC for 5 h in a 'cross-flow drier.' Dried cashew nut testa was gift from a Cashew nut processing unit (M/s Mangala cashews, Mangalore, Karnataka, India). The dried potato peels and cashew nut skin were powdered in a 'Disk Mill' (Glen Mills Inc, Clifton, NJ, USA) and passed through a 0.5 mm sieve to obtain a fine powder. Tamarind seed coat powder was kindly gifted by the Department of Grain Science and Technology, CFTRI, Mysore. The powdered samples were stored in airtight containers at 4ºC until further analysis.

2.3 Preparation of extracts

All the three powders were individually extracted with five parts of distilled ethanol in a rotatory shaker at 37ºC for 3h. Each of the extract was separated by centrifugation and the resultant extracts were stored in dark at 4ºC until use.

Cashew nut skin extract (10mg/ml) and Epicatechin (10mg/ml) were dissolved in water and passed through membrane filter (0.2 µ) before employing in islet studies.

2.4 Determination of yield of the extracts

All the three powders were individually extracted with five parts of distilled ethanol in a rotatory shaker at 37ºC for 3h. Each of the extract was separated by centrifugation, the supernatant filtered through Whatman No. 1. filter paper, and the filtrates thus obtained were concentrated using a rotatory evaporator. The yields of the extracts were determined gravimetrically.

2.5 Total polyphenols *(Singleton and Rossi, 1965)*

An aliquot of each extract (volume adjusted to 3ml with distilled water) was incubated with 0.5ml of 95% ethanol and 250µl of Folin's reagent (1:1diluted with distilled water) for 5 min at room temperature. To this, 0.5ml of Na_2CO_3 solution (5%) was added, mixed and the mixture was incubated for 60min at room temperature. The absorbance of the solution was read at 720nm against a reagent blank. Gallic acid (0.1mg/ml) was used as the standard and the total polyphenol content in the extract was expressed as mg gallic acid equivalent (GAE)/ g extract.

2.6 Preparation of β-carotene emulsion

β-carotene emulsion was prepared according to the procedure described by Taga et al., (1984). Briefly, β-carotene (3mg) was dissolved in 20ml of chloroform and an aliquot (3ml) of this solution was added to a conical flask containing 40mg linoleic acid and 400mg of tween-20. Chloroform in the flask was evaporated to dryness under nitrogen. 100ml of oxygenated distilled water was added to the flask, the mixture was shaken well and aliquots were taken for the antioxidant assay.

2.7 Preparation of $ABTS^{\cdot+}$ radical

ABTS radical was produced by reacting ABTS (7mM) with ammonium persulphate (2.45 mM) in 10 ml water and keeping the mixture in dark at room temperature for 12-16 h before use. The aqueous $ABTS^{\cdot+}$ solution was diluted with ethanol (1:100 v/v) to an absorbance of 0.7 (± 0.02) at 734 nm.

2.8 Pesticide solution

Stock solutions (1 M) of DM and DDVP were prepared in DMSO and further diluted with distilled water to obtain working solutions for *in vitro* assays. The pesticide solutions were passed through a sterile membrane filter (0.2 µ) before using in cell culture studies.

2.9 Animals and care

Adult male rats (CFT-Wistar strain, 12-14 week old, 280 ± 5g) were randomly drawn from the stock colony of our Institute animal house facility and were housed individually in polypropylene cages under standard housing conditions (controlled atmosphere with 12:12-hour light/dark cycles, 50% ± 5% humidity, and an ambient temperature of 25± 2º C). Rats were maintained on commercial pellet diet (M/s Saidurga Feeds and Foods Pvt. Ltd., Bangalore, India) *ad libitum* and had free access to water. All procedures with animals were conducted strictly in accordance with guidelines approved by the Institute Animal Ethical Committee, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

2.10 Preparation of pancreas and brain homogenates

Rats were sacrificed under mild ether anesthesia, pancreas and brain were excised, blotted free of blood, rinsed in ice-cold saline and homogenized (1% w/v) in 1.15% KCl. The homogenate was centrifuged at 3000rpm at 4°C for 10min and the supernatant used for the assays.

2.11 Pancreatic islet culture *(Sitasawad et al., 2000)*

Rat pancreatic islets were isolated by collagenase digestion and cultured as described earlier *(Section 2.5, Chapter I A)*.

2.12 Assessment of viability and specificity of islets *(Clark et al., 1994)*

Islet viability and specificity were determined by trypan blue dye exclusion assay and dithizone staining as described earlier *(Section 4.1 & 4.2, Chapter I A)*.

3.0 EXPERIMENTAL PROCEDURE

3.1 Antioxidant activity of extracts of potato peel, tamarind seed coat and cashew nut skin powder in chemical model systems *in vitro*

3.1.1 DPPH· radical scavenging (*Ohnishi et al., 1994)*

1.5 ml of DPPH solution (0.1mM in 95% EtOH) was incubated with various concentrations of each of the extract (PPE: 45-225µg; TSCE: 3-15µg; CSE: 3-17µg) and the final volume was adjusted to 1.75ml with 95% EtOH. The reaction mixture was shaken well and incubated for 20 min at room temperature and the absorbance of the resulting solution was read at 517nm against blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation

$$\text{Scavenging effect (\%)} = \left(1 - \frac{A_{\text{Sample (517nm)}}}{A_{\text{Control (517nm)}}}\right) \times 100$$

Four concentrations of each extract were used to determine the concentration-response. EC_{50} of each extract was obtained by plotting the percent DPPH remaining at the steady state (30min) of the reaction against the corresponding extract concentration. EC_{50} is the concentration of the antioxidant to quench 50% DPPH· under the assay conditions.

3.1.2 ABTS radical scavenging (Re et al., 1999)

Aliquots of each extract at varying concentrations (PPE: 9-45µg; TSCE: 1-4µg; CSE: 1-5µg) were added to $ABTS^{\cdot+}$ solution (2 ml), mixed and the absorbance was read at 734 nm after 5 min. The percentage inhibition of radical scavenging was calculated.

Four concentrations of each extract were used to determine the concentration-response. EC_{50} of each extract was obtained by plotting the percent $ABTS^{\cdot+}$ remaining at the steady state (30min) of the reaction against the corresponding extract concentration. EC_{50} is the concentration of the antioxidant to quench 50% $ABTS^{\cdot+}$ under the experimental conditions.

3.1.3 Superoxide radical scavenging (Nishikimi et al., 1972)

The reaction mixture, containing various concentrations of each of the extracts (PPE: 88-440µg; TSCE: 3-13µg; CSE: 5-22µg), NADH (100 µM) and NBT (100 µM) in tris- HCl (0.02 M pH 8.3) were taken in spectrophotometric cuvette and the reaction was started by adding the PMS (1 µM). The change in absorbance (∆A) was monitored for one min. The capability of scavenging the superoxide radical was calculated using the following equation:

Scavenging effect (%) = $[1- \Delta A_{Sample\ 560\ nm} / \Delta A_{Control\ 560\ nm}] \times 100$

Four concentrations of each extract were used to determine the concentration-response. EC_{50} of each extract was obtained by plotting the percent superoxide scavenging of the reaction against the corresponding extract concentration. EC_{50} is the concentration of the antioxidant to quench 50% superoxide scavenging under the experimental conditions.

3.1.4 Deoxyribose oxidation (Halliwell et al., 1987)

The reaction mixture, containing various concentrations of the extracts (PPE: 20-90µg; TSCE: 4-14µg; CSE: 5-20µg) was incubated with deoxyribose (3.75 mM), H_2O_2 (1mM), $FeCl_3$ (100 µM) in potassium phosphate buffer (20mM, pH 7.4) for 60 min at 37ºC. The reaction was terminated by adding 1 ml TBA

(1% w/v) and 1 ml TCA (2% w/v) followed by heating the tubes in a boiling water bath for 15 min. The contents were cooled and absorbance of the mixture was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

Four concentrations of each extract were used to determine the concentration-response. EC_{50} of each extract was obtained by plotting the percent inhibition of deoxyribose against the corresponding extract concentration. EC_{50} is the concentration of the antioxidant to quench 50% inhibition of hydroxyl radical under the experimental conditions.

3.1.5 Fe^{2+} chelation *(Decker and Welch, 1990)*

The reaction mixture, containing various concentrations of the extracts (PPE: 0.4-1.8mg; TSCE: 0.5-4mg; CSE: 1-7mg), $FeCl_2$ (2 mM), and ferrozine (5 mM) was adjusted to total volume of 0.8 ml with methanol, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against blank. EDTA (0.05-0.2 mg) was used as positive control. The ability of the extract to chelate ferrous ion was calculated using the following equation:

Chelating effect (%) = $[1- A_{Sample\ 560\ nm} / A_{Control\ 560\ nm}] \times 100$

Four concentrations of each extract were used to determine the dose effects. EC_{50} of each extract was obtained by plotting the percent chelating against the corresponding extract concentration. EC_{50} is the concentration of the antioxidant to quench 50% chelating under the experimental conditions.

3.1.6 β-carotene bleaching (*Taga et al., 1984)*

20 µg of each extract was added to 3 ml of β - carotene emulsion in test tube, the mixture was shaken well and incubated at 50ºC in water bath for various periods. Absorbance of the mixture was read at 470nm at, 10, 20, 30, 40 and 60 min intervals. Each sample was read against an emulsion prepared as described earlier but without β-carotene (blank). To correct any interference of color of extract in the β-carotene degradation rate, aliquots of extracts were

added to blank and absorbance was recorded. BHA (20 µg) was employed as model antioxidant for the purpose of comparison.

3.1.7 Linoleic acid oxidation *(Mitsuda et al., 1966)*

Linoleic acid emulsion (0.02 mol/l) was prepared by mixing and homogenizing linoleic acid (0.280g) and tween-20 (0.280g) in phosphate buffer (50ml, pH 7.0). The reaction mixture containing 100 µg of each extract, linoleic acid emulsion (2.5 ml), and phosphate buffer (2.3 ml, 0.2mol/l, pH 7.0) were mixed and incubated at 37ºC in dark. Aliquots (100µl) of the reaction mixture were taken at intervals of 20h or 120h and the degree of oxidation was measured by sequentially adding 75% ethanol (4.7ml), 30% ammonium thiocyanate, sample solution (0.1 ml) and ferrous chloride (0.1 ml, 20mmol/l in 3.5% HCl). After the mixture was incubated for 3 min, the peroxide value was determined by monitoring the absorbance at 500 nm. All tests were run in duplicate, and analysis of all samples were run in triplicate and averaged. BHA was used as positive control.

3.2 Antioxidant activity of extracts of potato peel, tamarind seed coat and cashew nut skin powder in biological model systems *in vitro*

3.2.1 Lipid peroxidation in rat brain homogenate *(Yen and Hsieh, 1998)*

Peroxidation was induced in rat brain homogenate by $FeCl_2$-H_2O_2, and the extent of protection offered by the extracts against induced peroxidation was measured. Briefly, brain homogenate (1%) was incubated with 0.5 mM each of $FeCl_2$ and H_2O_2 with varying concentrations of each extract (PPE: 50-200 µg; TSCE: 8-40 µg; CSE: 8-40 µg). After incubation at 37ºC for 60 min, TBARS formed in the incubation mixture was measured at 535 nm as described earlier *(Section 4.6, Chapter I A).*

3.3 High performance liquid chromatography (HPLC) identification of the active molecule from CSE

Ethanolic extract cashew nut skin (CSE) was applied on silica gel thin layer chromatography plate and resolved using chloroform: methanol (8:2). The antioxidant-active spot was visualized after spraying with β-carotene linoleate solution (9 mg of β-carotene dissolved in 30 ml of chloroform to which 2 drops of linoleic acid were added and then added to 60 ml of ethanol, Pratt & Miller, 1984). The β-carotene positive spot was scraped, extracted in acetonitrile and subjected to HPLC.

The HPLC (Hewlett Packard 1100 Series, Palo Alto, CA) was equipped with a quaternary pump fitted with a Zorbax C18 analytical column (25 cm X 4.6 cm I.D 5µ particle size) (M/s S. V. Scientific, Bangalore, India). Detection was done with an HP 1250m series variable wavelength detector at wavelength of 280 nm. The gradient mobile phase consisted of acetonitrile (A) and 1% trifluoroacetic acid (TFA) (B) with flow rate of 0.1ml /min. The elution program involved a linear gradient from 100 - 0 % for 15 min followed by 5 min equilibrium in a total program time of 25min. The sample and the standard were dissolved in mobile phase and 10µl of each was injected.

3.4 Protective effect of CSE and standard antioxidants (epicatechin and β-carotene) against DDVP-induced oxidative stress in rat pancreatic homogenate

Pancreatic homogenate (75µl) was pre-incubated with DCFH-DA (30 µl) and 25µl of CSE (1mg) / Epicatechin (1mM) / β-carotene (1mM) for 10 min. 1mM of DDVP was added to 130µl of the homogenate mixture. The total volume was made up to 300µl with phosphate buffer. The above mixture was incubated for 60 min in a multiwell plate in a shaker water bath at 37ºC. After incubation, supernatant was separated by centrifugation at 2000 rpm. The resultant DCF was measured as described earlier (*Section 4.5 Chapter I A*).

3.5 Protective effect of CSE and epicatechin against DM / DDVP-induced oxidative stress in rat pancreatic islet cells

500 isolated islets were incubated in 0.3ml of RPMI with varying concentrations (0.25, 0.5, 0.75 and 1 µM) of DM or DDVP with or without CSE or epicatechin (0.25, 0.5, 1 µg / ml) for 12 h at 37ºC in a CO_2 incubator gassed with 5 % CO_2 and relative humidity of 95%. After 12h of incubation, samples were centrifuged at 5000rpm to sediment the islets. The islets were washed 3 times with PBS and finally suspended in a known volume of PBS for the biochemical determinations. The supernatant was collected and stored at -20ºC for measurement of LDH.

3.5.1 Viability

Aliquots of islet suspension were used for determination of viability by MTT dye reduction assay as described below.

3.5.2 Lactate dehydrogenase (LDH) activity

Cell free supernatant from the incubation mixture was used for the determination of lactate dehydrogenase activity as described below.

3.5.3 Glutathione content

The suspended islets were pelletted and homogenized in 5% TCA in microcentrifuge tubes using micro pestle and centrifuged at 3000 rpm. The resultant supernatant was used for the determination of reduced glutathione as described below.

3.5.4 Reactive oxygen species

500 islets were pre-incubated with DCFH-DA, for 30min at 37ºC in a CO_2 incubator gassed with 5 % CO_2 and relative humidity of 95%. Following this the islets were exposed to varying concentrations of pesticides and CSE or epicatechin as described earlier in *Section 3.5 Chapter I A*. After incubation, the reaction mixture was centrifuged at 5000rpm to sediment the islets. They were

washed 3 times with PBS and finally suspended in a known volume of PBS. The islets were homogenized in a microcentrifuge tubes using a micropestle and centrifuged at 3000 rpm for 5 min at 4ºC and the supernatant was used for the determination of ROS as described below.

3.6 Statistical analysis

Mean and standard error (SE) (n=3) values were determined for all the parameters studied. Results were statistically analyzed by analysis of variance (ANOVA) using Statistica software (STATSOFT, USA). Duncan's multiple range test (DMRT) was performed to determine the significant difference among the samples. *P* value<0.01 were considered significant.

4.0 ASSAY METHODS

4.1 MTT dye reduction (Alley et al., 1998)

Viability of cultured islet cells was quantified using the MTT assay as described earlier *(Section 4.6, Chapter I A).*

4.2 Reactive oxygen species *(Keston and Brandt, 1965)*

Reactive oxygen species in isolated pancreatic islets were estimated as described earlier *(Section 4.4, Chapter I A).* Results were expressed as pmol DCF/ml in cell free supernatant.

4.3 Reduced Glutathione *(Benke et al., 1974)*

Reduced glutathione content in rat pancreatic islets were quantified as described earlier *(Section 4.7, Chapter I A).* Results were expressed as % decrease from control.

4.4 Lactate dehydrogenase (EC 1.1.1.27) *(Kornberg, 1974)*

Lactate dehydrogenase activity in the supernatant was estimated as described earlier *(Section 4.8, Chapter I A).* The results were expressed as nmoles of NADH oxidized / min/ mg protein.

5.0 RESULTS

5.1 Antioxidant activity of the extracts of the selected plant materials

5.1.1 Yield and total phenolic content

The yield and total phenolic content in crude ethanolic extract obtained from each powder are presented in **Table 3.1**. As clearly evident, significant differences in yield and total phenolics were evident among the extracts. The yield of crude ethanolic extract from cashew nut skin (CSE) was the highest (45.1%), followed by tamarind seed coat extract (TSCE) (27.5%) and least by potato peel extract (PPE) (8.5%). The content of polyphenolic compounds also varied markedly from a low of 28.4 mg GAE /g of PPE to a high of 673 mg GAE /g of TSCE.

5.1.2 DPPH$^{\cdot}$ / ABTS scavenging activity

The results of DPPH$^{\cdot}$ / ABTS scavenging activity of the three extracts along with that by the known antioxidant BHA is presented in **Table 3.2**. The scavenging potentials of the extracts are reported as µg of the extract that reduces the concentration of DPPH radical/ABTS cation in the reaction mixture by 50%. A lower value of EC_{50} indicates a higher antioxidant activity. Of the three extracts, CSE was the most effective radical scavenger, as effective as BHA.

Fig 3.1 and Fig 3.2 depicts the concentration-dependent decolorization of DPPH and $ABTS^{\cdot+}$ radical by PPE, TSCE and CSE along with the synthetic antioxidants such as BHA and BHT. As evident from the figures the radical scavenging ability of CSE was comparable to that of BHA (4.77 ± 0.02 and 1.37 ± 0.03 for DPPH and ABTS radical scavenging respectively). TSCE was slightly lesser efficient than CSE although it exhibited strong radical scavenging activity. Of the three extracts, PPE was found to be relatively a poor radical scavenger. The extent of scavenging of proton radical by TSCE and CSE was similar. The EC_{50} values for scavenging of DPPH radical was 91.92 ± 11.75, 7.53 ± 0.28 and 5.87 ± 0.94 µg/ml by PPE, TSCE and CSE respectively, and that for ABTS

scavenging was 24.33 ± 0.49, 1.6 ± 0.06 and 1.3 ± 0.02 µg/ml by PPE, TSCE and CSE respectively.

5.1.3 Superoxide scavenging activity

All the three extracts exhibited scavenging of superoxide radicals in a concentration-dependent manner. **Fig 3.3** depicts the concentration-response of inhibition of superoxide radical generated by ethanolic extracts of PPE, TSCE and CSE. The scavenging of superoxide radical also increased with increasing concentration of all the extracts, with EC_{50} of 263 ± 3.51, 7.69 ± 0.11 and 10.69 ± 0.13 (**Table 3.3**). The inhibitory effect of TSCE and CSE on superoxide radical was marked.

5.1.4 Deoxyribose oxidation

Protective effect of the three extracts on oxidative damage induced by Fe^{3+}/ H_2O_2 on deoxyribose as measured by the TBA is shown in **Fig 3.4**. The scavenging activity against hydroxyl radical increased with increasing amounts of extracts. As evident from EC_{50} in the **Table 3.3**, both TSCE (12.13 ± 0.26) and CSE (17.7 ± 0.05) inhibited deoxyribose oxidation at much lower concentrations as compared to PPE (88.56 ± 1.5).

5.1.5 Iron chelation

Table 3.3 shows the EC_{50} of ferrous ion-chelating effect of various extracts, which was significantly higher in PPE (1.69 ± 0.03) than TSCE (3.8 ± 0.5) and CSE (6.0 ±0.24) (**Fig. 3.5).**

5.1.6 β-carotene bleaching activity

Antioxidant activity of various extracts has been compared using β-carotene linoleate system and the data has been represented in **Fig. 3.6**. All the three extracts offered protection to varying extent. CSE exhibited marked antioxidant activity, nearly equal to that of BHA. Data on the effects of the extracts and BHA on the peroxidation of linoleic acid were investigated and the

results are presented in **Fig 3.7**. At a concentration of 100µg in the final reaction mixture, all the three extracts inhibited peroxidation for 120 h (five days). In summary, the inhibitory potential was as follows: BHA > CSE > PPE > TSCE.

5.1.7 Lipid peroxidation in rat brain homogenate

Inhibitory effect of different extracts on MDA production in rat brain homogenate induced by $FeCl_2/H_2O_2$ is shown in the **Fig. 3.8**. Addition of extracts to the Fe^{2+}-H_2O_2 system resulted in a concentration–dependent decrease in lipid peroxidation. As evident from the **Table 3.3**, marked inhibition of TBARS formation was noted with TSCE (33.33 ± 0.66) and CSE (24.66 ± 0.31) than PPE (336 ± 3.75).

5.1.8 HPLC analysis of CSE

The TLC chromatogram of CSE sprayed with β-carotene showed an active spot (bleached). The HPLC chromatogram (**Fig. 3.9**) of the active spot showed the presence of epicatechin as major component along with other polyphenols. The identities of the peaks were confirmed by determination of relative retention times and by spiking with the standard epicatechin. The relative percentage of epicatechin was 96 .2 %.

Table 3.1 Yield and total polyphenol content (TPC) in ethanolic extracts of Potato peel (PPE), Tamarind seed coat (TSCE) and Cashew nut skin (CSE)

Extract	Yield[1]	TPC[2]
PPE	$8.45^{a} \pm 0.32$	$28.40^{a} \pm 1.0$
TSCE	$27.50^{b} \pm 0.23$	$673.00^{b} \pm 15.0$
CSE	$45.08^{c} \pm 0.72$	$539.04^{c} \pm 35.0$

[1]g/ 100g powder ; [2]mg GAE/g extract
Values are mean ± SE of three determinations each
Mean in the same column with different superscript differ significantly (*p<0.05*)

Table 3.2 EC_{50} values of Potato peel extract (PPE), Tamarind seed coat extract (TSCE) and Cashew nut skin extract (CSE) against scavenging of DPPH and ABTS radicals

	EC_{50} (μg extract / ml)	
	DPPH radical	ABTS radical
PPE	$91.92^{b} \pm 11.75$	$24.33^{b} \pm 0.49$
TSCE	$7.53^{a} \pm 0.28$	$1.60^{a} \pm 0.06$
CSE	$5.87^{a} \pm 0.94$	$1.38^{a} \pm 0.02$
BHA	$4.77^{a} \pm 0.02$	$1.37^{a} \pm 0.03$

EC_{50} value in each assay was determined graphically by plotting each activity as a function of extract concentration.
Values are mean ± SE of three determinations each
Mean in the same column with different superscript differ significantly (*p<0.05*)

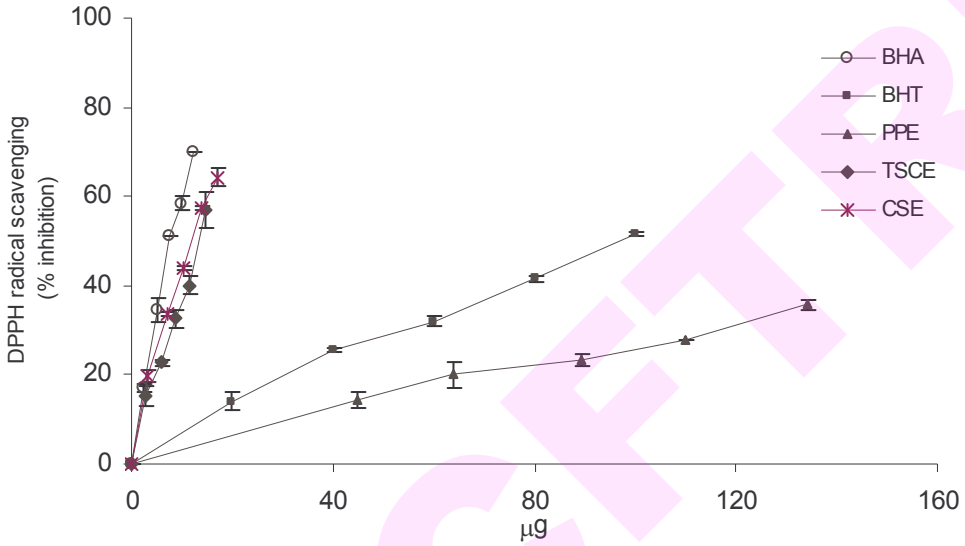


Fig. 3.1 DPPH radical scavenging effect of Potato peel (PPE), Tamarind seed coat (TSCE) and Cashew nut skin extract (CSE)

Values are mean ± SE of three determinations each

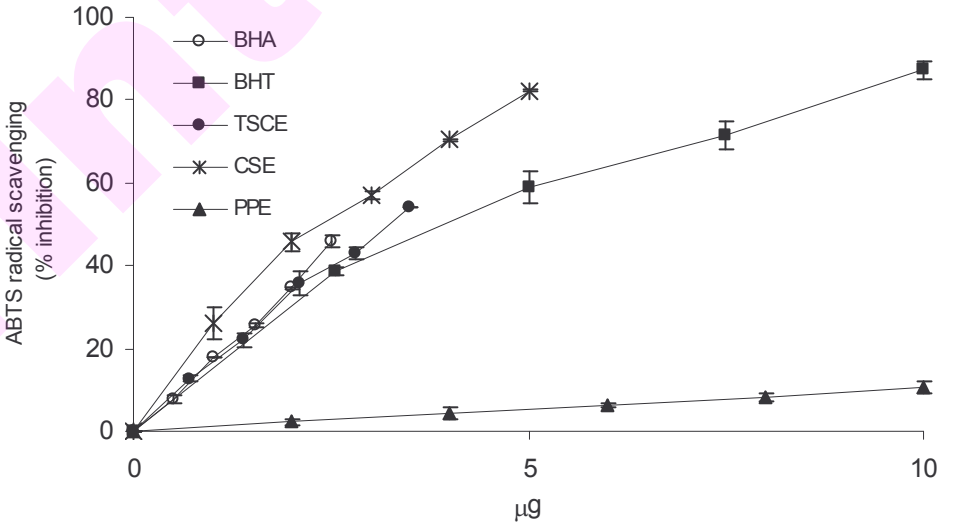


Fig. 3.2 ABTS radical scavenging effect of Potato peel (PPE), Tamarind seed coat (TSCE) and Cashew nut skin extract (CSE)

Values are mean ± SE of three determinations each

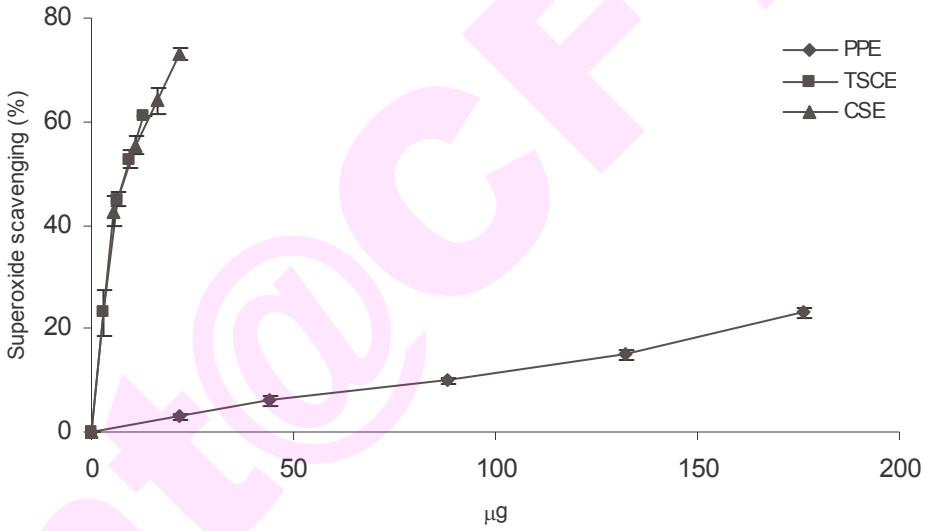


Fig. 3.3 Superoxide radical scavenging effect of Potato peel (PPE), Tamarind seed coat (TSCE) and Cashew nut skin extract (CSE)

Values are mean ± SE of three determinations each

Table 3.3 EC_{50} values of Potato peel extract (PPE), Tamarind seed coat extract (TSCE) and Cashew nut skin extract (CSE) against superoxide radical scavenging, deoxyribose oxidation, iron chelation and inhibition of lipid peroxidation in rat brain homogenate

	EC_{50}			
Extract	Superoxide radical scavenging*	Deoxyribose oxidation *	Iron chelation activity**	Inhibition of Lipid peroxidation*
PPE	$263^{c} \pm 3.51$	$88.56^{c} \pm 1.50$	$1.69^{a} \pm 0.03$	$336.00^{c} \pm 3.75$
TSCE	$7.69^{a} \pm 0.11$	$12.13^{a} \pm 0.26$	$3.80^{b} \pm 0.05$	$33.33^{b} \pm 0.66$
CSE	$10.69^{b} \pm 0.13$	$17.70^{b} \pm 0.05$	$6.00^{c} \pm 0.24$	$24.66^{c} \pm 0.32$

* EC_{50} : μg extract / ml ; ** EC_{50} : mg extract / ml
EC_{50} values in each assay were determined graphically by plotting each activity as a function of extract concentration.
Values are mean ± SE of three determinations each
Mean in the same column with different superscript differ significantly ($p<0.05$)

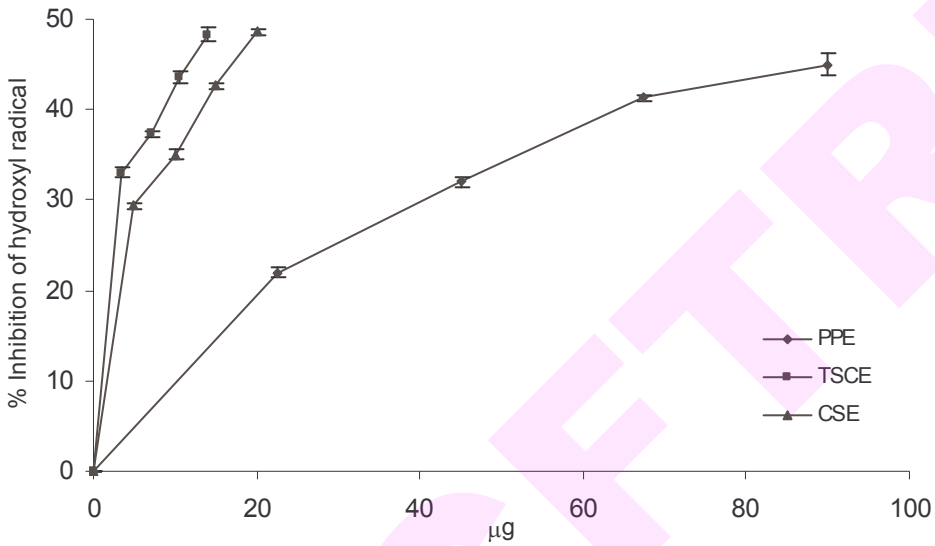


Fig. 3.4 Inhibitory effect of Potato peel (PPE), Tamarind seed coat (TSCE) and Cashew nut skin extract (CSE) on deoxyribose oxidative damage

Values are mean ± SE of three determinations each

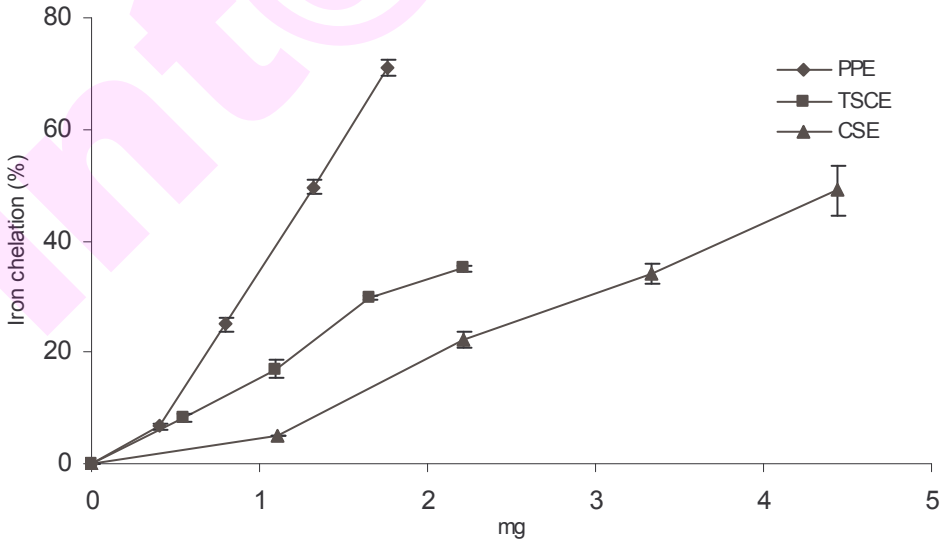


Fig. 3.5 Chelating effect of Potato peel (PPE), Tamarind seed coat (TSCE) and Cashew nut skin extract (CSE) on Fe^{2+} ion

Values are mean ± SE of three determinations each

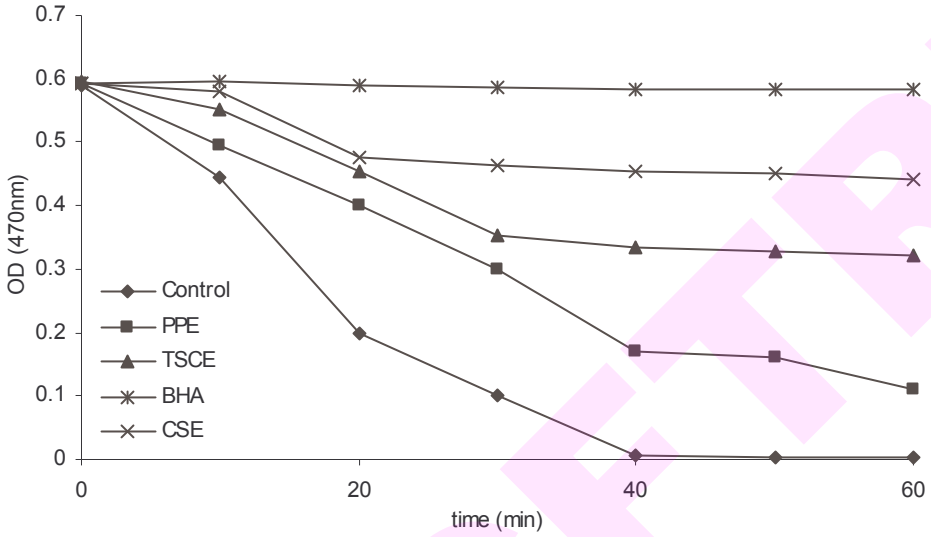


Fig. 3.6 Antioxidant activity of Potato peel extract (PPE), Tamarind seed coat extract (TSCE) and Cashew nut skin extract (CSE) in β-carotene-linoleic acid system

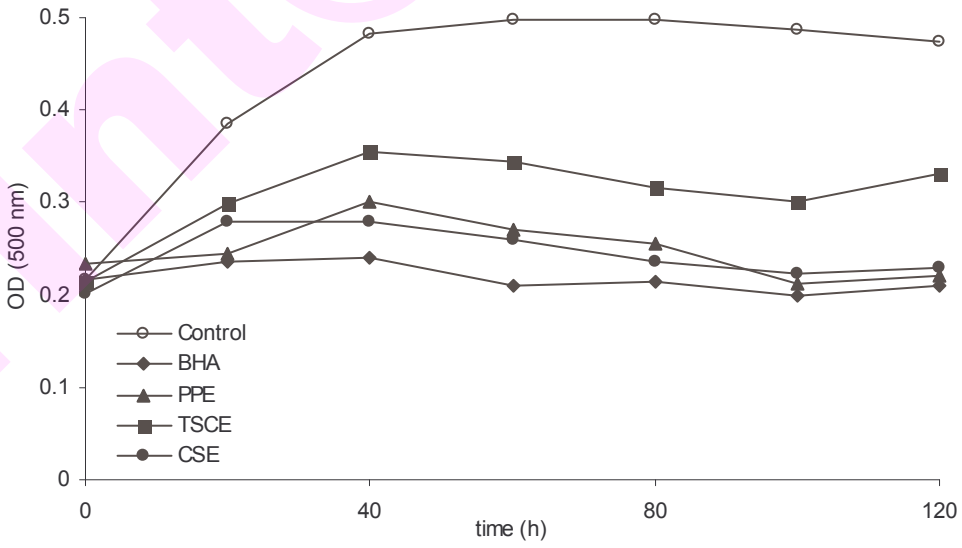


Fig. 3.7 Antioxidant activity of Potato peel extract (PPE), Tamarind seed coat extract (TSCE) and Cashew nut skin extract (CSE) in linoleic acid oxidation system

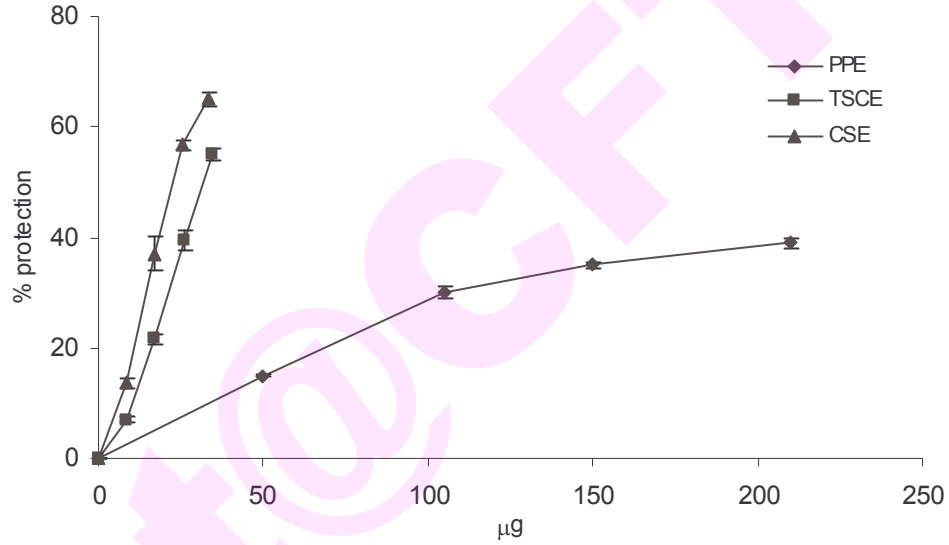


Fig. 3.8 Inhibition of Fe^{2+}-H_2O_2 induced lipid peroxidation in rat brain homogenate by Potato peel (PPE), Tamarind seed coat (TSP) and Cashew nut skin extract (CSE)

Values are mean ± SE of three determinations each

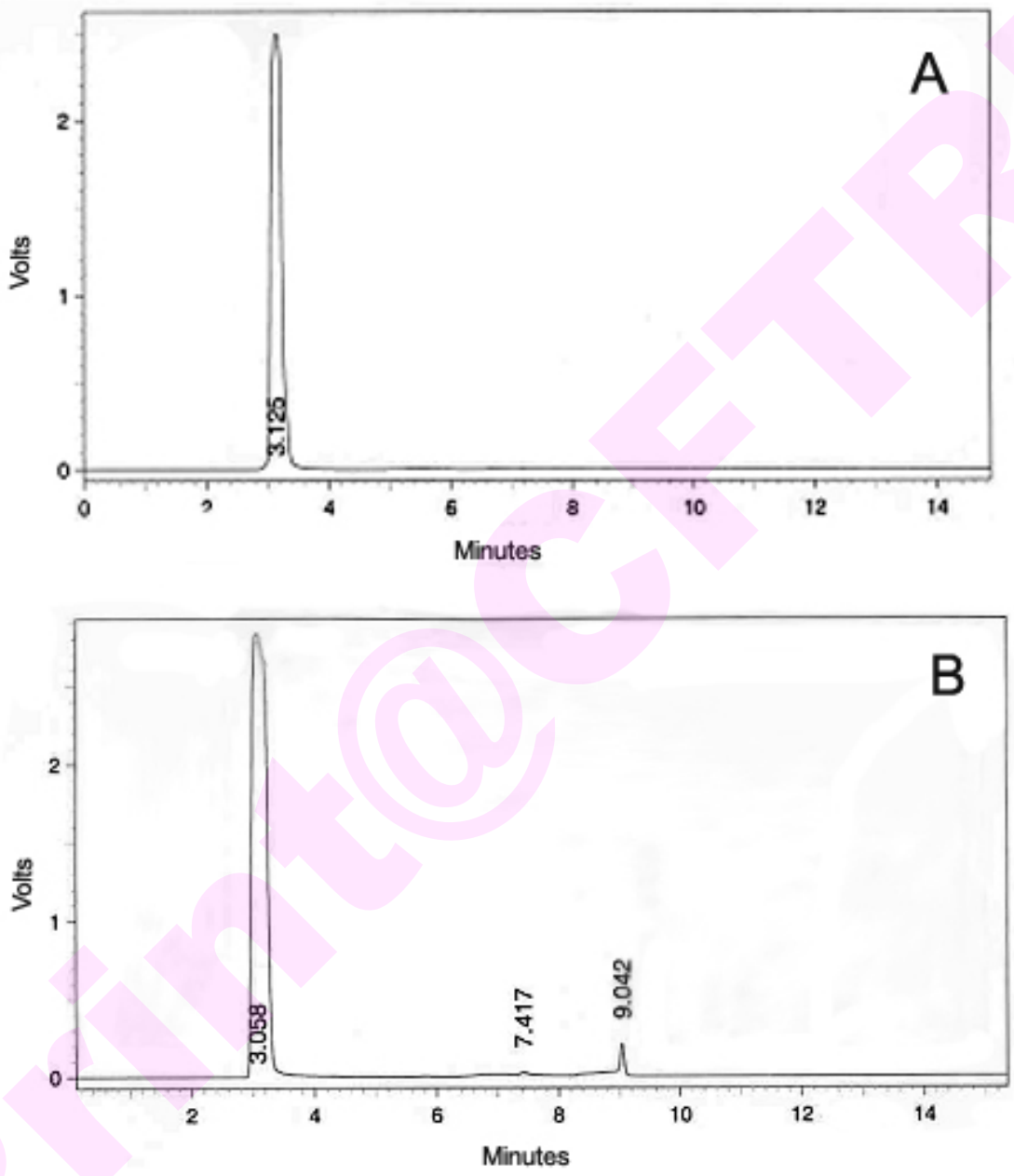


Fig. 3.9 HPLC chromatogram (at 280nm) of (A) standard epicatechin (B) antioxidant fraction of CSE showing presence of epicatechin

5.2 Protective effect of CSE and standard antioxidants (epicatechin and β-carotene) against DDVP-induced oxidative stress in rat pancreatic homogenate

Data on the effect of CSE and standard antioxidants (epicatechin and β-carotene) against DDVP-induced oxidative stress in rat pancreatic homogenate is presented in **Fig 3.10.** DDVP induced significant enhancement in ROS generation in rat pancreatic homogenate while pre-treatment with CSE / epicatechin significantly reduced the extent of ROS generated by DDVP. β-carotene (1 mM) did not offer any protection to pancreatic homogenate from DDVP induced ROS generation.

5.3 Protective effect of CSE and epicatechin against DM / DDVP-induced oxidative stress in rat pancreatic islets

5.3.1 MTT dye reduction

Protection offered by cashew nut skin extract (CSE) and epicatechin to pancreatic islets exposed to varying concentrations of DDVP for 12h in terms of MTT dye reduction is depicted in **Fig. 3.11 A & B.** CSE and epicatechin *per se* showed elevated MTT dye reduction in rat pancreatic islets while, both the compounds protected pancreatic islets from DDVP-induced cell death at all concentrations since decrease in MTT dye reduction was evident in presence of CSE and epicatechin. However, epicatechin offered greater protection than CSE. Protection to a similar extent was also offered by CSE and epicatechin against dimethoate toxicity in pancreatic islets (**Fig. 3.12 A & B**).

5.3.2 LDH leakage

Lactate dehydrogenase activity in rat pancreatic islets exposed to DDVP and DM was evaluated as an index of cytotoxicity (**Fig 3.13 & 3.14**). LDH activity, which increased by 4-5 folds in the presence of dimethoate and DDVP, was reduced to normal levels in the presence of both CSE and epicatechin at all the tested concentrations.

5.3.3 Oxidative stress in islets

We also quantified ROS levels in DDVP and DM treated pancreatic islets 30 min after addition of DCFH-DA. **Fig. 3.15** and **3.16** show the levels of ROS generation after treatment with DDVP and DM and the concentration-dependent protection offered by CSE and epicatechin. Exposure to varying concentrations of DDVP and DM for 12 h decreased intracellular GSH content in rat pancreatic islets. A marked decrease in intracellular GSH was evident at 0.25 µM of DDVP and DM (**Fig. 3.17 & 3.18**). Treatment with CSE and epicatechin protected pancreatic islets from loss of intracellular GSH and a dose-dependent protection to islets cells was evident at all the tested concentrations of CSE and epicatechin.

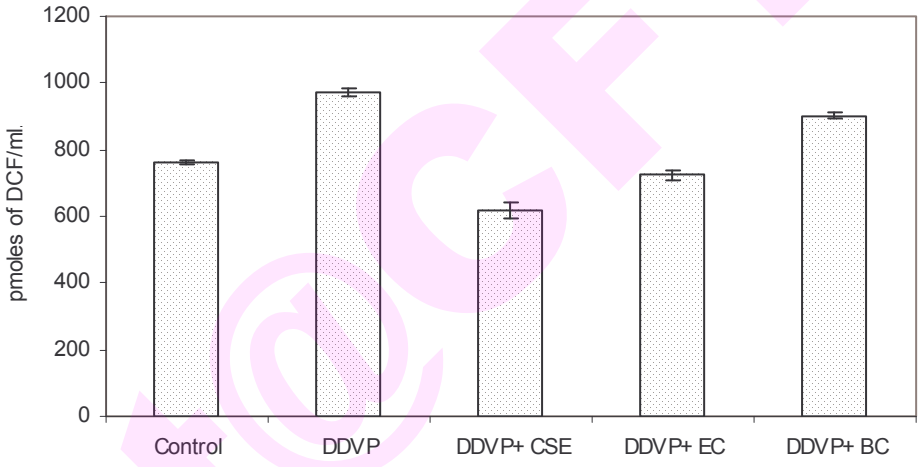


Fig 3.10 Extent of ROS generated in rat pancreatic homogenate treated with DDVP ± CSE, epicatechin or β-carotene

CSE: Cashew nut skin extract (1mg); EC: epicatehcin (1mM); BC: β-carotene (1mM)
Values are mean ± SE of three determinations

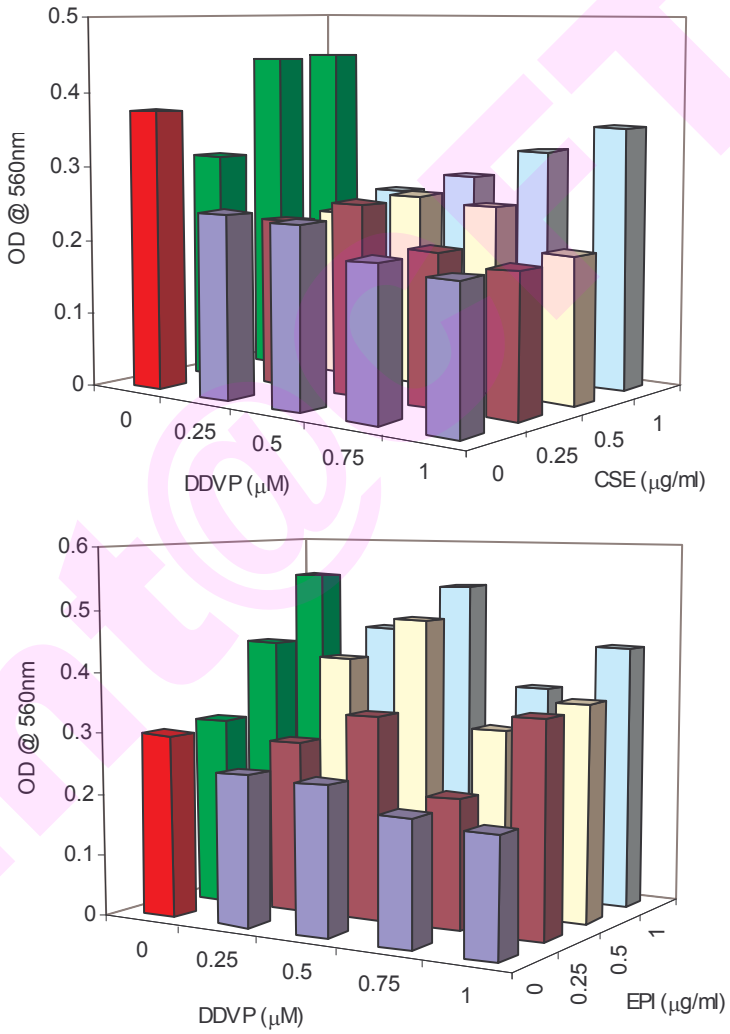


Fig. 3.11 MTT dye reduction in pancreatic islets treated with DDVP ± (A) CSE ; (B) Epicatechin (EPI)

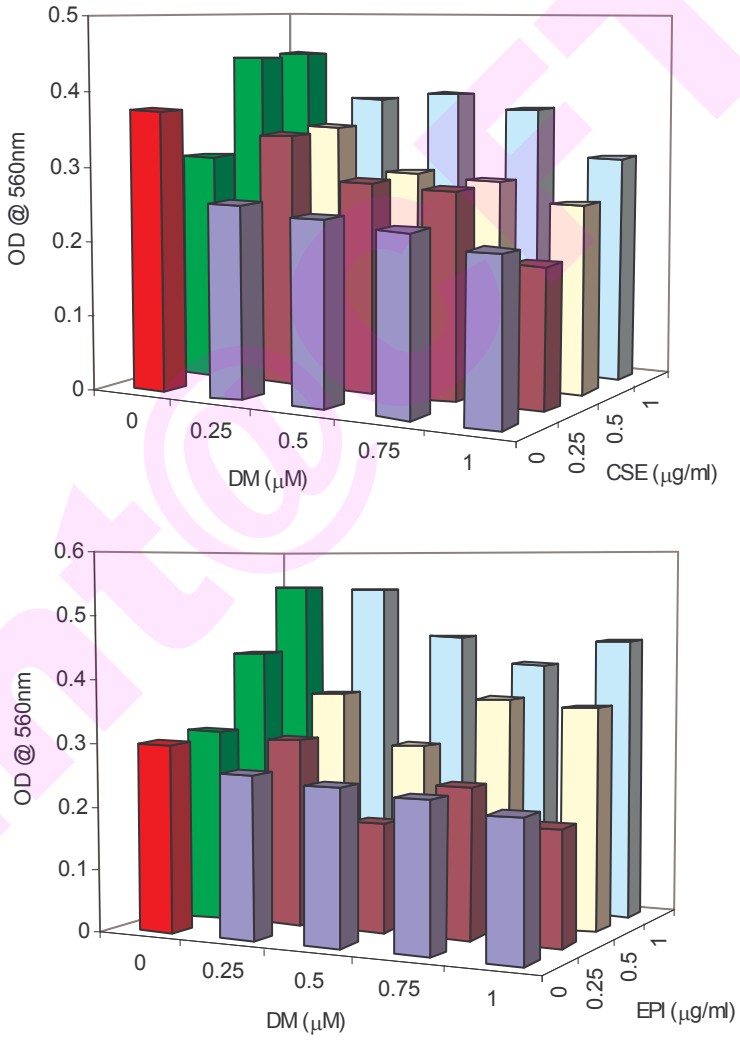


Fig. 3.12 MTT dye reduction in pancreatic islets treated with dimethoate (DM) ± (A) CSE ; (B) Epicatechin (EPI)

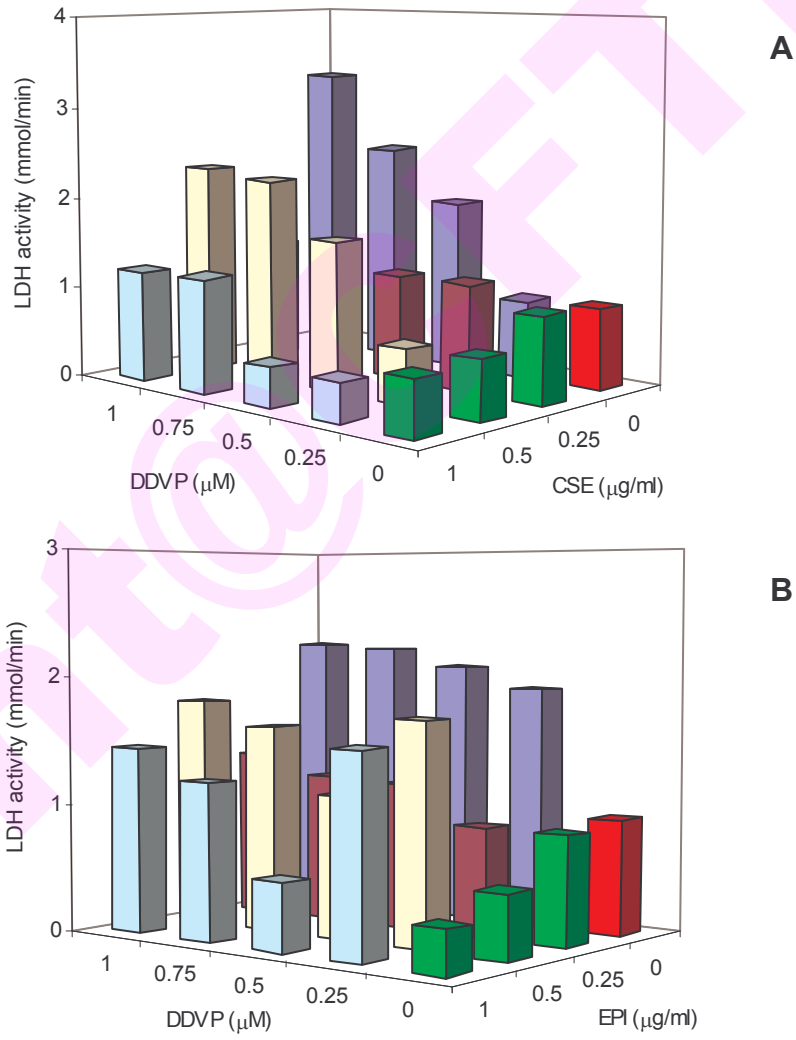


Fig. 3.13 LDH leakage in rat pancreatic islets treated with DDVP ± (A) CSE ; (B) Epicatechin (EPI)

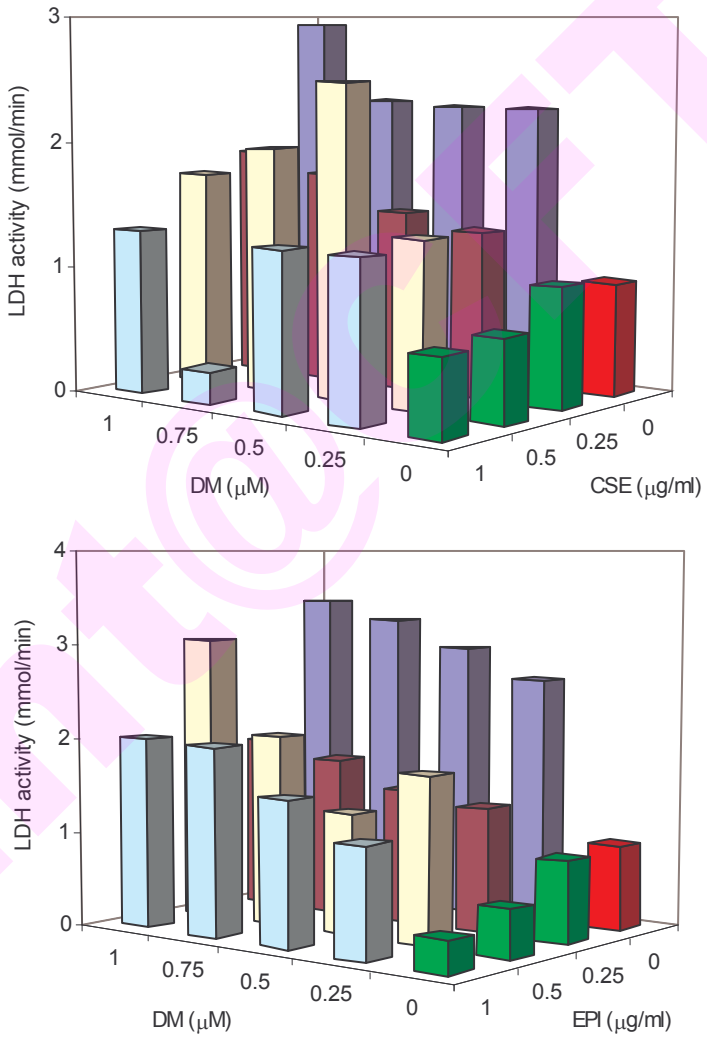


Fig. 3.14 LDH leakage in rat pancreatic islets treated with dimethoate (DM) ± (A) CSE ; (B) Epicatechin (EPI)

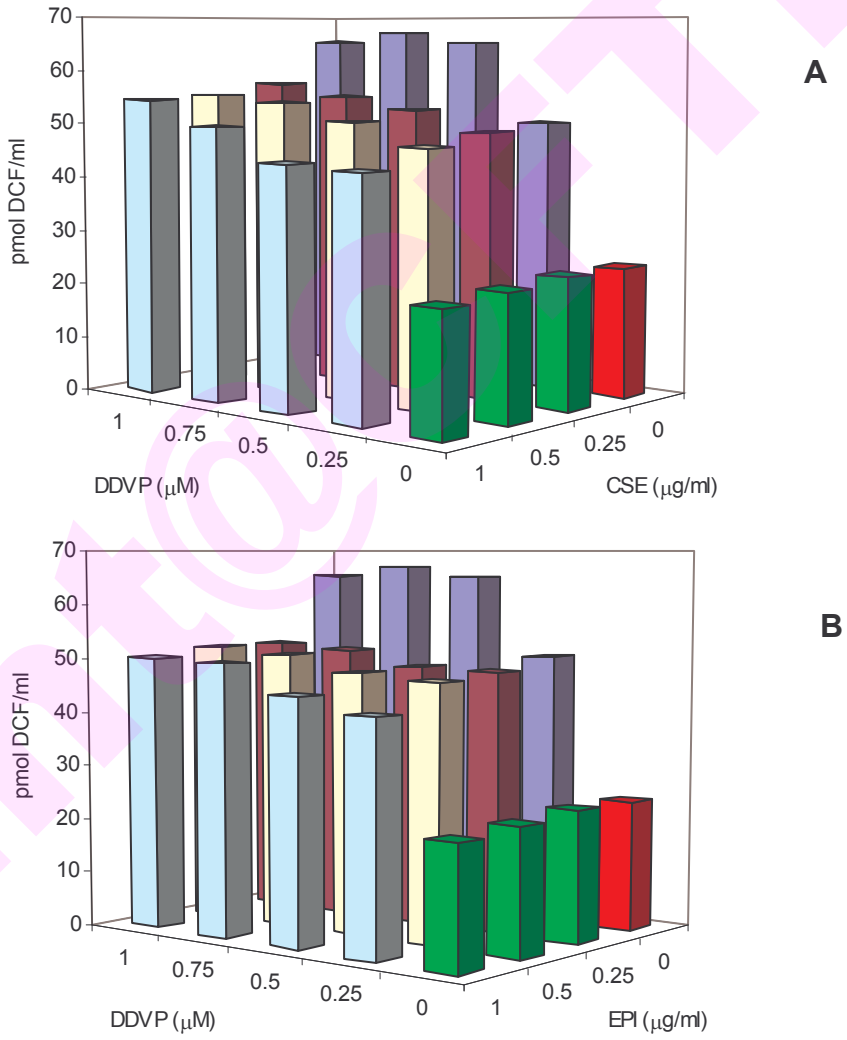


Fig. 3.15 ROS generation in rat pancreatic islets treated with DDVP ± (A) CSE ; (B) Epicatechin (EPI)

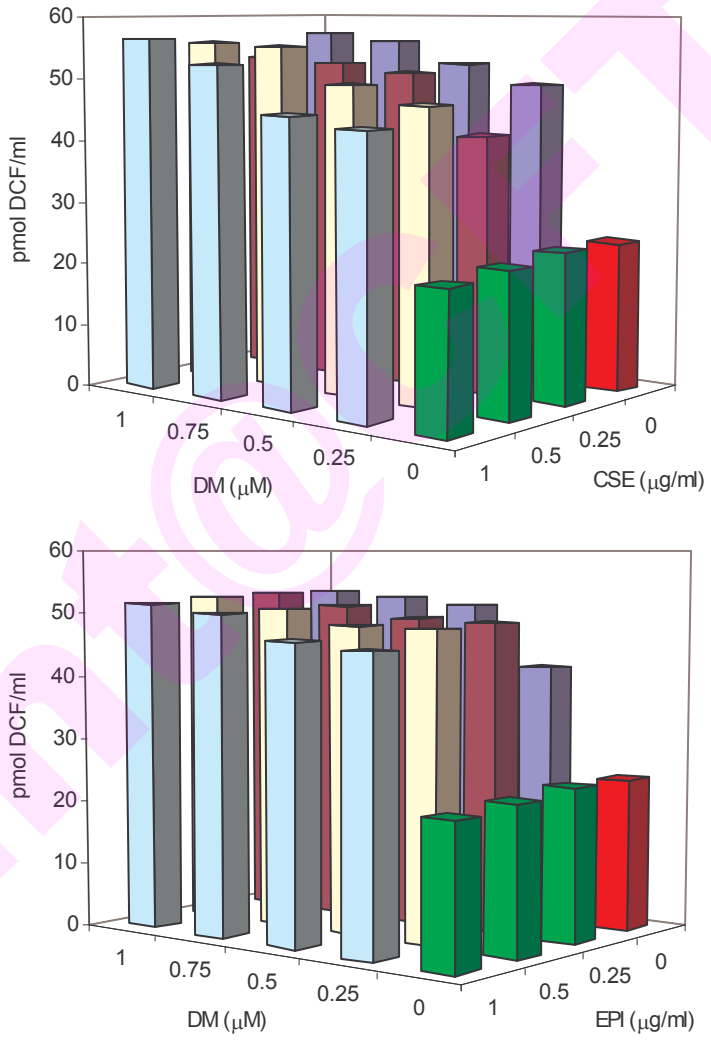


Fig. 3.16 ROS generation in rat pancreatic islets treated with dimethoate (DM) ± (A) CSE ; (B) Epicatechin (EPI)

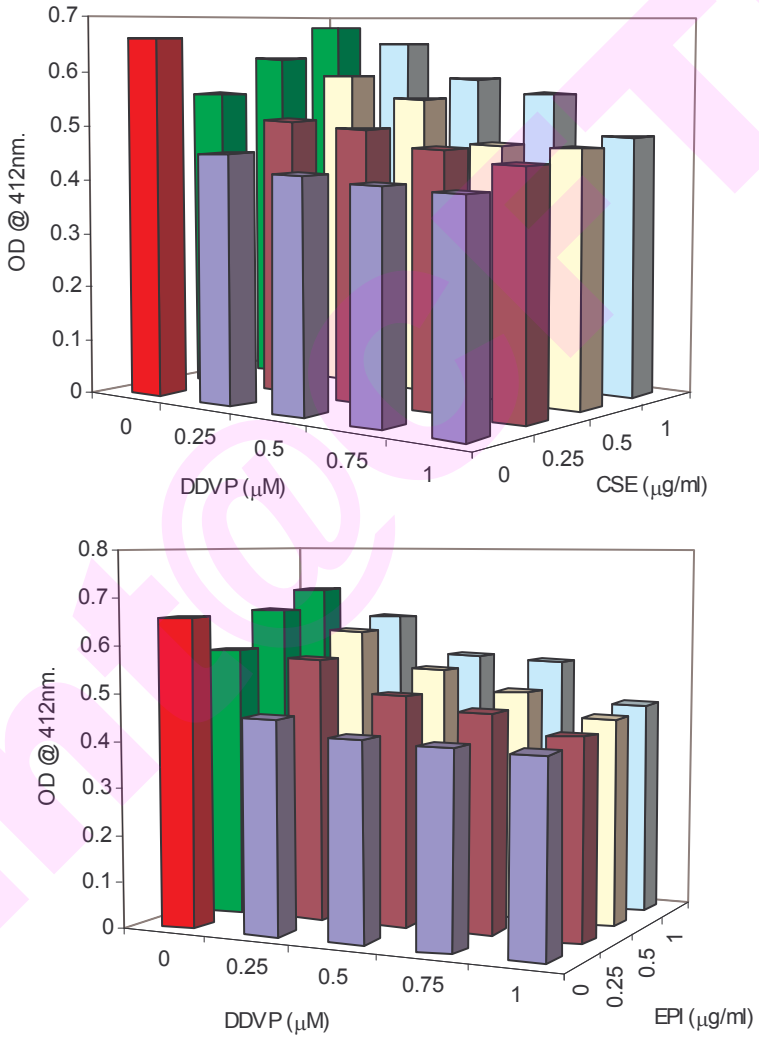


Fig. 3.17 Intracellular GSH content in rat pancreatic islets treated with DDVP ± (A) CSE ; (B) Epicatechin (EPI)

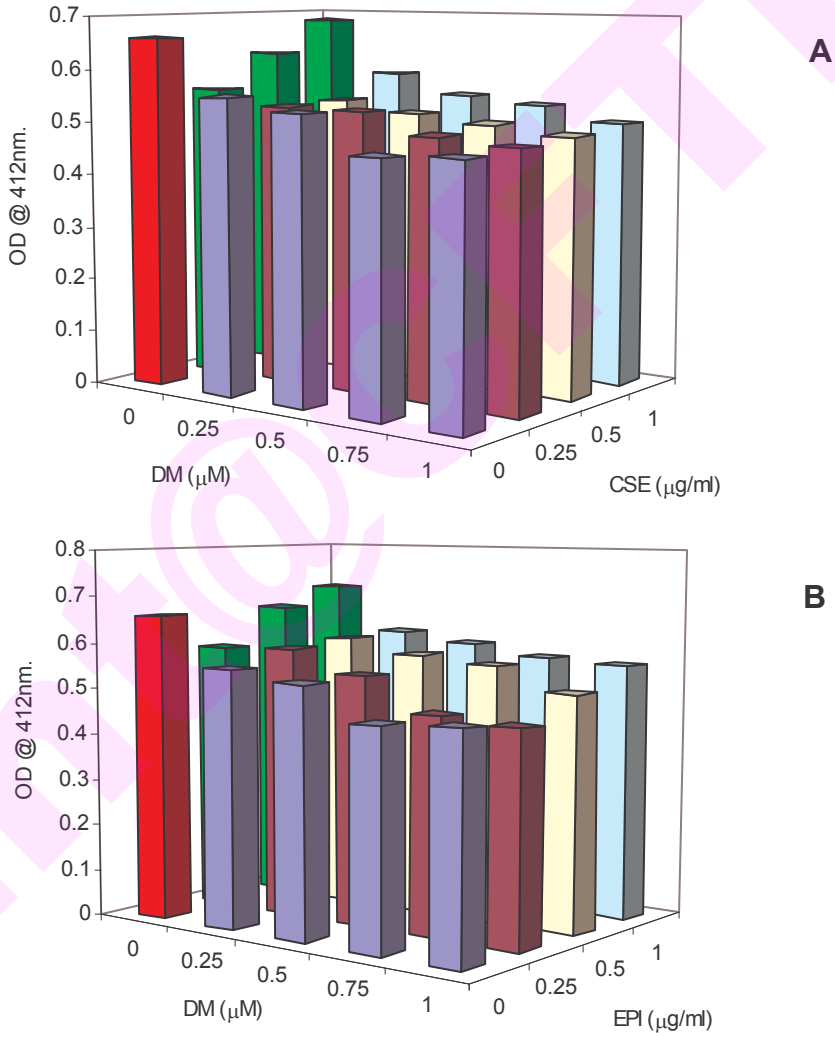


Fig. 3.18 Intracellular GSH content in rat pancreatic islets treated with dimethoate (DM) ± (A) CSE ; (B) Epicatechin (EPI)

6.0 DISCUSSION

We selected three novel plant products which are basically food processing wastes whose antioxidant activities in water extract or extract in other solvents have been reported. In this study, a comparative investigation on the antioxidant efficacy of the ethanolic extracts of the products has been carried out to select the most potent one. We selected ethanol as the solvent since it is a safer solvent than other solvents from a toxicological point of view and hence suitable for food industry.

Yield of the crude ethanolic extract of cashew nut skin (CSE) was the highest followed by that of tamarind seed coat (TSCE) and least by that of potato peel (PPE). However, this yield of PPE was comparable to that reported by Rehaman et al., (2004) for ethanolic extract of potato peel (10.1%). The yield of TSCE obtained in the present work is in accordance with that reported earlier for methanolic extract of raw tamarind seed coat (Siddhuraju, 2007). The content of polyphenolic compounds in our extracts ranged markedly from a low of 28.4 mg/g for PPE to a high of 673 mg/g of TSCE. We had earlier obtained TPC of 3.93 mg GAE/g potato peel powder in water extract which was marginally higher than that observed in the present study (2.4 mg GAE/g powder). Recently Kannatt et al., (2005) reported phenolics in ethanolic extract of potato peel as 0.875 mg catechin equivalents/g of peel.

The antioxidant activities of tamarind seed coat extracted in various solvents have been reported earlier (Tsuda et al., 1994), however, none of these studies reported the TPC in ethanolic extract of tamarind seed coat. TPC in our ethanolic extract was lower (18.5 g/100g powder) than that reported by Siddhuraju (2007) for methanolic extract of tamarind seed coat (32.4 g/100 g dry matter). Although the yield of crude ethanolic extract from tamarind seed coat in our study was second highest of the three extracts, it had the highest level of polyphenolic content.

There are various methods and modifications for evaluation of antioxidant activity of compounds and of these DPPH assay, ABTS assay, metal chelating, superoxide ($O_2^{-\cdot}$) and hydroxyl radical ($OH^{\cdot}$) radical quenching, β-carotene bleaching, linoleic acid oxidation and inhibition of lipid peroxidation are most commonly used for establishing the antioxidant activities of extracts (Amarowicz et al., 2000). We employed these assays to compare the antioxidant activities of the three extracts.

DPPH radical scavenging assay is commonly employed for evaluating the ability of antioxidants to scavenge free radicals and is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The change in absorbance at 517nm is used as the measure of the scavenging effect of a particular extract for DPPH radicals (Naczk et al., 2003). The absorbance at 517nm decreases as the reaction between antioxidant molecules and DPPH radical progresses. Hence, more rapidly the absorbance decreases, the more potent is the antioxidant activity of the extract in terms of its hydrogen atom-donating capacity (Amarowicz et al., 2004). The scavenging effect on the DPPH radical by an antioxidant, eg., phenolic compounds, depends on the chemical structure and the concentration of the antioxidant (Adom and Liu, 2002) and the effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability (Chen and Ho, 1995). The free and combined hydoxycinnamic compounds show a free radical scavenging activity greater than that of the corresponding hydroxybenzoics (Natella et al., 1999). Another antioxidant activity screening method applicable for both lipophilic and hydrophilic antioxidants is ABTS radical cation decolorization assay where the pre-formed radical monocation of 2,2'-azinobis-(3-ethylebenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) is generated by oxidation of ABTS with ammonium persulfate and is reduced in the presence of such hydrogen-donating antioxidants (Re et al., 1999).

In the present study, we have compared the ability of the ethanolic extracts of PPE, TSCE and CSE in scavenging radicals such as DPPH and ABTS. CSE appeared to be the most effective radical scavenger, as effective as BHA while, TSCE although exhibited strong radical scavenging activity, was slightly lesser efficient than CSE. Of the three extracts, PPE was found to be a relatively poor radical scavenger. Earlier, Siddhuraju (2007) reported significant DPPH radical and ABTS cation scavenging ability of the methanolic extracts of raw and dry heated seed coats of tamarind. A concentration-dependent inhibition of the DPPH radical scavenging by aqueous extract of potato peel (Singh and Rajini, 2004) has been recently reported from our laboratory.

It is reported that superoxide decomposes to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids (Dahl and Richardson, 1978). Superoxide also indirectly initiates lipid peroxidation as a result of superoxide and hydrogen peroxide, which serve as precursors of highly reactive hydroxyl radicals (Meyer and Isaksen, 1995). In the present study, all the three extracts possessed superoxide radicals scavenging activity in a dose-dependent manner. The inhibitory effect of TSCE and CSE on superoxide radical was marked. Our earlier studies with aqueous extract of PPE showed dose-dependent inhibition of superoxide radicals (Singh and Rajini, 2004). Siddhuraju (2007) reported superoxide scavenging activity of methanolic extract of tamarind seed coat extracts at high concentrations ranging from 50-200 µg /ml where as in the present study, the ethanolic extract exhibited a very strong superoxide scavenging activity indicating the greater potency of the ethanolic extract of tamarind seed coat compared to the methanolic extract. Furthermore, the ability of TSCE in scavenging the superoxide was greater than that by CSE.

The deoxyribose assay is a simple method to assess the extent of reaction of most biological molecules with hydroxyl radicals (Halliwell et al., 1987). Deoxyribose has often been used to measure the formation of $^{\cdot}OH$

radical in biochemical systems (Gutteridge, 1981*).* The assay is based on the principle that the $^{\cdot}OH$ radicals generated by Fenton systems attack deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde, which is measured as a MDA-TBA chromogen. Hence, the amount of color formation at 532 nm, is proportional to the rate of attack of $^{\cdot}OH$ on deoxyribose. Hydroxyl radical is the most reactive radical known and it can attack and damage almost every molecule found in living cells (Halliwell, 1991). Hydroxyl ($^{\cdot}OH$) and alkoxyl ($LO^{\cdot}$) radicals are believed to be the major causative species for iron-induced free radical injury in biological systems (Halliwell and Gutteridge, 1990). When hydroxyl radical attacks DNA, free radical chain reactions occur and lead to chemical alteration of deoxyribose and purines and pyrimidines. This can lead to mutations and DNA strand breakage. In addition, hydroxyl radicals stimulate the free-radical chain reaction known as lipid peroxidation, which leads to the production of toxic decomposition products (Halliwell, 1991). Consequently, the ability to diminish color formation has been adapted not only as a measurement of antioxidant properties, but also as an assay of protection of deoxyribose from oxidative damage.

The $^{\cdot}OH$ scavenging effects of the three extracts were investigated at various concentrations in the model system wherein the mixture of $FeCl_3$ and H_2O_2 was incubated with deoxyribose in phosphate buffer (pH 7.4). The ability of the three extracts to quench the hydroxyl radical seemed to relate directly to the prevention of propagation of the process of lipid peroxidation. Zhao and Jung (1995) reported that any hydroxyl radical scavenger added to the reaction mixture would compete with deoxyribose for hydroxyl radicals to an extent depending on its rate constant for reaction with hydroxyl radicals and its concentration relative to deoxyribose. According to the results obtained, the rate constant of TSCE and CSE for reaction with OH radicals was apparently greater than that of deoxyribose.

Iron, a transition metal, is capable of generating free radicals from peroxides by the Fenton reaction and is implicated in many diseases (Halliwell and Gutteridge, 1990). Fe^{2+} has also been shown to produce oxyradicals and lipid peroxidation, and reduction of Fe^{2+} concentrations in the Fenton reaction would protect against oxidative damage. Smith et al., (1992) earlier reported that those molecules that can inhibit deoxyribose degradation are also those that can chelate iron ions and render them inactive or poorly active in Fenton reaction. Plant extracts enriched in phenolic compounds are capable of complexing with and stabilize transition metal ions, rendering them unable to participate in metal-catalyzed initiation and hydroperoxide decomposition reactions (Gordon, 1990). The iron chelating ability of polyphenols is well known (Bravo, 1998) and phenolic acids such as caffeic acid, chlorogenic acid and related compounds are demonstrated to be good chelators of Fe^{3+} (Kono et al., 1998). Ferrozine can quantitatively form complexes with Fe^{2+} and in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator. PPE, TSCE and CSE were assessed for its ability to compete with ferrozine for iron (II) ions in the solution. Chelating effect was obtained with relatively very high amounts of the extracts when compared with that by EDTA. However, the results demonstrate that the extracts possessed metal chelating capacity by which they could probably reduce the concentration of the catalyzing metal in lipid peroxidation. Interestingly, PPE appeared to be relatively a more potent iron chelator followed by TSCE and CSE.

The antioxidant ability of any compound/ extract is also reported to be directly correlated with the reducing power (Tanaka et al., 1988). The reducing properties are generally associated with the presence of reductones (Duh, 1998), which exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones/ reductants are also

reported to react with certain precursors of peroxide, thus preventing peroxide formation. In the reducing power assay, the presence of reductones (antioxidants) in the sample results in the reduction of the Fe^{2+}/ ferricyanide complex to the ferrous form (Yen and Chen, 1995). The Fe^{2+} can therefore be monitored by measuring the color formed at 700nm, and increased absorbance indicates an increase in reducing power and hence the antioxidant activity. Our results with PPE also indicate that the significant antioxidant activity of the extract may also be due to its reducing power and the power to terminate free radical chain reaction.

The antioxidant assay using the discoloration of β-carotene is another widely used assay system because β-carotene is extremely susceptible to free radical mediated oxidation and β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant (Unten et al., 1997). The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color that can be monitored spectrophotometrically. The presence of any antioxidant can hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system. In the present study, all the extracts showed varying degrees of antioxidant activity in β-carotene bleaching assay, when compared with the control. A decrease in absorbance was due to the oxidation of linoleic acid and β-carotene. It showed the slowest decrease in absorbance of β-carotene in the presence of BHA followed by that in CSE, TSCE and PPE. Thus, all the three extracts offered protection to various extents. In this system also, CSE exhibited marked antioxidant activity, nearly equal to that of BHA.

The order of inhibitory potential on the peroxidation of linoleic acid was: BHA > CSE > PPE > TSCE. Siddhuraju (2007) reported that methanolic extracts from seed coat of tamarind had inhibitory action at 500 µg at final concentration in this assay system of linoleic acid peroxidation, however in the present study the ethanolic extract from tamarind seed coat had a very good inhibitory action on linoleic acid peroxidation even at 100µg which was on par with PPE, CSE and BHA. Many hydrolysable tannins from *Obeckia chinensis* have been reported to have potential antioxidative efficiency in the linoleic acid –thiocyanate system (Su et al., 1988).

The evidence presented herein suggests that the presence of polyphenolics as major constituents in the extract may be responsible for their antioxidant activity. PPE had lesser TPC and so perhaps the lesser antioxidant activity in the model systems employed. TSCE and CSE had higher TPC and their activities were on par with synthetic antioxidants and they had almost equal antioxidant activities. Our earlier studies (Singh, 2002) had revealed four major phenolic acids viz., chlorogenic acid, caffeic acid, protocatechuic acid, and gallic acid which might be responsible for its antioxidant activity. Our HPLC data has shown that epicatechin present in CSE may be responsible for its strong antioxidant activity. This is in accordance with the earlier study of Mathew and Parpia, (1970) who using two-dimensional paper chromatography had shown the presence of (+) catechin and (-) epicatechin as the major polyphenols of cashew kernel testa, accounting for about 6 and 7.5% respectively. Together, they were reported to represent more than 40% of total polyphenols. Tamarind seed coat reportedly contains four antioxidative components viz., (-) epicatechin, 2-hydorxy-3', 4'-dihydroxyacetophenone; methyl3, 4-dihydroxybenzoate and 3,4-dihydroxyphenyl acetate (Tsuda et al., 1994). On the basis of the results of this study, it is clear that all the three by-product extracts possess differing degrees of antioxidant activity in various antioxidant systems *in vitro*. There may be several other factors and

components contributing to these varying activities. Since all the extracts were derived from waste by products, they could serve as a source of inexpensive natural antioxidants. However, their efficacy as antioxidants *in vivo* has to be established along with their safety.

In biological systems, lipid peroxidation (oxidative degradation of polyunsaturated fatty acid in the cell membranes) generates a number of degradation products, such as malondialdehyde (MDA), and is found to be an important cause of cell membrane destruction and cell damage (Yoshikawa, et al., 1997). MDA, one of the major products of lipid peroxidation, has been extensively studied and measured as a marker of oxidative stress (Janero, 1990). Inhibition of LPO may be considered as one of the tests to determine the antioxidant activity of a compound (Cos et al., 2000). Marked inhibition of TBARS formation was noted with TSCE and CSE than with PPE, while Singh and Rajini (2004) reported the inhibitory response of aqueous extract of PPE between 0.75 mg and 5 mg. The extracts of seed coats of red beans and black beans, which contain phenolic substances, have been reported to have strong antioxidant activity against lipid peroxidation (Muanza et al., 1998).

Since of the three extracts, CSE was the most potent, we selected CSE for further studies in *in vitro* systems. The protective effect of CSE and standard antioxidants viz., epicatechin and β-carotene was studied in rat pancreatic homogenate and rat islets exposed to DM and DDVP in order to see the extent of protection offered by the antioxidants *in vitro* to pancreatic tissue. The concentrations of DDVP and DM employed in this study were those shown to induce cell death and OS in pancreatic homogenate and islets in our earlier study (*Chapter IA*). Incubation of isolated rat pancreatic islets with DDVP caused loss of cell viability as evident by LDH leakage into the medium, along with a marked decrease in glutathione and increased ROS production. The islets exposed to CSE and epicatechin showed normal islet morphology, integrity and viability indicating non-toxic nature of CSE and epicatechin at the

tested concentrations. However CSE / epicatechin treatment along with either DM or DDVP protected the islets from OS and cell death. Epicatechin has been demonstrated to protect pancreatic islets both *in vitro* and *in vivo* from toxic effects of STZ (Kim et al., 2003). Further, epicatechin, a member of a group of phenolic compounds collectively known as 'catechins' that are present in tea and belong to the flavonoid family, has been reported to possess insulin-like activity (Rizvi and Zaid, 2001).

Based on the results of our studies it can be concluded that PPE, TSCE and CSE are novel sources of potent biological antioxidants. Of the three, CSE, being 'epicatechin-rich', is a powerful antioxidant and it also possesses the propensity to offset DM and DDVP induced oxidative damage in rat pancreatic tissue *in vitro.*

7.0 SUMMARY

1. Ethanolic extracts of the three materials differed in their yield ranging from 8.45 ± 0.32 to 45.08 ± 0.72 g /100 g powder.
2. The phenolic content of the extracts ranged from 28.4 ± 1.0 to 673 ± 15 mg GAE/ g extract. Cashew nut skin yielded relatively higher extract with higher total polyphenols as compared to both potato peel and tamarind seed coat.
3. DPPH and ABTS radical scavenging ability of CSE was comparable to that of BHA. TSCE was slightly lesser efficient than CSE while PPE was found to be a relatively poor radical scavenger.
4. All the three extracts exhibited scavenging of superoxide radicals in a concentration-dependent manner.
5. Both CSE and TSCE inhibited deoxyribose oxidation at much lower concentrations as compared to PPE.
6. PPE was a potent iron chelator than TSCE and CSE.

7. In β-carotene bleaching and peroxidation of linoliec acid, the inhibitory potential of the extracts and BHA was: BHA>CSE>PPE>TSCE.
8. Marked inhibition of TBARS formation in rat brain homogenate was offered by TSCE and CSE than PPE.
9. HPLC analysis of the 'antioxidant rich ethanolic extract of CSE' showed epicatechin to be one of the major components and hence CSE was selected for further studies.
10. The potential of CSE, epicatechin and β-carotene were studied for their ability to protect rat pancreatic homogenate and isolated rat pancreatic islets against DM/ DDVP toxicity *in vitro*.
11. CSE and epicatechin significantly reduced the extent of ROS generated by DDVP in pancreatic homogenate, while β-carotene (1mM) failed to offer any protection.
12. A marked decrease in the MTT dye reduction was evident in the islets exposed to DM and DDVP, while treatment with CSE and epicatechin elevated the MTT dye reduction in the pesticide treated islet cells.
13. LDH activity was increased by 4-5 folds on exposure to DM and DDVP, while this activity was restored to normal levels in the presence of CSE and epicatechin at the tested concentrations.
14. Both CSE and epicatechin effectively offset the DDVP/ DM induced ROS generation in the system.

SECTION B

AMELIORATIVE EFFECT OF CASHEW NUT SKIN EXTRACT ON OPI-INDUCED PANCREATIC DAMAGE AND ALTERED GLUCOSE HOMEOSTASIS

1.0 INTRODUCTION

Free radicals play an important role in the toxicity of pesticides (Abdollahi et al., 2004b). Pesticides stimulate peroxidation of cellular membranes by different mechanisms: direct initiation by free radicals produced by metabolism of the chemical, indirect initiation by the production of reactive forms of oxygen during their metabolism, inhibition of enzymatic systems of defense involved in the control of reactive oxidizing entities, and destruction of natural antioxidants (Banerjee et al., 1999). It has been well demonstrated that the enzymes associated with antioxidant defense mechanism are altered under the influence of OPI and that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced oxidative stress (Akhgari et al., 2003; Ranjbar et al., 2002, 2005; Abdollahi et al., 2004b). Our studies (*Chapter IB, Chapter II A*) have clearly demonstrated the involvement of oxidative stress in both dimethoate and dichlorvos induced pancreatic toxicity and ensuing alteration in glucose homeostasis in rats.

Although animals have their own antioxidant defense systems, the defense can be externally strengthened. This might be especially true for the pancreas, since it has a relatively weak intrinsic defense system against oxidative stress (Tiedge et al., 1997). Earlier studies (Altuntas et al., 2002a, b; Gultekin et al., 2001) have shown that antioxidant vitamins E and C could decrease lipid peroxidation caused by various OPI. Furthermore, certain studies have demonstrated that activities of pancreatic enzymes altered by OPI were partially prevented by vitamins E and C (Mollaoglu et al., 2003; Gokalp et al., 2003). Antioxidants have also been shown to exert beneficial effects on

β-cell function in diabetes and thus prevent or delay β-cell dysfunction by providing protection against glucose toxicity (Kaneto et al., 1999).

At present there is considerable interest in free radical mediated damage to biological system due to pesticide exposure, but there is lack of consensus as to which determinations are best used to quantify future risk of sub chronic xenobiotic exposure, and the use of natural plant products as antioxidant interventions. A study of some commonly used plant products as antioxidants against xenobiotic-induced oxidative stress therefore appears to be of interest.

Earlier (*Chapter III A*), we screened extracts of three plant products for their antioxidant activities in chemical and biological model systems *in vitro*. Our studies clearly demonstrated that ethanolic extract of cashew nut skin (CSE) is a potent antioxidant, rich in epicatechin and confers protection to islet cells *in vitro* against DM induced oxidative damage. Recently, administration of epicatechin has been demonstrated to protect pancreatic islets against STZ-induced damage in rats (Kim et al., 2003). Hence, we selected cashew nut skin extract for studying its ameliorative effect on dimethoate induced pancreatic damage and altered glucose homeostasis *in vivo* in experimental rats.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Thiobarbituric acid (TBA), reduced glutathione (GSH), oxidized glutathione (GSSG), xanthine oxidase, glutathione reductase (GR), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), 2',7'-Dichlorofluorescein (DCF), *p*-nitrophenyl acetate (PNPA), *p*-nitrophenol (PNP) and cytochrome 'C' were procured from M/s Sigma Chemical Co., (St. Louis, MO, USA). Xanthine, ethylenediamine tetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), nicotinamide adenine dinucleotide phosphate-reduced (NADPH), nicotinamide adenine dinucleotide–reduced (NADH), trichloroacetic acid (TCA), 1-chloro-2,4- dinitrobenzene (CDNB),

2,6-dichlorophenol indophenol (DCPIP), methylene blue and acetylthiocholine iodide were procured from M/s Sisco Research Lab., (Mumbai, India). Dimethoate (Technical grade, 97.4 % pure) was a gift from M/s Hyderabad Chemical Supplies Ltd., (Hyderabad, India). Glucometer ("Accu-Check") was procured from M/s Roche diagnostics, (GmbH, Mannheim, Germany). Amylase kit was procured from M/s Span diagnostics (Mumbai, India). All other chemicals used were of analytical grade.

2.2 Animals and care

Adult male rats (CFT-Wistar strain, 8 week old, 180 ± 5g) were randomly drawn from the stock colony of our Institute animal house facility and were housed individually in polypropylene cages under standard housing conditions (controlled atmosphere with 12:12-hour light/dark cycles, 50% ± 5% humidity, and an ambient temperature of 25± 2º C). The rats were acclimatized for 1 week prior to the start of the experiment. Rats were maintained on a commercial pellet diet (M/s Saidurga Feeds and Foods Pvt. Ltd., Bangalore, India) *ad libitum* and had free access to water. All procedures with animals were conducted strictly in accordance with guidelines approved by the Institute Animal Ethical Committee, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. During the experiments, maximum care was taken to minimize animal suffering and in addition, the number of rats used was kept at minimum.

2.3 Preparation of cashew nut skin extract

Cashew nut skin extract (CSE) was prepared as described earlier (*Section 2.3, Chapter III A*).

2.4 Pesticide solution

Stock solution of dimethoate (20mg/ml) was prepared in normal saline and used for intragastric (oral) administration.

3.0 EXPERIMENTAL PROCEDURE

3.1 Amelioration of Dimethoate toxicity by CSE

Rats were grouped by randomized design into four groups (n=6). They were maintained on pellet diet and water *ad libitum.* Rats of Group I (control) received saline daily for eight weeks while, rats of the second group were administered oral dose of DM (40mg/kg b.w./d, 1/10 of LD_{50}; 400mg/kg b.w.) in saline for eight weeks. Rats of group III received CSE in saline at 20mg/kg b.w./day for eight weeks. Rats of group IV received DM (40mg/kg b.w) daily and after 1h, they were administered with CSE at 20mg/kg b.w/d in saline for eight weeks. Random blood glucose was measured weekly in rats of all the groups. All the experimental rats were subjected to oral glucose tolerance test at the end of the eight weeks and were sacrificed. Immediately after euthanizing, blood was collected, for separation of serum, pancreas was excised, washed in ice-cold saline and stored at -20º C until further use. The biochemical analyses included- blood glucose, lipase and amylase in serum and pancreas , markers of oxidative stress, AChE and diaphorase in pancreas.

3.2 Preparation of pancreatic homogenates

10 or 20 % (w/v) homogenates of pancreas were prepared in various buffers as per the requirements of a given assay and the homogenate was centrifuged at 9000 x *g* at 4º C for 20 min. The supernatant was used for various biochemical analyses.

3.3 Serum

Blood was drawn by cardiac puncture into tubes and processed separately for obtaining serum as described earlier (*Section 3.5, Chapter II A*).

3.4 Oral glucose tolerance test

Oral glucose tolerance was performed on rats from all the experimental groups as described earlier (*Section 3.3, Chapter II A)*.

4.0 ASSAY METHODS

4.1 Blood glucose

Blood glucose was estimated using glucometer as described earlier (*Section 4.1, Chapter I B*).

4.2 Lipase (EC 3.1.1.3) *(Young et al., 1978)*

Lipase activity was estimated by monitoring the hydrolysis of para nitrophenyl acetate to para nitrophenol as described earlier *(Section 4.2, Chapter II B).*

4.3 Amylase (EC 3.2.1.1) *(Street and Close, 1956)*

The enzyme activity was measured using a commercial kit as described earlier (*Section 4.3, Chapter II A*).

4.4 Reactive oxygen species (ROS) *(Keston and Brandt, 1965)*

ROS in tissue was estimated by DCFH-DA oxidation method as described earlier (*Section 4.8, Chapter II A*).

4.5 Lipid peroxidation *(Buege and Aust, 1978)*

Lipid peroxidation in pancreas was estimated by measuring the thiobarbituric acid reactive substances as described earlier (*Section 4.9, Chapter II A*).

4.6 Reduced glutathione (GSH) *(Benke et al., 1974)*

Reduced glutathione in pancreas was estimated as described earlier (*Section 4.3, Chapter I B*).

4.7 Acetylcholinesterase (EC 3.1.1.7) *(Ellman et al., 1961)*

Acetylcholinesterase enzyme in serum and other tissues viz., brain, liver, adrenal and pancreas was measured as described earlier (*Section 4.2, Chapter I B*).

4.8 Antioxidant enzymes in pancreas

The activities of antioxidant enzymes viz, CAT, SOD, GST, GPX in pancreas was determined according to the procedures described earlier (*Section 4.0, Chapter IIA*).

4.9 DT-diaphorase (EC 1.6.99.3) *(Ernestr, 1967)*

DT-diaphorase was measured using NADH as the electron donor and 2,6-dicholoropenolindophenol as the electron acceptor as described earlier (*Section 4.21, Chapter II A*).

4.10 NADPH Diaphorase (EC 1.6.99.1) *(Huennckens et al., 1957)*

This method is based on reduction of methylene blue to leukomethylene blue during the transfer of hydrogen from NADPH to NADPH-diaphorase. Assay was conducted as described in (*Section 4.22, Chapter II A*)

4.11 Protein estimation *(Lowry et al., 1951)*

Protein content of tissue homogenate and serum was estimated as described earlier (*Section 4.4, Chapter I B*).

4.12 Statistical analysis

Mean and standard error values were determined for all the parameters studied. Results were statistically analyzed by analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was performed to determine the significant difference among the groups. Statistica software from STATSOFT, USA was used to analyze the data. Results were expressed as mean ± SE from six rats in each group. The p values < 0.05 were considered significant.

5.0 RESULTS

5.1 Amelioration of Dimethoate toxicity by CSE

5.1.1 Signs and symptoms

Administration of CSE did not affect the body weight gain in rats or induced any signs of toxicity. DM failed to induce any distinctive clinical signs of toxicity or mortality.

5.1.2 Blood glucose levels

Data on blood glucose levels in rats of various groups at various sampling periods is presented in **Fig. 3.19 A**. CSE supplements *per se* did not alter the blood glucose levels in rats while, blood glucose levels among DM treated rats were significantly elevated (119 ± 5 mg/dl vs. 92 ± 4 mg/dl) at all sampling weeks. However, DM treated rats receiving CSE supplements, showed blood glucose levels which were comparable to those of controls (102 ± 6 mg/dl vs. 92 ± 4 mg/dl).

5.1.3 Oral glucose tolerance

Blood glucose levels monitored in various groups of rats over 3 h period following glucose overload are presented in **Fig. 3.19 B**. Blood glucose levels in rats of all groups remained elevated up to 60 min. Subsequently, in control rats it decreased towards normal levels at further time points. No significant differences in blood glucose levels were seen among control and rats supplemented with CSE at all time points during glucose tolerance test. However, blood glucose levels of DM treated rats were consistently higher (129 ± 6 mg/dl) at all time points. Interestingly, the blood glucose level of DM treated rats receiving CSE supplement was restored to normal level at 90 min and beyond.

5.1.4 Pancreatic damage markers

Activities of marker enzymes of pancreatic damage such as lipase and amylase in the experimental rats are depicted in the **Fig. 3.20 & 3.21**. CSE supplements *per se* did not alter either serum or pancreatic enzyme activities. Significantly ($p< 0.05$) higher activities of serum lipase (47 %) and amylase (3.6 fold) were evident in DM treated rats while, the activities of lipase (2.4 folds) and amylase (12%) in pancreas were significantly lowered. In DM treated rats supplemented with CSE, only serum lipase activity (5.96 ± 0.13 nmol PNP/min/mg protein) was restored to normal levels. Further, serum amylase activity which was significantly elevated in DM treated rats were normalized in DM + CSE group (49.7 ± 0.69 units) and similarly the activity of amylase in pancreas was restored to normal levels.

5.1.5 Oxidative stress in pancreas

CSE *per se* had no effect on both pancreatic ROS and TBARS levels as they were comparable to those of control rats (**Fig. 3.22 A & B**). Treatment with DM caused significant increase in both ROS (1.88 ± 0.16 pmoles of DCF) as well as TBARS (38 ± 0.75 nmoles of MDA) levels in pancreas. In DM+CSE rats, a dramatic restoration in ROS levels in pancreas was observed (100 % protection) while the TBARS levels were only marginally (14 %) lowered.

5.1.6 Antioxidant levels in pancreas

Reduced glutathione levels and activities of various antioxidant enzymes in pancreas of rats of various groups are presented in **Table 3.4 & Fig. 3.23**. CSE supplements *per se* had no measurable effect on GSH levels and on antioxidant enzymes. In DM treated rats, the GSH levels were significantly decreased (66%) compared to those of controls (3.36 ± 0.12 mg/g tissue). CSE supplementation in DM treated rats significantly offset the decrease in GSH levels (2.83 ± 0.08 mg/g tissue) in pancreas. In DM treated rats there was a marked elevation in the activities of GST (2-fold), GR (1.5- fold) and SOD

(2- folds). While the activity of GPX was markedly diminished (by 50%), activity of CAT was significantly diminished (by 42 %). Interestingly, in DM+CSE rats, activities of all the antioxidant enzymes were restored to normalcy.

5.1.7 AChE and phase II enzymes in pancreas

Data on the effect of CSE supplements on pancreatic AChE and Phase II Xenobiotic metabolizing enzymes viz., NADPH-diaphorase and DT-diaphorase is presented in **Table 3.5 & Fig. 3.24**. While CSE *per se* had no effect on AChE and phase II enzymes, DM treatment significantly reduced the pancreatic AChE activity (71% inhibition). CSE supplements in DM treated rats offered significant protection to AChE activity as only 57% inhibition in the activity of AChE was observed. Rats treated with DM showed enhanced activities of NADPH-diaphorase (92%) and DT-diaphorase (40%). Interestingly, in DM+CSE rats activities of both xenobiotic metabolizing enzymes were restored to normalcy.

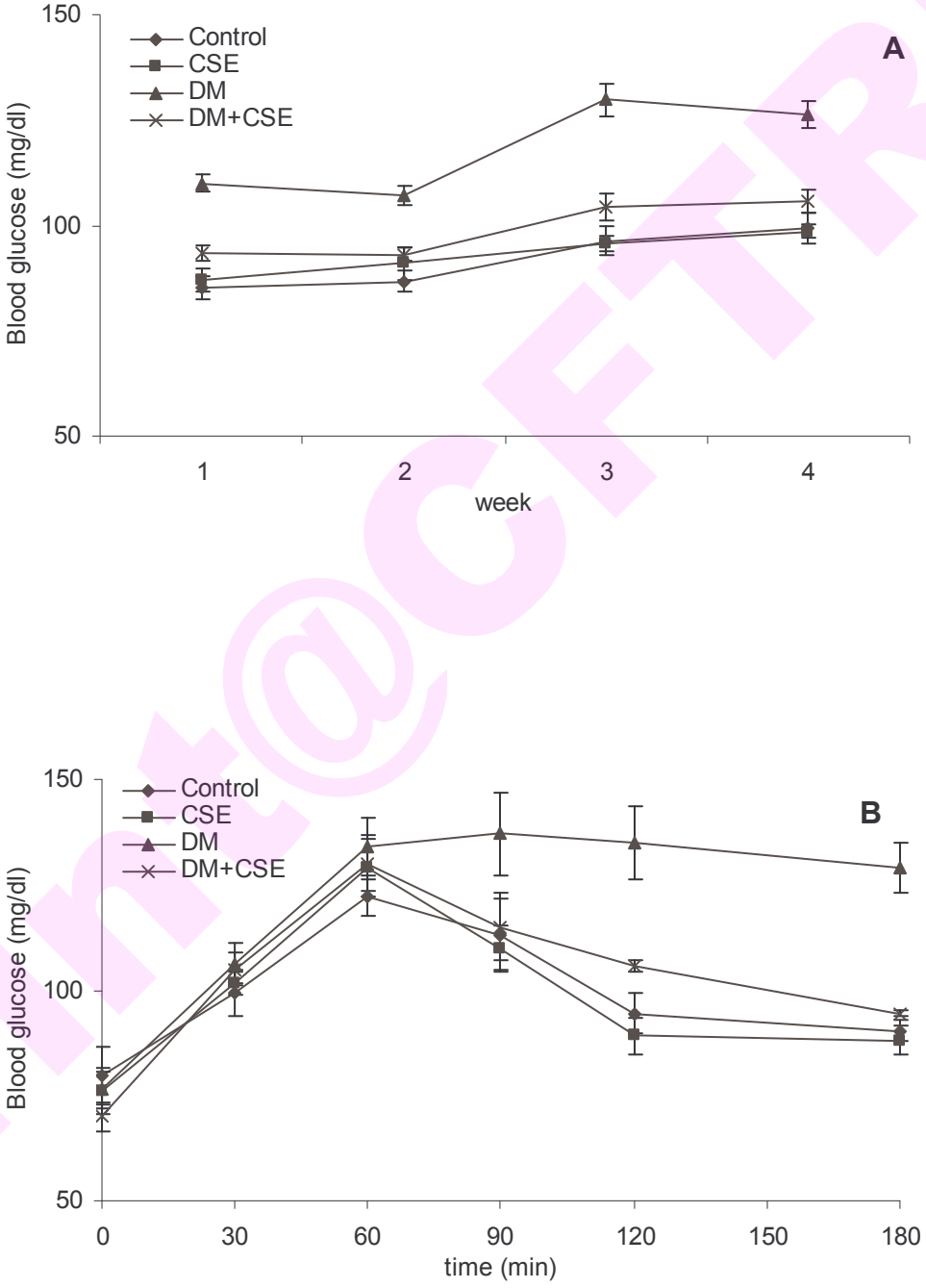


Fig. 3.19 Effect of cashew nut skin extract (CSE) on (A) blood glucose and (B) oral glucose tolerance in control and dimethoate (DM : 40 mg/ kg b.w/d for 8 weeks) treated rats

Values are mean ± SEM (n=6)

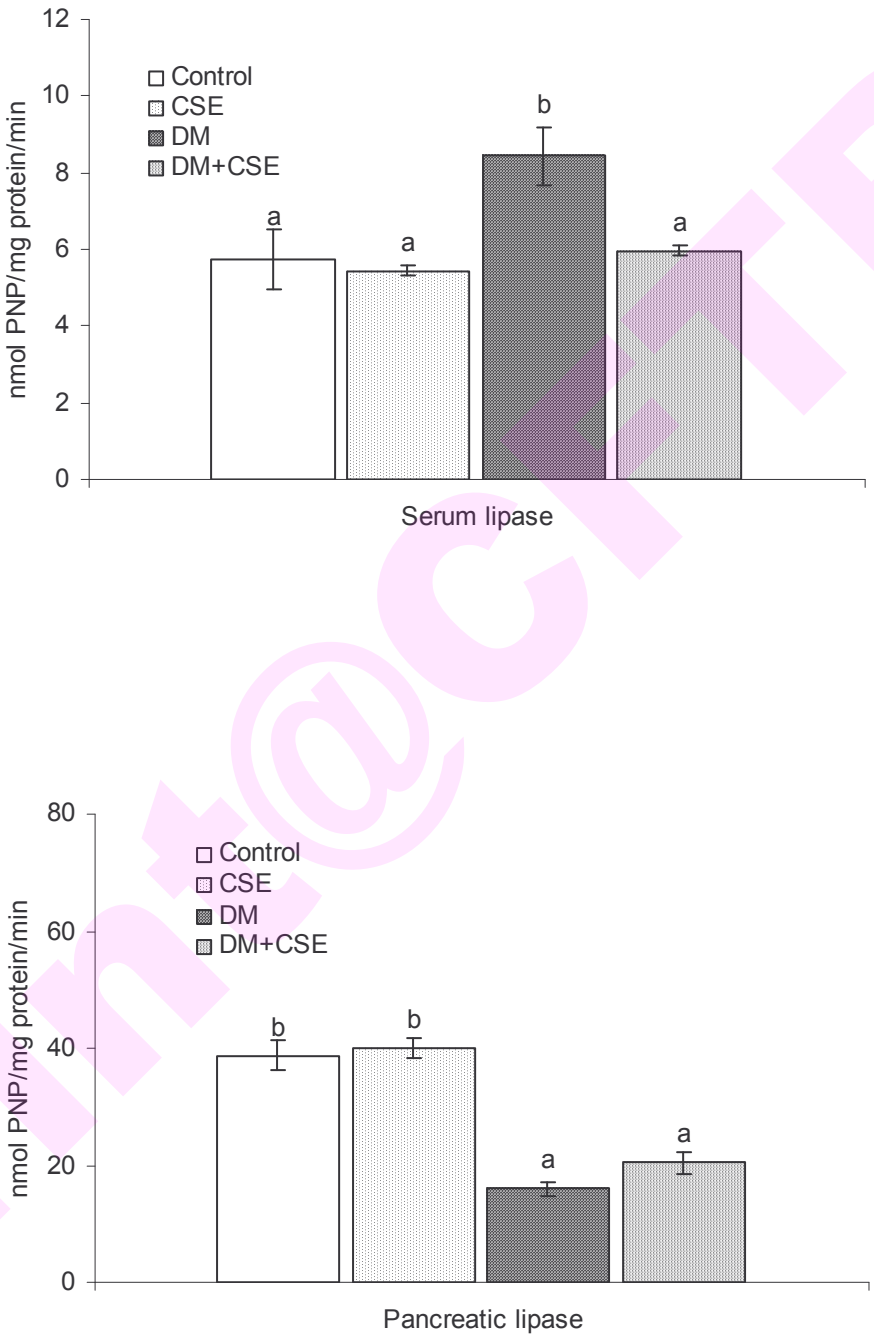


Fig. 3.20 Effect of cashew nut skin extract (CSE) on serum and pancreatic lipase in control and dimethoate (DM : 40 mg/ kg b.w/d for 8 weeks) treated rats

Values are mean ± SEM (n=6). The bars with different letter are significantly different p< *0.05* by DMRT

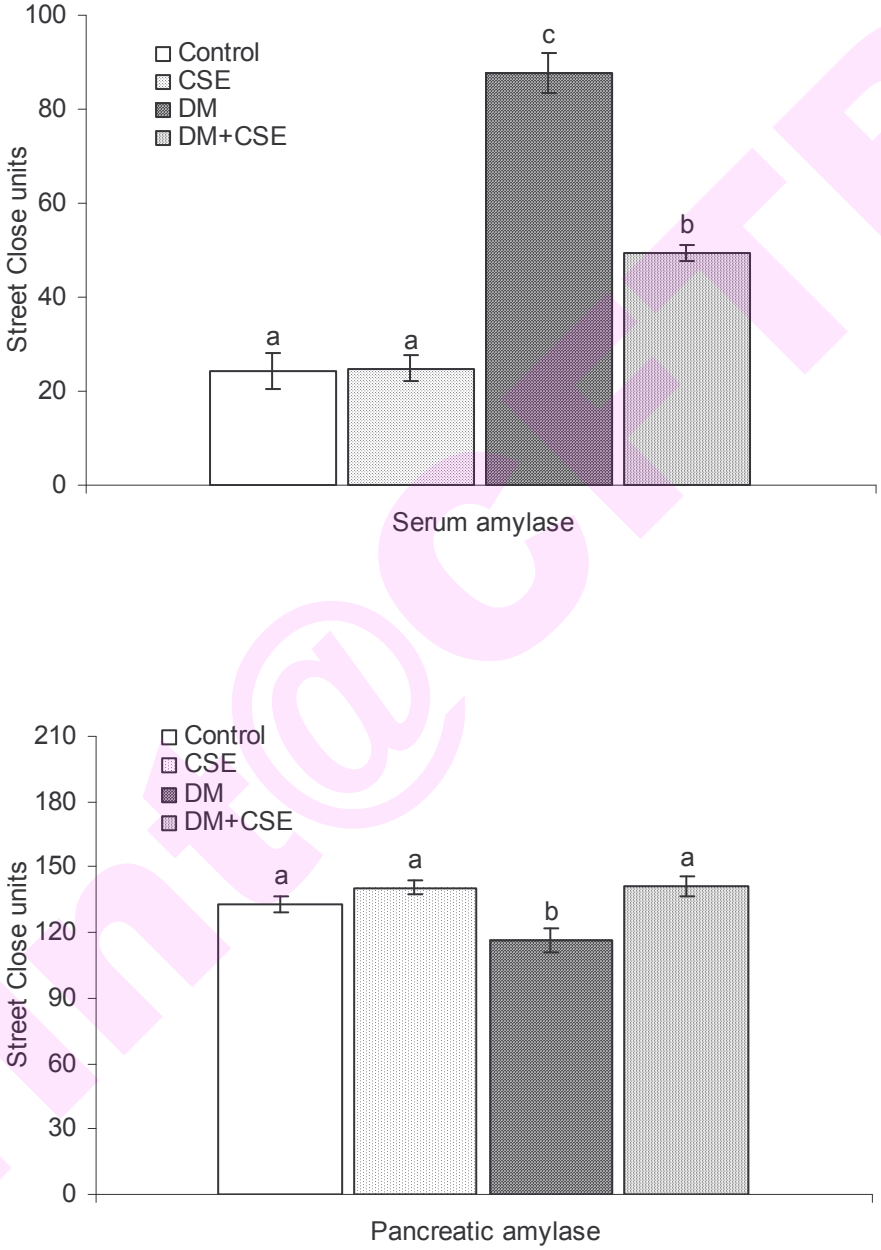


Fig. 3.21 Effect of cashew nut skin extract (CSE) on serum and pancreatic amylase in control and dimethoate (DM : 40 mg/ kg b.w/d for 8 weeks) treated rats

Values are mean ± SEM (n=6). The bars with different letter are significantly different p< *0.05* by DMRT

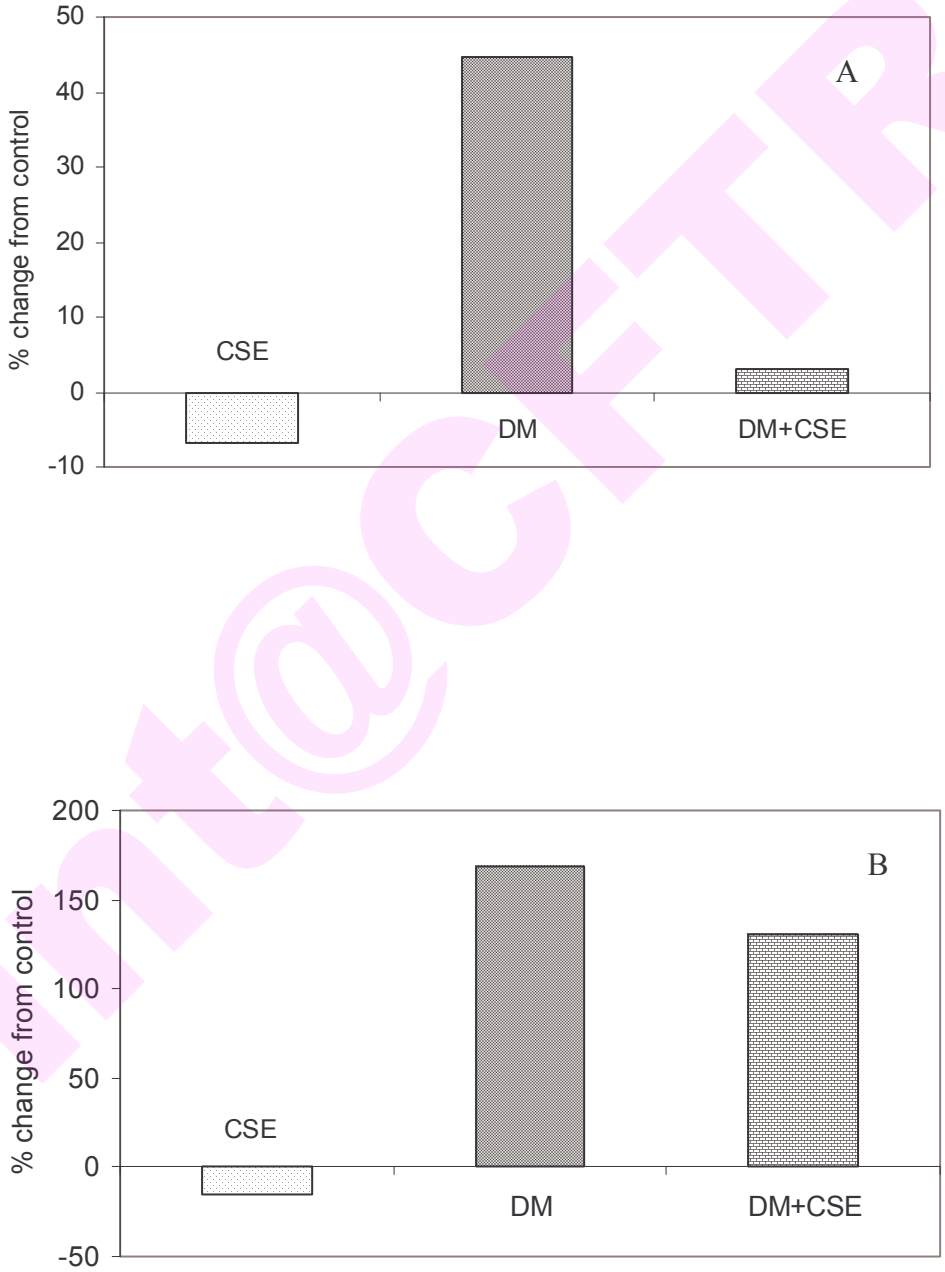


Fig. 3.22 Effect of cashew nut skin extract (CSE) on (A) ROS generation and (B) extent of lipid peroxidation in dimethoate (DM : 40 mg/ kg b.w/d for 8 weeks) treated rats

Values are mean ± SEM (n=6). The bars with different letter are significantly different p< *0.05* by DMRT

Table 3.4 Reduced glutathione levels and antioxidant enzyme activities in rats administered dimethoate (DM : 40 mg/kg b.w/d) and CSE (20 mg/kg b.w/d) for 8 weeks

	CAT[1]	SOD[2]	GR[3]	GPX[3]	GST[3]	GSH[4]
Control	9.07[a] ± 0.98	24.62[a] ± 0.79	17.50[a] ± 1.60	27.18[b] ± 5.24	0.029[a] ±0.004	3.36[a] ± 0.12
CSE	8.98[a] ± 0.85	25.44[a] ± 0.54	16.89[a] ± 1.42	26.14[b] ± 3.73	0.031[a] ±0.003	3.48[a] ± 0.11
DM	5.23[b] ± 1.09	51.48[b] ± 6.23	25.30[b] ± 1.30	13.85[a] ± 2.20	0.065[b] ±0.003	2.21[c] ± 0.14
DM+CSE	9.98[a] ± 1.09	28.69[a] ± 5.60	20.56[a] ± 1.20	16.68[ab] ± 1.18	0.092[c] ±0.004	2.83[b] ± 0.08

[1]µmol/min/mg protein; [2]units/mg protein; [3]nmol/min/mg protein; [4]mg/g tissue
Values are mean ± SEM (n=6)
Mean in the same column with different superscript differ significantly ($p<0.05$)

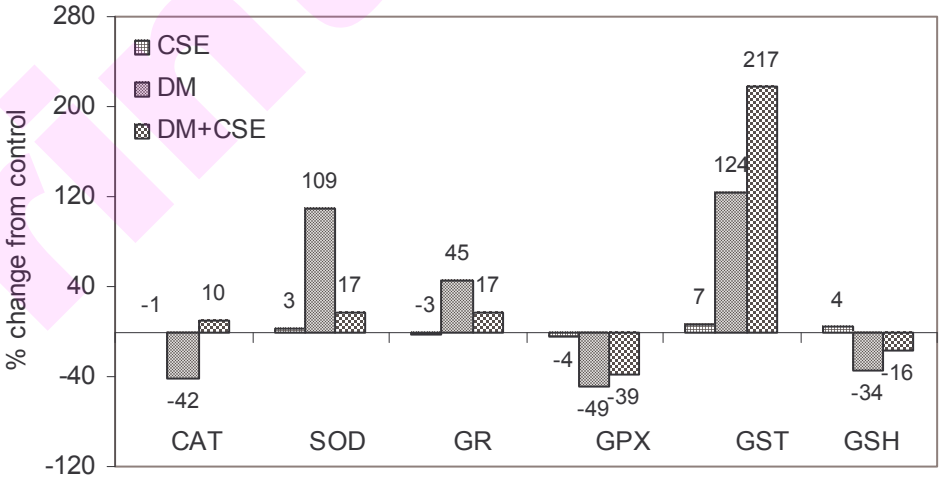


Fig. 3.23 Effect of CSE supplementation on GSH and antioxidant enzymes in control and dimethoate (DM : 40 mg/ kg b.w/d for 8 weeks) treated rats

Table 3.5 AChE and xenobiotic metabolizing enzyme activities in rats administered dimethoate (40 mg/kg b.w/d) and CSE (20 mg/kg b.w/d) for 8 weeks

	AChE[1]	NADPH-diaphorase[2]	DT-diaphorase[3]
Control	4.96[b] ± 0.47	8.78[a] ± 1.27	0.62[a] ± 0.06
CSE	5.03[b] ± 0.86	9.12[a] ± 1.42	0.60[a] ± 0.02
DM	1.43[a] ± 0.21	16.87[b] ± 0.37	0.87[c] ± 0.03
DM+CSE	2.11[a] ± 0.80	11.17[a] ± 0.279	0.72[b] ± 0.03

[1]µmol/min/mg protein; [2]nmol of NADPH utilized/min/mg protein;
[3]µmol of NADH utilized/min/mg protein;
Values are mean ± SEM (n=6); Mean in the same column with different superscript differ significantly (*p<0.05*)

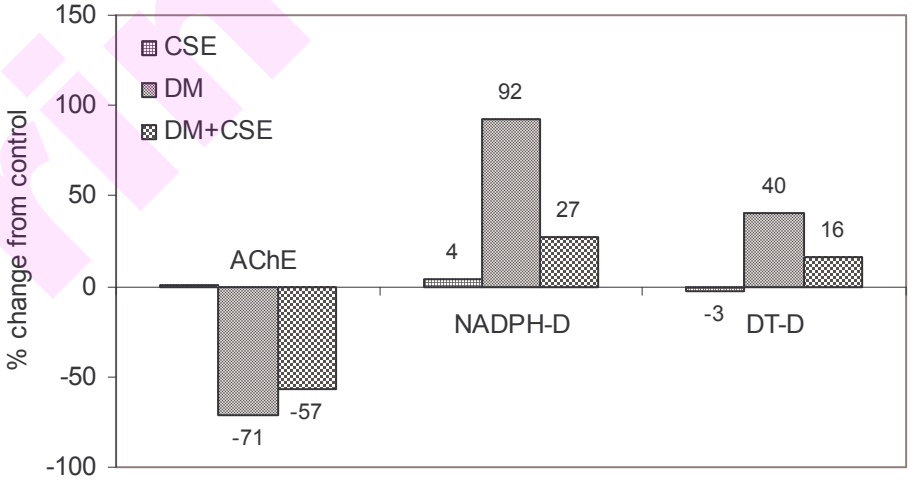


Fig. 3.24 Effect of CSE supplementation on AChE and XME activity in control and dimethoate (DM : 40 mg/ kg b.w/d for 8 weeks) treated rats

6.0 DISCUSSION

Organophosphorus insecticides (OPI) have been claimed to have harmful effects on the endocrinal and other biochemical functions of the pancreas (Hsiao et al., 1996; Sikk et al., 1985). In the present study, administration of repeated doses of dimethoate for eight weeks resulted in a significant increase in random blood glucose levels in rats. Further, DM treatment also impaired glucose tolerance, and induced biochemical alterations in pancreatic tissue. Majority of the studies evaluating the effects of OP on glucose homeostasis have shown that exposure to OP induces hyperglycemia. In our earlier study, we observed an increase in blood glucose to the same extent in rats administered DM at the same dosage for 4 weeks (40 mg/kg b.w /d; *Chapter IIA*). Increase in blood glucose has also been reported due to intoxication in rats by acephate (Deotare and Chakrabarti, 1981), fenthion (Ikizceli et al., 2005) malathion (Abdollahi et al., 2004a), in mice by diazinon (Seifert, 2001) and in humans due to OP poisoning (Meller et al., 1981).

The main mechanism of action of OP occurs through the inhibition of neuronal cholinesterase activity, a key enzyme involved in neurotransmission. However, cholinergic action of OPI is also reported to result in hyperglycemia due to hyperesthesia, intermittent spasm, muscular tremors and convulsions (Rahimi and Abdollahi, 2007). Furthermore, it has been reported that ACh is a potent secretagogue of both insulin and glucagons (Seifert, 2001; Duttaroy et al., 2004). In the present study we observed marked inhibition in AChE activity in pancreas of DM treated rats. Interestingly, the extent of AChE inhibition was similar in rats when exposed to DM for 4 weeks (*Chapter IIA*). Probably, as proposed by Teichert-Kulizenwska et al., (1981) the cholinergic effect of dimethoate may perhaps involve changes in the insulin /glucagon ratio.

Exposure to OPI has been reported to evoke an imbalance in the cellular oxidative status and also lead to increased activity of enzymes associated with detoxification arsenal of experimental animals (Ranjbar et al., 2005). Our study

indicates that sub chronic administration of dimethoate causes pancreatic damage as shown by increases pancreatic damage enzymes, amylase and lipase in serum. This finding indicates that DM causes acute pancreatitis since increase in lipase activity by 2 fold is specific for the diagnosis of pancreatitis. Administration of another OPI, Diazinon has also been shown to increase serum lipase and amylase activity in rats (Gokalp et al., 2005). CSE supplements normalized the levels of these enzymes in serum which suggests that CSE offered protection against DM induced pancreatic damage.

It has been well demonstrated that the enzymes associated with antioxidant defense mechanism are altered under the influence of OPI and that LPO is one of the molecular mechanisms involved in OP-induced tissue damage (Akhgari et al., 2003; Abdollahi et al., 2004; Ranjbar et al., 2005). In the present study, we observed significant increase in the ROS production in pancreas of rats treated with dimethoate along with a marked reduction in GSH. The reduction was significantly greater than that seen in rats administered DM at the same dosage for 4 weeks (*Chapter IIA*), suggesting increased oxidative stress in pancreas due to prolonged exposure to DM. The decrease in GSH clearly suggests that there is a net suppression in the total antioxidant capacity in the pancreatic tissue.

Oxidative damage primarily occurs through production of ROS, including hydroxyl radicals, which subsequently react with biological molecules, causing damage to membranes and other tissues (Banerjee et al., 1999). The increase in ROS generation and subsequent increase in levels of LPO in the pancreatic tissue upon pesticide exposure points to the underlying membrane damage stemming from a substantial loss of membrane integrity in the pancreatic tissue. The increase in serum amylase and lipase and decrease in the pancreatic tissue amylase and lipase is probably an outcome of this phenomenon.

There are conflicting data regarding the effect of OPI on antioxidant enzymes. We observed that administration of DM (20 mg /kg b.w /d) both for 4 weeks (*Chapter II A*) or 8 weeks (present study), increased the activities of SOD, GST and GR and decreased the activity of GPX. However, treatment for 4 weeks resulted in increased activity of CAT, while treatment for 8 weeks resulted in significant reduction in CAT activity. Ahmed et al., (2000) reported that malathion caused highly significant increase in CAT activity. Increased activity of CAT reflects an activation of the compensatory mechanism due to the effects of pesticides on cells and its degree depends on the magnitude of the oxidative stress and hence on the dose of the stressor. The elevated activity of CAT is due to the adaptive response to the generated free radicals, indicating the failure of the total antioxidant defense mechanism to protect the tissues from damage caused by pesticides as evidenced by increased lipid peroxidation. The superoxide generated is dealt with by enhanced SOD and is converted to H_2O_2 by CAT or GPX.

Activities of GSH-independent phase II detoxifying enzymes like DT-diaphorase and NADPH-diaphorase were also significantly altered in the pesticide-treated rats. The activities of NADPH-D and DT-diaphorase were further increased (3-4 fold) in the rats of the present study compared to those administered DM for 4 weeks (*Chapter IIA*).

Our other question was that if oxidative stress has a role in DM-induced pancreatic damage and the ensuing altered glucose homeostasis and whether an antioxidant rich natural extract can ameliorate the effect. In the present study, we employed an ethanolic extract of cashew nut skin (CSE) to study its ameliorative potential. Our earlier studies (*Section A Chapter III*) had clearly demonstrated the strong radical quenching potential of CSE. HPLC analysis of the antioxidant fraction of the extract revealed the predominant presence of epicatechin. Epicatechin, a polyphenolic compound and a green/black tea constituent, has been shown to protect pancreatic islets from alloxan and

streptozotocin, inducing restoration of blood glucose levels and by promoting β-cell regeneration (Kim et al., 2003). Further, in experimental acute pancreatitis, green tea catechins have been demonstrated to restore serum amylase levels and the concentrations of lipid peroxide (Takabayashi et al., 1995; Takabayashi and Harada, 1997). Black tea and green tea are shown to selectively induce phase I and Phase II metabolic enzymes which increase the formation and excretion of detoxified metabolites resulting from xenobiotic metabolism (Frei and Higdon, 2003). Black tea is shown to have protective effect on pesticide-induced toxicity in mice liver (Noguchi et al., 2000).

CSE supplements along with DM administration appeared to significantly decrease oxidative stress in rat pancreas, since CSE supplemented rats showed significantly lower ROS generation and lipid peroxidation in pancreas. The results of the biochemical assays show that administration of CSE ameliorates the pancreatic damage besides improving the activities of antioxidant enzymes, CAT, GPX and GST. The latter two components of phase II metabolism play an important role in detoxification, by catalyzing the conjugation of electrophiles with glutathione and thereby facilitating clearance of the pesticide from the body. The reversal in the antioxidant defenses caused by CSE clearly demonstrates its antioxidant action as well as proves beyond doubt that the DM-induced pancreatic damage involves oxidative stress.

Further, CSE treatment also decreased the levels of serum lipase and amylase and increased the levels of pancreatic lipase and amylase in DM treated rats. DM-induced hyperglycemia and also impaired glucose tolerance were reversed by CSE supplementation. However, CSE supplementation did not significantly reverse AChE inhibition in pancreas induced by DM. These findings also strengthen the fact that oxidative stress mechanisms are involved in DM-induced pancreatic damage and altered glucose homeostasis.

Our findings indicate that the hyperglycemia and impaired glucose tolerance evoked by DM in rats probably occurs as a result of oxidative damage

in pancreas and rule out the cholinergic involvement in DM-induced pancreatic damage /dysfunction. Further, our finding also establishes the protective role of an antioxidant-rich extract from a waste by product, cashew nut skin, against OPI induced toxicity with special reference to pancreatic damage and altered glucose homeostasis. Our results emphasize the need to understand the significant beneficial effects of various phytochemicals present in natural food stuffs, in order to utilize their potential to ameliorate pesticide toxicity *in vivo.*

7.0 SUMMARY

1. CSE supplements *per se* did not affect the blood glucose levels in rats while DM treated rats showed significantly elevated blood glucose levels at all sampling weeks. However, DM treated rats receiving CSE supplements, showed blood glucose levels, which were comparable to those of normal controls.
2. In DM+CSE rats, only serum lipase activity was restored to control values.
3. While the activity of pancreatic amylase was restored to normal levels, Serum amylase activity that was significantly elevated in DM treated rats was reduced significantly in DM + CSE group.
4. In CSE + DM rats, a dramatic reduction in ROS levels was evident in pancreas (100% protection), while the TBARS levels were only marginally (14%) lowered.
5. CSE supplements in DM treated rats also significantly offset the decrease in GSH levels (2.83 ± 0.08 mg/g tissue) and restored the activities of all antioxidant enzymes to normalcy.
6. CSE supplements in DM treated rats offered only marginal protection against insecticide induced to AChE inhibition.
7. Collectively, these data convincingly demonstrate that the DM-mediated oxidative impairments could be effectively countered by oral supplements of antioxidant-rich extract of cashew nut skin.

CONCLUSIONS

1. Of the selected OPI screened for their oxidative stress inducing potential *in vitro*, only ethion (ET), dimethoate (DM) and dichlorvos (DDVP) were found to enhance ROS generation, while only DDVP significantly increased lipid peroxidation *in vitro*. Both DM and DDVP were found to equitoxic to rat pancreatic islets and *Caenorhabditis elegans.*

2. An acute (oral) dose of DM (40 mg/kg b.w) elevated blood glucose levels significantly in adult male rats while, a single dose of DDVP (10 mg/kg b.w) failed to increase the blood glucose level to a significant extent. Interestingly, repeated oral doses of DDVP (10 or 20 mg/kg b.w /d for 5 or 10 d) induced a significant increase in blood glucose levels.

3. Repeated oral doses of Dimethoate (20 and 40 mg/kg b.w/d for 30 d) induced an elevation in blood glucose levels and also altered glucose tolerance in male rats. At these dosages, DM also induced marked oxidative stress in pancreas.

4. Elevated blood glucose levels in rats administered repeated oral doses of DDVP (20 mg/kg b.w /d for 10 d) was associated with significant perturbations in various biochemical markers in pancreas which are suggestive of pancreatic oxidative damage. Biochemical dysfunctions related to carbohydrate metabolism were also evident in DDVP treated rats.

5. Rats pre-treated with multiple oral doses of DDVP followed by a sub-diabetogenic dose of Streptozotocin showed much higher blood glucose and oxidative damage in pancreas in comparison with rats treated with DDVP or STZ alone suggesting that exposure to OPI can render the animal susceptible to diabetes.

6. Ethanolic extracts of novel plant-derived food processing wastes such as potato peel (PPE), tamarind seed coat (TSCE) and cashew nut skin (CSE)

varied in their yield and total polyphenolic content. Cashew nut skin yielded relatively higher extract with higher total polyphenols as compared to both potato peel and tamarind seed coat. All the extracts exhibited significant antioxidant activity *in vitro* in the different assay systems. However, CSE and TSCE were equipotent and their activity was comparable to that of BHA. HPLC analysis of the 'antioxidant rich ethanolic extract of CSE' showed epicatechin to be one of the major antioxidant components.

7. CSE and epicatechin significantly reduced the degree of ROS generated by DDVP *in vitro* in rat pancreatic homogenate while, β-carotene (1mM) failed to offer any protection. Both CSE and epicatechin effectively offset DDVP/ DM induced ROS generation in rat pancreatic islets *in vitro.*

8. Oral supplementation of rats with CSE had no appreciable effect on blood glucose levels. However, rats administered repeated oral doses of DDVP, along with CSE supplements, showed blood glucose levels, which were comparable to those of controls. The degree of pancreatic oxidative damage was also significantly attenuated in these rats.

9. In conclusion, the data obtained from the present series of investigations provide evidences on the effects of OPI on pancreatic function and their possible role in the development / progression of metabolic disorder like diabetes. Supportive evidences are also presented to demonstrate the involvement of oxidative stress in OPI induced pancreatic dysfunction and ensuing hyperglycemia. Our studies also demonstrate that OPI-induced pancreatic damage and hyperglycemia could be significantly abrogated by antioxidant-rich phytochemicals.

10. Thus, these studies suggest the potential of OPI in induction of diabetes in those who are occupationally exposed to OP pesticides (such as pesticides formulating employees or farmers who use OP pesticides) as well as the general public who may be exposed to OPI residues through food and water.

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Altered glucose homeostasis and oxidative impairment in pancreas of rats subjected to dimethoate intoxication

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Abstract

The primary objective of this study was to investigate the effect of repeated sublethal doses of dimethoate (DM), an organophosphorus insecticide on glucose homeostasis, oxidative stress induction in pancreas and pancreatic damage in adult rats. Daily oral administration of DM (20 and 40 mg/kg b.w.) for 30 days induced a significant increase in blood glucose levels which was associated with impaired glucose tolerance. DM treatment resulted in elevated levels of pancreatic tissue specific markers such as activities of amylase and lipase in serum and pancreatic tissue indicating pancreatic dysfunction. Further, the activities of DT-diaphorase and NADPH-diaphorase in pancreas of DM treated rats were also found to be elevated. Interestingly, these biochemical dysfunctions were accompanied by a marked dose-related enhancement of lipid peroxidation and ROS levels in the pancreatic tissue indicating significant induction of oxidative damage. Additional evidence such as depletion in reduced glutathione levels and significant alterations in enzymic antioxidant defenses in pancreas among DM treated rats suggested induction of oxidative stress. Taken together, these findings provide experimental evidence that dimethoate at subchronic oral doses has the propensity to impair glucose homeostasis, induce significant pancreatic damage and also provide an account of the associated oxidative damage to pancreatic tissue in adult rats.

Keywords: Dimethoate; Organophosphorus insecticide; Oxidative impairment; Pancreas; Blood glucose; Pancreatic enzymes; Lipid peroxidation; Antioxidant enzymes

1. Introduction

The widespread use of pesticides in public health and agricultural programs has caused severe environmental pollution and potential health hazards, including acute and chronic cases of human poisoning. Organophosphate insecticides (OPI) constitute one of the most widely used classes of pesticides being employed for both agricultural and landscape pest control. Use of OPI has increased considerably due to their low toxicity and low persistence in the mammalian system compared to organochlorine pesticides. OPI are primarily recognized for their ability to induce toxicity in mammals through inhibition of acetylcholinesterase (AChE) and subsequent activation of cholinergic receptors (Costa, 2006).

Various complications have been reported in OPI intoxication cases (Hsiao et al., 1996). In fact, the OPI are currently responsible for more poisonings than any other single class of pesticides (Sultatos, 1994; Gulr et al., 1996). Hyperglycemia has been widely reported as one of the adverse effects in poisoning by OPI in humans and animals (Abdollahi et al., 2003; Hagar et al., 2002; Seifert, 2001; Shoba and Prakash, 2000; Kalender et

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al., 2004). Although the precise mechanism/s of OP-induced hyperglycemia is not known, it is speculated to be due to inhibition of acetylcholinesterase of central and peripheral synapses that act in the endocrine regulation of glucose metabolism (Matin and Siddiqui, 1982; Kant et al., 1988). Acute pancreatitis is also well known complication of OP poisoning in both humans and animals (Dressel et al., 1979; Frick et al., 1987; Hsiao et al., 1996). Epidemiological findings indicate that the incidence of pancreatitis is high in OPI intoxication based on various pathophysiological reports (Gokalp et al., 2005). However, the precise molecular mechanisms underlying OPI-induced acute pancreatitis are still undefined, although it is believed to involve obstruction of pancreatic ducts and/or enhanced reactive oxygen species (Dressel et al., 1982; Sultatos, 1994; Sevillano et al., 2003). Involvement of oxidative stress following acute exposure to OPI has been reported recently (Banerjee et al., 2001) and it has been demonstrated unequivocally that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced cytotoxicity (Ranjbar et al., 2002; Akhgari et al., 2003; Abdollahi et al., 2004a).

Dimethoate (*O*,*O*-dimethyl *S*-*N*-methyl carbomyl methyl phosphorodithioate) (DM) is one of the most important OPI used extensively on a large number of crops against several pests. The residue of DM and its analog were found in number of foods including cow's milk (Srivastava and Raizada, 1996). While data on acute, subchronic and chronic toxicity of DM in laboratory animals are well documented, its potential to alter glucose homeostasis and impair the endocrine function of pancreas in mammals is less understood. Interestingly, DM is reported to cause various toxic effects on rat pancreas following chronic exposure (Hagar et al., 2002) as well as pancreatitis in humans following dermal exposure (Panieri et al., 1997). In view of the above, in the present study, we have focussed our attention on the potential of DM to cause alterations in glucose homeostasis and also on the toxic effects in pancreas of adult rats subjected to daily oral administration at sublethal doses for 1 month. Further, we have examined the various oxidative impairments in pancreas in terms of lipid peroxidation, generation of reactive oxygen species (ROS), response of antioxidant enzymes and examined its correlation with pancreatic acetylcholinesterase activity.

2. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA), xanthine oxidase, ethylenediamine tetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), glutathione reductase (GR), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2′,7′-dichlorofluorescein (DCF) and cytochrome *C* were procured from M/s Sigma Chemical Co. (St. Louis, MO, USA). Xanthine, NADPH, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), reduced glutathione (GSH), oxidized glutathione (GSSG), 1-chloro-2,4-dinitrobenzene (CDNB), and acetylthiocholine iodide were procured from M/s Sisco Research Lab (Mumbai, India). All other chemicals used were of analytical grade. Dimethoate, technical grade (97.4% pure) was a gift from M/s Hyderabad Chemical Supplies Ltd., Hyderabad, India.

2.2. Animals and care

Adult male rats (CFT-Wistar strain, 12–14-week old, 280 ± 5 g) were randomly drawn from the stock colony of our institute animal house facility and were housed individually in polypropylene cages under standard housing conditions (controlled atmosphere with 12:12-h light/dark cycles, 50 ± 5% humidity and an ambient temperature of 25 ± 2 °C). The rats were acclimatized for 1 week prior to the start of the experiment. Rats were maintained on commercial pellet diet (Gold Mohur, supplied by M/s Lipton India Pvt Ltd.) *ad libitum* and had free access to water. All procedures with animals were conducted strictly in accordance with approved guidelines by the Institute Animal Ethical Committee, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. During the experiments, maximum care was taken to minimize animal suffering and in addition, the number of rats used was kept at minimum.

2.3. Animal treatment and experimental protocol

Adult male rats were grouped by randomized design into three groups ($n = 6$). Rats of the first group (negative control) received saline daily, while rats of the treatment groups were orally administered dimethoate (DM) at dosages of 20 and 40 mg/kg b.w./day (corresponding to 1/20 and 1/10 of LD50 value: 400 mg/kg b.w., determined in a preliminary study) (0900–1100 h) for 30 days to non-fasted rats. Both control and DM treated rats were subjected to oral glucose tolerance test at the end of the 30 days and were subsequently killed. Body weights were recorded weekly and at autopsy. After the treatment period, the rats were sacrificed and blood was collected for separation of serum. Pancreas and other vital organs were excised and their weights were recorded. Pancreas was rinsed in ice-cold saline, homogenized to obtain 10% homogenate in phosphate buffer (0.1 M, pH 7.4) and the homogenate was centrifuged at 9000 × *g* at 4 °C for 20 min. The supernatant was used for various biochemical analyses.

2.4. Oral glucose tolerance test

Twenty-four hours after the last dose of DM, oral glucose tolerance was conducted in control and dimethoate treated rats.

Blood samples were collected from tail vein of rats (control and treated groups) which were fasted overnight to obtain baseline blood glucose levels. Subsequently, rats of both control and DM treated groups were orally administered a bolus of glucose (3 g/kg b.w.). Blood was collected from tail vein of these rats at intervals of 30 min up to 3 h for estimation of glucose. Blood glucose was estimated using a commercial glucometer, 'Accu-Check' procured from M/s Roche diagnostics, Mumbai, India.

2.5. Biochemical measurements

2.5.1. Assessment of pancreatic damage

Lipase activity was estimated by monitoring the hydrolysis of *p*-nitrophenyl acetate to *p*-nitro phenol as described by Young et al. (1978). Enzyme activity was calculated as nmol PNP released using a standard graph and enzyme activity is expressed as nmol PNP released min^{-1} mg^{-1} protein. Amylase activity was measured according to the method of Street and Close (1956) using a commercially available kit procured from M/s Span diagnostics, Mumbai, India. The assay is based on the degradation of starch by the enzyme, amylase into reducing dextrins and small oligosaccharides. Iodine solution is reacted to give a blue coloration, which is read at 620 nm. The relative decrease in blue color is the measure of enzyme activity, which was expressed as Street-Close units.

2.5.2. Acetylcholinesterase activity

The acetylcholinesterase (AChE) (EC. 3.1.1.7) activity was assayed in pancreatic homogenate by the method of Ellman et al. (1961) using acetylthiocholine iodide as the substrate and the enzyme activity was expressed as μmol of substrate hydrolyzed min^{-1} mg^{-1} protein.

2.5.3. Glutathione levels

Reduced glutathione (GSH) levels in pancreatic homogenate was quantified by the method of Benke et al. (1974). Glutathione concentration was calculated from a standard graph and results were expressed as mg GSH/g tissue.

2.5.4. Oxidative stress markers

ROS generated in pancreas was determined by DCFH oxidation (Keston and Brandt, 1965). The amount of DCF (resulting from the ROS mediated oxidation of DCFH, which is produced by hydrolytic cleavage of DCFH-DA by cellular esterases) was estimated in pancreatic homogenate and results were expressed as pmol of DCFmg^{-1} protein min^{-1}. Lipid peroxidation was estimated in pancreas by measuring the thiobarbituric acid reactive substances according to the method of Buege and Aust (1978). The amount of thiobarbituric acid reactive substances (TBARS) was determined using the molar extinction coefficient of 1.56×10^{-5} M^{-1} cm^{-1} and the results were expressed as nmol MDA min^{-1} mg^{-1} tissue.

2.5.5. Activity of antioxidant enzymes

Catalase (EC. 1.11.1.6) activity in pancreatic homogenate was assayed by monitoring the decomposition of H_2O_2 at 240 nm as described by Aebi (1984) and expressed as μmol of H_2O_2 decomposed min^{-1} mg^{-1} protein. Superoxide dismutase (SOD) (EC. 1.15.1.1) was assayed employing the method of Flohe and Otting (1984). The enzyme activity was expressed as units of SOD/mg protein. One unit was defined as the amount of enzyme that decreases the initial rate of cytochrome *C* reduction to 50% of its maximal value for the particular sample being analyzed. The activity of glutathione reductase (EC. 1.6.4.2) was determined by monitoring the oxidation of NADPH in the presence of GSSG and expressed as nmol NADPH oxidized min^{-1} mg^{-1} protein by the method of Carlberg and Manervik (1975). Activity of glutathione peroxidase (EC. 1.11.1.9) was determined using method of Paglia and Valentine (1967) and was expressed as nmol of GSH oxidized min^{-1} mg^{-1} protein. Glutathione-*S*-transferase activity was measured by the method of Rotruck et al. (1973) and the results were expressed as μmol adduct formed min^{-1} mg^{-1} protein.

2.5.6. Phase II enzymes

The activity DT-diaphorase was assayed as described by Ernestr et al. (1962) which involved measurement of reduction of NADH at 550 nm as the electron donor and 2,6-dichloropenol indophenol as the electron acceptor. The enzyme activity was calculated using the extinction coefficient 21 mM^{-1} cm^{-1}. NADPH-diaphorase activity was assayed as described by Huennekens et al. (1957). This method is based on reduction of methylene blue to leukomethylene blue during the transfer of hydrogen from NADPH to NADPH-diaphorase.

2.5.7. Protein

Protein content in tissue homogenate was measured as described by Lowry et al. (1951) using bovine serum albumin (BSA) as the standard.

2.6. Statistical analysis

Mean and standard error values were determined for all the parameters and the results were expressed as mean ± S.E. from six rats in each group. The data were analyzed employing analysis of variance (ANOVA) using Statistica software (STATSOFT, USA). Tukey test for multiple comparisons was performed to determine the significant differences among the groups. P values <0.01 were considered significant.

3. Results

3.1. Growth and organ weight

Repeated oral doses of DM failed to induce any distinctive clinical signs of toxicity or mortality. Data on the body weights along with the relative organ weights are presented in Table 1. While the weight gain in con-

Table 1
Body weight and relative organ weights of adult male rats administered oral doses of dimethoate for 30 days

	Control	Dimethoate (mg/kg b.w.)	
	0	20	40
Initial body weight (g)	282.67 ± 9.04	287.28 ± 7.41	287.00 ± 5.51
Final body weight (g)	333.33 ± 9.27	327.67 ± 4.23	309.00 ± 7.03[a,b]
Pancreas (mg/100 g b.w.)	129.05 ± 6.43	144.25 ± 7.96[a]	170.73 ± 6.74[a,b]
Liver (g/100 g b.w.)	3.08 ± 0.21	3.25 ± 0.32	3.56 ± 0.51
Kidney (g/100 g b.w.)	0.75 ± 0.10	0.74 ± 0.08	0.82 ± 0.06
Adrenals (mg/100 g b.w.)	15.26 ± 1.28	15.41 ± 1.97	14.89 ± 2.17

Values are mean ± S.E.M. ($n = 6$).
[a] Comparison of control and other groups.
[b] Comparison of dimethoate 20 mg/kg b.w. treated group with dimethoate 40 mg/kg b.w. treated group.

trol group at end of 30 days was 17%, rats treated with the higher dose of DM showed only 7.6% weight gain. There was a marked increase (32%) in the pancreatic weight in rats administered higher dose of DM (170.73 ± 6.74 mg versus 129.05 ± 6.43 mg). However, only marginal increases in liver and kidney weights were observed in rats administered with higher dose of DM.

3.2. Oral glucose tolerance test

Data on the blood glucose levels monitored in control and DM treated rats following glucose overload over a 3 h period is presented in Fig. 1. Fasting glucose levels of rats in all the three groups were similar. There was no significant difference in blood glucose levels measured at 30 min. However, the DM treated group showed higher glucose level (135 mg/dl) at 60 min and beyond. The glucose levels among the control rats returned to normalcy (90 mg/dl) at the end of 3 h suggesting the normal glucose tolerance. On the other hand DM treated rats showed higher levels of glucose (125 and 129 mg/dl) at the end of 3 h indicating altered glucose tolerance.

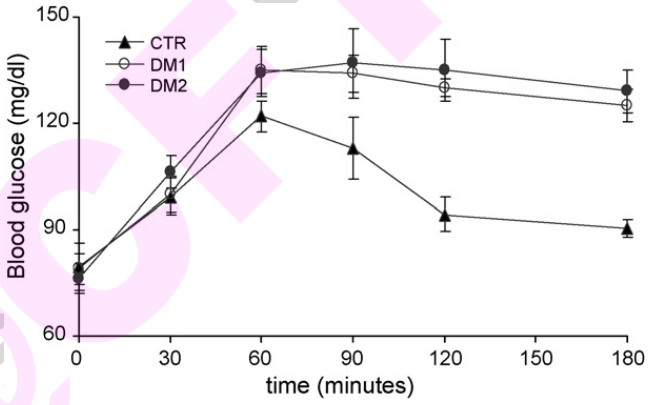

Fig. 1. Oral glucose tolerance in control (CTR) and dimethoate treated rats (DM1: dimethoate 20 mg/kg b.w.; DM2: dimethoate 40 mg/kg b.w.). Values are mean ± S.E.M. ($n = 6$).

3.3. Blood glucose, acetylcholinesterase and reduced glutathione

Data on the blood glucose levels, pancreatic acetylcholinesterase activity and reduced glutathione levels in control and DM treated rats are presented in Table 2. The blood glucose levels were significantly elevated in DM treated rats, and a dose-dependent increase was

Table 2
Blood glucose, acetylcholinesterase activity and reduced glutathione levels in pancreas of adult male rats administered with oral doses of dimethoate for 30 days

	Control	Dimethoate (mg/kg b.w.)	
	0	20	40
Initial blood glucose (mg/dl)	85.33 ± 3.85	87.34 ± 5.23	85.00 ± 5.30
Final blood glucose (mg/dl)	91.33 ± 2.41	105.28 ± 3.57[a]	138.67 ± 5.70[a,b]
AChE (nmol/min/mg protein)	4.96 ± 1.47	2.94 ± 1.75	0.43 ± 0.21[a,b]
GSH (mg/g tissue)	1.11 ± 0.02	0.99 ± 0.05[a]	0.91 ± 0.07[a,b]

Values are mean ± S.E.M. ($n = 6$).
[a] Comparison of control and other groups.
[b] Comparison of dimethoate 20 mg/kg b.w. treated group with dimethoate 40 mg/kg b.w. treated group.

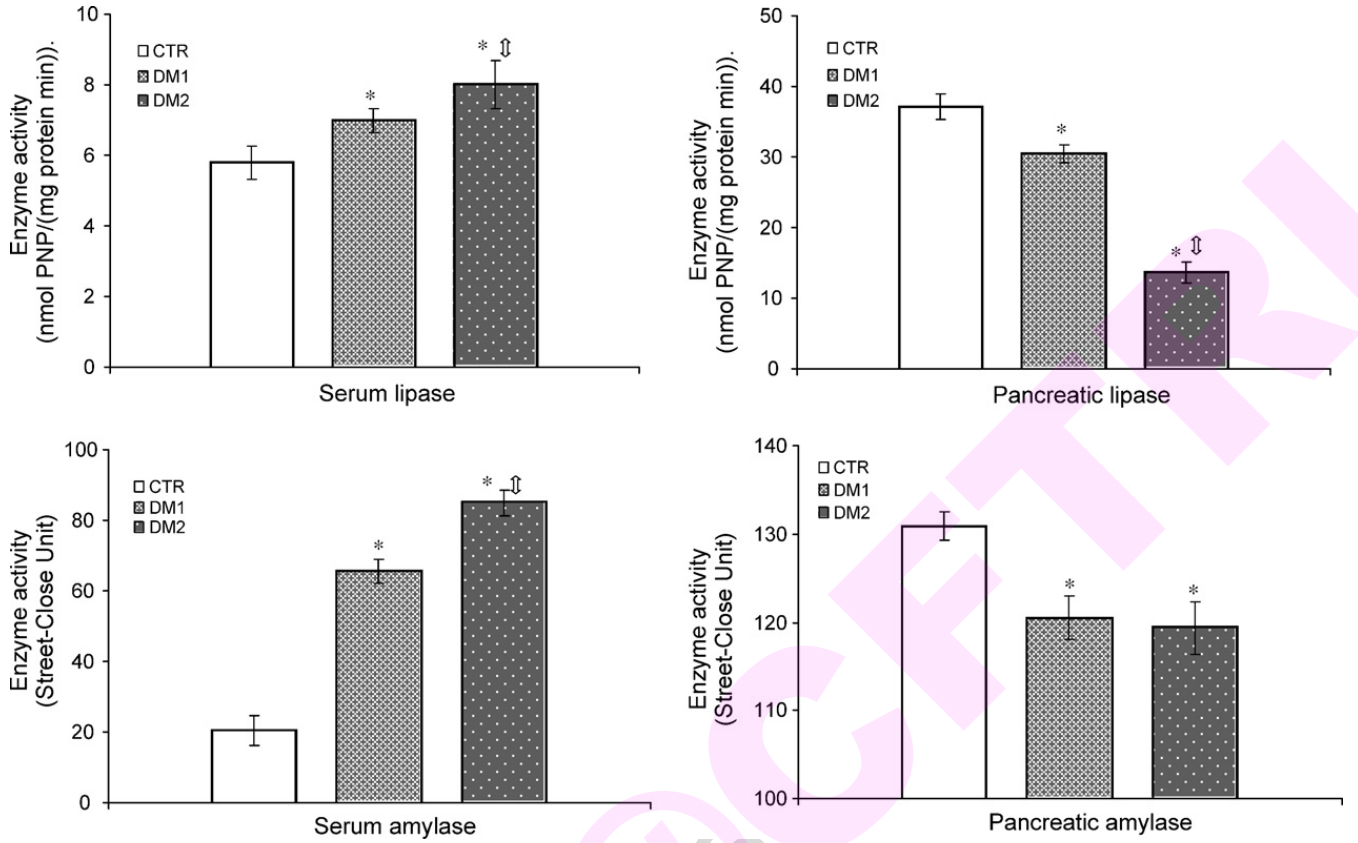

Fig. 2. Activities of lipase and amylase enzymes in serum and pancreas of control (CTR) and dimethoate (DM) treated rats; (DM1: dimethoate 20 mg/kg b.w.; DM2: dimethoate 40 mg/kg b.w.). Values are mean ± S.E.M. ($n = 6$). * Comparison of control and other groups ($P < 0.01$); ⇕ Comparison of dimethoate 20 mg/kg b.w. treated group with dimehtoate 40 mg/kg b.w. treated group ($P < 0.01$).

evident. The percent increase in glucose levels was 15 and 51% at the lower and higher doses, respectively. Further, the activity of AChE in pancreatic tissue was markedly reduced (40 and 90%) among DM treated rats. While the decrease in the reduced GSH was marginal in the lower dose (10%), it was 18% at the higher dose.

3.4. Pancreatic damage

The activity of enzymes viz., amylase and lipase in serum and pancreatic tissue following DM treatment is presented in Fig. 2. There was significant increase in serum lipase activity (20 and 38%) in DM treated rats with respect to the enzyme activity in control rats (5.79 ± 0.26 nmol PNP/(mg protein min)). However, the pancreatic tissue lipase activity was significantly decreased in rats administered with DM (18 and 63%) with respect to control (37.1 nmol PNP/(mg protein min)). While serum amylase activity was increased (2–3-fold) in DM treated rats, the pancreatic amylase activity was marginally decreased in DM treated rats.

3.5. Induction of oxidative damage in pancreas

The ROS and TBARS levels determined in the pancreatic tissue are presented in Fig. 3. There was a dose-related elevation in the ROS levels in the DM treated rats. While, the percentage increase at the lower dose was 66%, a dramatic (150%) increase was evident at the higher dose. Concomitantly, a dose-related increase in TBARS levels was observed in DM treated rats. There was 2.5- and 3.7-fold increase in TBARS level at lower and higher doses of DM, respectively.

3.6. Activities of antioxidant enzymes and phase II enzymes in pancreas

Data on the activities of various antioxidant enzymes in pancreas following DM treatment is presented in Table 3. In general the activities of SOD, CAT, GR and GST were significantly elevated among DM treated rats compared to the control group. At the higher dose, the activities of SOD, CAT, GR and GST were increased by 112, 64, 45 and 100% over the controls. However, the GPx activity was diminished (50%) at the higher dose.

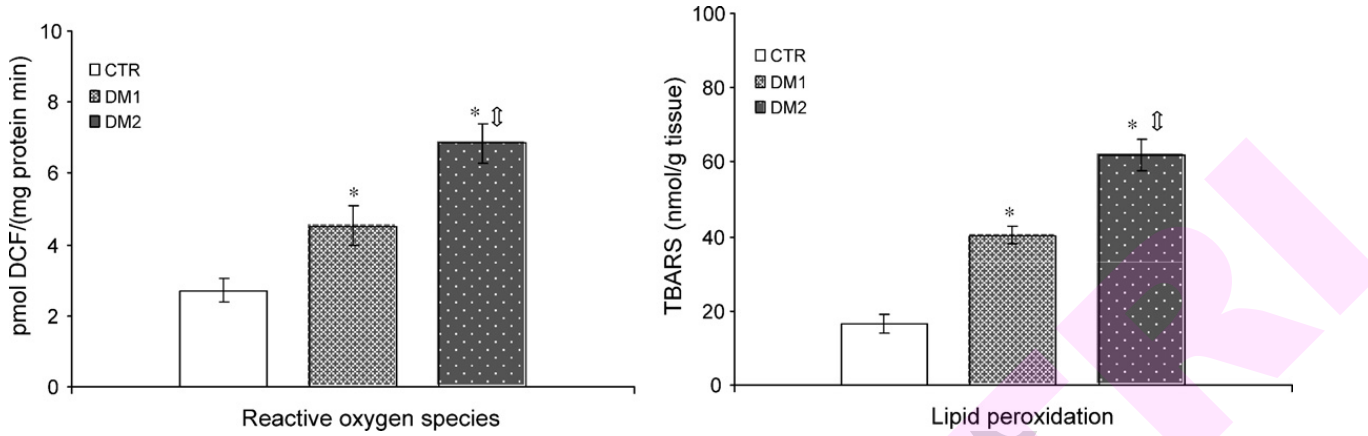

Fig. 3. ROS and TBARS levels in pancreas of control and dimethoate treated rats (CTR : control; DM1: dimethoate 20 mg/kg b.w.; DM2: dimethoate 40 mg/kg b.w.). Values are mean ± S.E.M. ($n = 6$). * Comparison of control and other groups ($P < 0.01$); ⇕ Comparison of dimethoate 20 mg/kg b.w. treated group with dimehtoate 40 mg/kg b.w. treated group ($P < 0.01$).

Table 3
Activities of antioxidant enzymes in pancreas of adult male rats administered with oral doses of dimethoate for 30 days

Dosage (mg/kg b.w.)	Enzyme activity				
	SOD (units/mg protein)	CAT (μmol/ mg protein/min)	GPX (nmol/ mg protein/min)	GR (nmol/ mg protein/min)	GST (μmol/ mg protein/min)
0	26.42 ± 2.2	9.38 ± 0.31	27.18 ± 5.24	17.50 ± 1.60	0.03 ± 0.004
20	42.72 ± 0.38[a]	10.24 ± 0.32	25.23 ± 3.89	19.72 ± 2.03	0.04 ± 0.003[a]
40	56.23 ± 1.18[a,b]	15.44 ± 0.51[a,b]	13.85 ± 2.20[a,b]	25.30 ± 1.30[a,b]	0.06 ± 0.003[a,b]

Values are mean ± S.E.M. ($n = 6$).
[a] Comparison of control and other groups.
[b] Comparison of dimethoate 20 mg/kg b.w. treated group with dimethoate 40 mg/kg b.w. treated group.

The activities of phase II enzymes such as NADPH-daphorase and DT-diaphorase are presented in Table 4. There was 27% increase in the activity of NADPH-diaphorase and a 16% increase in the activity of DT-diaphorase in the rats treated with higher dose of DM.

Table 4
Activities of phase II enzymes in pancreas of adult male rats administered with oral doses of dimethoate for 30 days

Dosage (mg/kg b.w.)	Enzyme activity	
	NADPH-D (nmol of NADPH utilized/ mg protein/min)	DT-diaphorase (μmol of NADH utilized/ mg protein/min)
0	8.78 ± 0.27	0.62 ± 0.05
20	9.12 ± 0.37	0.60 ± 0.02
40	11.17 ± 0.42[a,b]	0.72 ± 0.02[a,b]

Values are mean ± S.E.M. ($n = 6$).
[a] Comparison of control and other groups.
[b] Comparison of dimethoate 20 mg/kg b.w. treated; group with dimethoate 40 mg/kg b.w. treated group.

4. Discussion

The mechanisms involved in the blood glucose alterations following OPI exposure have been under investigation in the recent years since hyperglycemia is one of the prominent side effects of OPI poisoning in humans (Namba et al., 1971; Hayes et al., 1978; Meller et al., 1981). The ability of OPI to cause hyperglycemia has also been confirmed in laboratory animals (Matin and Siddiqui, 1982; Fletcher et al., 1988). Organophosphate pesticides have been demonstrated to impair the endocrinal and biochemical functions of pancreas (Sikk et al., 1985; Hsiao et al., 1996). Hence, the present study addressed the potential of dimethoate (DM), an OPI to induce alteration in glucose homeostasis and examined whether oxidative stress plays an important role in DM-induced pancreatic dysfunction.

In the present study oral administration of DM for 30 days resulted in significant reduction in body weight at the higher dose. Reduction in body weight in experimental animals due to OPI intoxication is a commonly reported phenomenon (Chung et al., 2002; Kalender

et al., 2006). This occurs probably due to decreased food intake in these animals. Dimethoate treatment also resulted in significant increase in pancreas weights at both the doses. This is a clear indication of the effect of the insecticide on pancreas. Increased weights of pancreas may probably be due to edema and inflammation as has been reported in several cases of OP poisoning in rats (Ikizceli et al., 2005).

Our results showed that oral administration of DM for 30 days induced significant elevation in blood glucose in rats. Further, we observed significant alteration in the oral glucose tolerance among DM-treated rats. These data are in agreement with the earlier reports, which have shown elevated blood glucose levels after administration of DM (Hagar et al., 2002). Hyperglycemic effect has also been reported in experimental animals following to acute and chronic OPI exposures (Begum and Vijayaraghavan, 1999; Sarin and Gill, 1999; Abdollahi et al., 2004b; Pournourmohammadi et al., 2005). Hyperglycemia has been known to occur as a consequence of increased accumulation of acetylcholine (ACh) at the nerve endings following AChE inhibition (Anam and Maitra, 1995). ACh, the major parasympathetic neurotransmitter, is released from intra pancreatic nerve endings. The effects of ACh at the β-cells of the pancreas are mediated by muscarinic cholinergic receptors (Gilon and Henquin, 2001).

DM is an OPI that exerts its neurotoxicity by binding to, and phosphorylating the enzyme, AChE in the central and peripheral nervous systems. DM undergoes oxidative desulfuration to its more potent cholinesterase inhibiting oxon-omethoate (Hassan et al., 1969). In the present study, we observed significant AChE inhibition in pancreatic tissue following administration of DM, which is indicative of pesticide intoxication. The possible pathogenetic sequel speculated for the pancreatic insult in OP poisoning is excessive cholinergic stimulation of the pancreas and ductal hypertension (Hsiao et al., 1996; Sahin et al., 2002). Pancreas is a sensitive organ and hence pressure elevation can cause severe tissue damage.

Several studies have demonstrated acute pancreatitis after oral exposure to various OPI (Dressel et al., 1979; Moore and James, 1988; Hsiao et al., 1996). Increase in lipase activity by two-fold is specific for the diagnosis for pancreatitis. Increased serum lipase activity has been reported after administration of methidathion, an OPI (Mollaoglu et al., 2003). In the present study, DM administration significantly increased both activities of lipase and amylase in serum. This finding is suggestive of the potential of DM to cause acute pancreatitis. Our results corroborate with earlier reports of acute pancreatitis in humans after accidental cutaneous exposure to DM (Marsh et al., 1988). Another biochemical marker, often employed to evaluate pancreatitis, is the increased amylase activity and DM treatment significantly elevated serum amylase activity supporting the evidence for pancreatic damage. Similar increase in amylase activity has been reported in dogs after diazinon administration (Dressel et al., 1982).

Another major question addressed in the present investigation was whether DM-induced pancreatic dysfunction is mediated through oxidative stress. Oxidative stress has been implicated to be an important component of the mechanism of toxicity of several OPI (Bagchi et al., 1995; Yamano and Morita, 1995; Yang and Dettbarn, 1996; Kalender et al., 2005; Ogutcu et al., 2006). Active oxygen metabolism plays a vital role in the normal functioning of the pancreas and several key enzymes in reactive oxygen species (ROS) defense are usually low in pancreatic islets compared to other tissues suggesting islet cells are uniquely susceptible to oxidative stress-induced damage (Grankvist et al., 1981; Kakkar et al., 1998). Further, the low levels of antioxidant enzyme gene expression may account for exquisite sensitivity of β-cell to ROS and free radical induced damage leading to β-cell death and type-1 diabetes (Ho and Bray, 1999). It has been observed that during chronic exposure to OPI, ROS generated consume and exhaust antioxidant molecules present in the body which results in lower antioxidant capacity while subchronic exposure (30 days) of rats to dimethoate has been shown to result in enhanced lipid peroxidation antioxidant defences (Ranjbar et al., 2002). This can be interpreted to mean that in subchronic exposures, the body is capable of defending itself by overproduction of antioxidants, an ability which fails in a chronic exposure scenario (Akhgari et al., 2003; Handy et al., 2002). The increased levels of ROS and TBARS in pancreas in the present study suggests that DM treatment induced significant oxidative stress in the organ.

Depletion of GSH, the most abundant cellular non-protein thiol, is associated with oxidative stress and cytotoxicity of pro-oxidant xenobiotic. Glutathione is presumed to be an important endogenous defence against the peroxidative destruction of cellular membranes. GSH can act either to detoxify activated oxygen species such as H_2O_2 or to reduce lipid peroxides themselves (Freeman and Crapo, 1982). Tissue GSH concentration reflects the potential for detoxification and the levels may decrease due to an increased use of glutathione to detoxify. Enhanced status of lipid peroxidation and concomitant depletion of GSH pools in pancreas in the present study provides further evidence for the occurrence of the oxidative stress.

Cytosolic free radicals are either removed non-enzymatically or by antioxidant enzymes such as SOD and GPX, which oxidize GSH to GSSG. GSSG is then reduced back to GSH by GR through oxidation of NADPH to $NADP^+$, which is recycled by the pentose phosphate pathway. Furthermore, glutathione-*S*-transferase (GST) catalyzes the conjugation of GSH to OPI, leading to its detoxification and elimination (Zeigler, 1985). Our results showed increased activity of SOD and CAT in the pancreas of DM treated rats. CAT is present in all major organs in the body of animals and human beings and is especially concentrated in liver and erythrocytes. Pancreas also has certain amount of CAT activity and the elevated activity of CAT in DM treated rats may be due to an adaptive response to the generated free radicals (Koner et al., 1997). Superoxide dismutase is a ubiquitous chain breaking antioxidant and is found in all aerobic organisms. It is a metalloprotein widely distributed in all cells and plays a protective role against ROS-induced oxidative damage. The increased activity of SOD in DM treated rats probably indicates an activation of the compensatory mechanism through the effects of DM on progenitor cells. Activities of the GSH-dependent phase II detoxifying enzymes in pancreas, GR and GST were also significantly increased in DM-treated rats. GST are detoxifying enzymes that catalyze the conjugation of variety of electrophilic substrate to the thiol group of GSH, producing less toxic forms (Hayes and Pulford, 1995). The increase in GST activity in the pancreatic tissue among DM treated rats indicate the role played by this system in detoxification of DM.

In conclusion, our results indicate that dimethoate has the propensity to significantly alter glucose homeostasis and the associated oxidative impairments observed in pancreas may wholly or in part contribute towards the development of pancreatitis in adult rats. However, further studies are required to understand the molecular mechanism/s underlying the dimethoate induced pancreatic dysfunction.

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The efficacy of cashew nut (*Anacardium occidentale* L.) skin extract as a free radical scavenger

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Abstract

The free radical scavenging activity of ethanolic extracts of cashew nut (*Anacardium occidentale*, L.) skin powder (CSP) was evaluated by employing various *in vitro* antioxidant assay systems. The yield of the extract as well as the total phenolic content was also determined. The yield of ethanolic extract of the skin powder was quite high (0.45 g/g powder) with a total phenolic content of 243 mg/g extract. The cashew nut skin extract (CSE) demonstrated promising antioxidant activity with EC_{50} of 1.30 ± 0.02 μg/ml in 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay, 10.69 ± 1.13 μg/ml in superoxide scavenging assay, 17.70 ± 0.05 μg/ml in deoxyribose oxidation assay, 24.66 ± 0.32 μg/ml in lipid peroxidation (LPO) assay and 6.00 mg/ml in iron chelation assay. To identify the compounds in the CSE responsible for the antioxidant activity, thin layer chromatography (TLC) was performed with the extract. The spot showing protection towards β-carotene bleaching was extracted and analyzed by high performance liquid chromatography (HPLC); epicatechin was found to be the major polyphenol present. The results of the present study suggest that cashew nut skin, a byproduct of cashew processing industry, can be used as an economical source of natural antioxidants.

Keywords: Cashew nut skin; Ethanolic extract; Radical scavenging; Antioxidant activity; ABTS; Total polyphenolics; Brain homogenate; Epicatechin

1. Introduction

Reactive oxygen species (ROS) are implicated in numerous pathophysiological events such as aging, cancer, atherosclerosis and diabetes (Halliwell, Guteridge, & Cross, 1992). Natural antioxidants from fruits and vegetables are reported to provide substantial protection that slows down the process of oxidative damage caused by ROS (Jacob & Burri, 1996). Hence there has been growing interest in natural antioxidants of plant origin since they also find use as nutracueticals due to their impact on the status of human health and disease prevention (Nagochi & Nikki, 2000). Several fruits, nuts, seeds, leaves, roots and barks have been exploited as potential sources of natural antioxidants (Schuler, 1990). There are considerably higher ratios of byproducts arising from seed/ nut processing plants, and hence it would be beneficial if they could be exploited as a source of natural antioxidants. Certain byproducts of agro-industries such as seed testa (Huang, Yen, Chang, Yen, & Duh, 2003), hulls (Yen & Duh, 1994), coats (Chang, Yen, Huang, & Duh, 2002) and peels (Larrauri, Ruperez, & Saura-Calixto, 1998; Singh & Rajini, 2004) have been reported to possess significant antioxidant activity.

The seed coat/testa which forms a protective barrier for the cotyledon in seeds, is reported to have the highest concentration of phenolic compounds (Duenas, Hernandez, & Estrella, 2004, 2006; Duenas, Sun, Hernandez, Estrella, & Spranger, 2003; Shahidi, Chavan, Naczk, & Amarowicz, 2001). Several recent studies have shown that extracts of almond skins (Sang et al., 2002; Siriwardhana & Shahidi, 2002; Chen, Milbury, Lapsley, & Blumberg, 2005), peanut skins (Lou et al., 2004; Lou et al., 1999), hazelnut testa and green leafy cover (Alasalwar, Karamac, Amarowicz, & Shahidi, 2006; Senter, Horvat, & Forbus, 1983), canola/ rapeseed hulls (Amarowicz, Naczk, Zadernowski, & Shah-

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idi, 2000), tamarind pericarp (Sudjaroen et al., 2005) and seed coats of lentils (Duenas et al., 2006; Duenas et al., 2003; Troszynska and Kubicka, 2001) possess strong antioxidant activities. Other authors have observed high antioxidant activity in lentils, faba beans and peas, mainly in the seed coat, due to the presence of large amounts of phenolic compounds in this part of the seed (Amarowicz et al., 2000; Nilsson, Stegmark, & Akesson, 2004; Shahidi et al., 2001; Takahata, Ohnishi-Kameyan, Furuta, Takahasi, & Suda, 2001; Troszynska & Ciska, 2002).

Cashew nut (*Anacardium occidentale* L.) is a major cash crop in the world. India is the largest producer and exporter of cashew kernel, accounting for almost 50% of world export (Paramashivappa, Kumar, Vithayathil, & Rao, 2001). Cashew nut shell liquid, a byproduct obtained during the processing of cashew nuts is reported to possess antioxidant activity (Singh, Kale, & Rao, 2004). The kernel of cashew nut valued in trade is covered with a thin reddish-brown skin or testa. The testa has been reported to be a good source of hydrolysable tannins (Pillai, Kedlaya, & Selvarangan, 1963) with catechin and epicatechin as the major polyphenols (Mathew & Parpia, 1970). However, there are no reports describing the radical scavenging activities of extracts of the skin of cashew nut.

The purpose of this study was to determine whether the ethanolic extracts of cashew nut skin exhibit radical scavenging activity in a set of antioxidant assay systems. Attempt was also made to identify the major active fraction in the extract responsible for the antioxidant activity.

2. Materials and methods

2.1. Materials

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), nitro blue terazolium (NBT), phenazine methosulphate (PMS), hydrogen peroxide (H_2O_2), 2-thiobarbituric acid (TBA), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA) and deoxyribose (DR) were purchased from M/s Sisco Research Laboratories, Mumbai, India. All other reagents used were of analytical grade.

2.2. Preparation of ethanolic extract of cashew skin powder

Dried cashew nut skins were a gift from a cashew processing unit (M/s Mangala Cashews, Mangalore, India). The skins were sun dried and pulverized in a multi-mill and passed through a 0.5 mm sieve to obtain a fine powder. The skin powder (CSP) was mixed with five parts of ethanol and kept in a rotatory shaker at 37 °C for 3 h. The extract was separated by centrifugation, and the resultant cashew nut skin extract (CSE) was stored in dark at 4 °C until further use within a week.

2.3. Determination of total polyphenolic compounds

The total phenolic content in the CSE was quantified using the Folin-Ciocalteu reagent according to Singleton and Rossi (1965). Aliquots of the sample with volumes adjusted to 3.0 ml with distilled water were incubated with 0.5 ml 95% ethanol and 0.25 ml of Folins reagent (1:1 diluted with distilled water) for 5 min at room temperature. Na_2CO_3 (5%) solution (0.5 ml) was added, mixed and the mixture held for 60 min at room temperature. The absorbance of the solution was then measured at 720 nm against reagent blank using a spectrophotometer. Gallic acid (0.1 mg/ml) was used as the standard and the phenolic content in the extract was expressed as milligram equivalents of gallic acid (GAE) per gram CSP.

2.4. ABTS radical scavenging activity

The radical scavenging activity of the CSE was determined by employing the $ABTS^{\cdot+}$ decolorization assay (Re et al., 1999). $ABTS^{\cdot+}$ was produced by reacting ABTS (7 mM) and ammonium persulphate (2.45 mM) in 10 ml water and keeping the mixture in the dark at room temperature for 12–16 h before use. The aqueous $ABTS^{\cdot+}$ solution was diluted with ethanol (1:100 v/v) to an absorbance of 0.7 (±0.02) at 734 nm. Aliquots of extract (20 μl) were added to $ABTS^{\cdot+}$ solution (2 ml), mixed and the absorbance was read at 734 nm after 5 min. The percentage inhibition of radical scavenging was calculated.

2.5. Superoxide-radical scavenging assay

The superoxide scavenging ability of the extract was assessed by the method of Nishikimi, Rao, and Yagi (1972). The reaction mixture, containing CSE (5.5–22 μg), NADH (100 μM) and NBT (100 μM) in tris–HCl (0.02 M pH 8.3), was added to a spectrophotometric cuvette and the reaction was started by adding PMS (1 μM). The change in absorbance (ΔA) was monitored for one min and the capability of the extract in scavenging superoxide radical was calculated using the following equation:

$$\text{Scavenging effect}(\%) = [1 - \Delta A_{\text{Sample 560 nm}}/\Delta A_{\text{Control 560 nm}}] \times 100$$

2.6. Deoxyribose oxidation assay

The reaction mixture, containing CSE (5–20 μg), was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 μM), EDTA (100 μM) and ascorbic acid

(100 μM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C (Halliwell, Gutteridge, & Aruoma, 1987). The reaction was terminated by adding 1 ml TBA (1% w/v) and 1 ml TCA (2% w/v) and then heating tubes in a boiling water bath for 15 min. The contents were cooled and the absorbance of the mixture containing the thiobarbituric acid reactive substances (TBARS) was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

2.7. Metal ion chelating assay

The Fe^{2+}-chelating ability of the extract was monitored by measuring the ferrous iron–ferrozine complex at 562 nm according to the method of Decker and Welch (1990). Briefly, the reaction mixture, containing CSE (1–5 mg), $FeCl_2$ (2 mM), and ferrozine (5 mM), was adjusted to total volume of 0.8 ml with methanol, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against the blank. EDTA (5–25 μg) was used as positive control. The ability of the extract to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating effect}(\%) = [1 - A_{\text{Sample 560 nm}}/A_{\text{Control 560 nm}}] \times 100$$

2.8. Antioxidant assay using β-carotene linoleate model system

The antioxidant activity was evaluated in a β-carotene-linoleate model system based on the procedure of Taga, Miller, and Pratt (1984). CSE (20 μg) was added to 3 ml of β-carotene emulsion and the mixture was shaken well and incubated at 50 °C in a water bath for various periods. The absorbance of the mixture was read at 470 nm at 10, 20, 30, 40 and 60-min intervals. Each sample was read against an emulsion prepared as described earlier but without β-carotene (blank). BHA and BHT (20 μg) were employed as model antioxidants for the purpose of comparison.

2.9. Inhibition of induced lipid peroxidation in rat brain homogenate

Rat brain excised from male Wistar rats (120–125 g) was homogenized (10% w/v) in potassium chloride solution (0.154 M). The homogenate was centrifuged at 800*g* at 4 °C for 10 min and the supernatant used for the assay. Peroxidation was induced in the brain homogenate by $FeCl_2$–H_2O_2 (Yen & Hsieh, 1998). Briefly, brain homogenate (1%) was incubated with 0.5 mM each of $FeCl_2$ and H_2O_2 with or without CSE (8.4–33.6μg). After incubation at 37 °C for 60 min, the TBARS formed in the incubation mixture was measured at 535 nm (Buege & Aust, 1978).

2.10. High performance liquid chromatography (HPLC) characterization of the active molecule from CSE

The antioxidant rich ethanolic extract (CSE) was applied on silica gel thin layer chromatography plate (TLC pre-coated plates, M/s Merck KGaA, Darmstadt, Germany) and resolved using chloroform: methanol (8:2, v/v). The antioxidant-active spot was visualized after spraying with β-carotene linoleate solution (9 mg of β-carotene dissolved in 30 ml of chloroform to which 2 drops of linoleic acid were added and then added to 60 ml of ethanol, Pratt & Miller, 1984). The β-carotene positive spot was scraped, extracted in acetonitrile and subjected to HPLC.

The HPLC (Hewlett Packard 1100 Series, Palo Alto, CA) was equipped with a quaternary pump fitted with a Zorbax C18 analytical column (25 cm × 4.6 cm I.D 5 μ particle size) (M/s S. V. Scientific, Bangalore, India). Detection was done by an HP 1250 m series variable wavelength detector at wavelength of 280 nm. The gradient mobile phase consisted of acetonitrile (A) and 1% trifluoroacetic acid (TFA) (B) with flow rate of 0.1 ml/min. The elution program involved a linear gradient from 100 - 0% for 15 min followed by 5 min equilibrium in a total program time of 25 min. The sample and the standard were dissolved in mobile phase and 10 μl of each was injected.

2.11. Statistical analysis

All the data are expressed as mean ± standard error (SE) ($n = 3$) and the results were processed by the Programme: Microsoft Office Excel 2002.

3. Results and discussion

The CSP on extraction with ethanol yielded a large amount of extract (0.45 g/g powder). The total phenolic content of CSE, expressed as gallic acid equivalents (GAE) was 243 mg GAE/g CSP. Earlier studies have suggested that polyphenolic compounds are associated with antioxidant activity and play an important role in preventing lipid peroxidation (Kirakosyan et al., 2003). However, the polyphenolic compounds in the pericarp or seed coats of nuts have been scantily reported, and no studies have explored the phenolics from cashew nut skins and their antioxidant activity. The present investigation shows that cashew nut skin contains considerable amounts of phenolics clearly indicating that CSP could be a potential source of natural antioxidants.

In this study, the potential of CSE to scavenge free radical was assessed by its ability to quench $ABTS^{\cdot+}$ synthetic free radical. The stable nitrogen-centered free radical $ABTS^{\cdot+}$ is frequently used for the estimation of free radical scavenging ability (Re et al., 1999). Fig. 1 depicts the concentration-dependent decolorization of $ABTS^{\cdot+}$ by CSE in comparison with BHA. As evident from the figure, CSE was equally potent as BHA in radical scavenging.

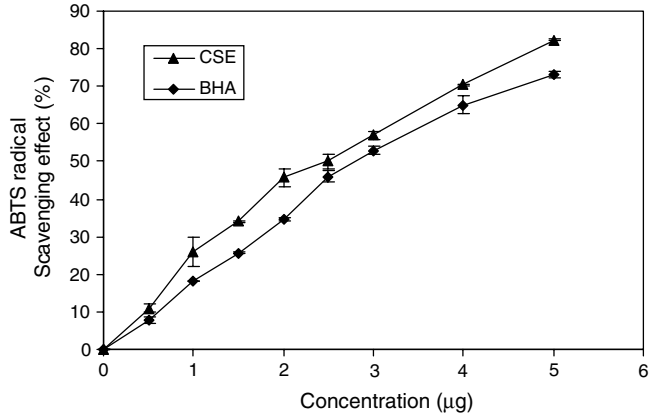

Fig. 1. ABTS radical scavenging effect of Cashew nut skin extract (CSE). Values are mean ± SE of three determinations.

Superoxide anion derived from dissolved oxygen by PMS-NADH coupling reduces NBT (Nishikimi et al., 1972). The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Fig. 2 shows the percentage inhibition of superoxide radical generation by various concentrations of CSE. Apparently the scavenging of the superoxide radical also increased with increasing CSE concentration, with an EC_{50} of 10.69 µg/ml (Table 1). This data shows that CSE is a potent scavenger of superoxide radicals.

Scavenging activity of CSE on hydroxyl radical is shown in Fig. 3. The scavenging activity against hydroxyl radical for CSE (0–20 µg) ranged from 0% to 50%, indicating that the scavenging effect increased with increasing amounts of CSE. The hydroxyl radical is the most reactive radical known to initiate lipid peroxidation (Halliwell, 1991). The ability of CSE to quench the hydroxyl radical seems to relate directly to the prevention of propagation of lipid peroxidation (LPO).

Plant extracts enriched in phenolic compounds are capable of complexing with and stabilize transition metal ions, rendering them unable to participate in metal-catalyzed initiation and hydroperoxide decomposition reactions (Gordon, 1990). Metal chelation is an important antioxidant property (Kehrer, 2000), and hence CSE was assessed for its ability to compete with ferozzine for iron (II) ions in the solution. As evident from Fig. 4, CSE was capable of reducing iron (III). This study revealed that CSE (EC_{50} 6 mg/ml, Table 1) had a relatively lower Fe^{2+} binding compared with that of EDTA.

Heat induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid is often employed as an antioxidant assay. In this particular model, β-carotene undergoes rapid discoloration in the absence of an antioxidant (Taga et al., 1984). The presence of a phenolic antioxidant

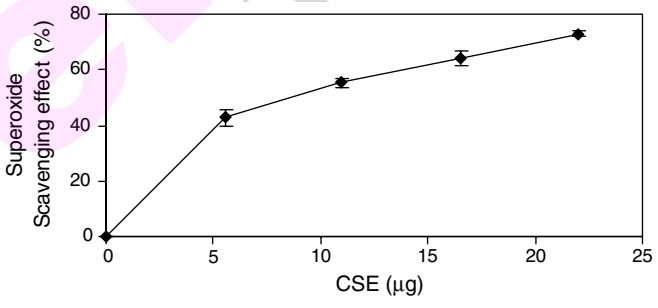

Fig. 2. Superoxide radical scavenging effect of CSE. Values are mean ± SE of three determinations.

Table 1
EC_{50} of CSE in various antioxidant assay systems

Antioxidant assay	EC_{50}[a]
ABTS[b]	1.30 ± 0.02
Superoxide scavenging[b]	10.69 ± 1.13
Deoxyribose oxidation[b]	17.70 ± 0.05
Iron chelation[c]	6.00 ± 0.24
LPO in rat brain homogenate[b]	24.66 ± 0.32

LPO: Lipid peroxidation.
Values are mean ± SE of three determinations.
[a] The EC_{50} value in each assay was determined graphically by plotting each activity as a function of CSE concentration (µg or mg).
[b] µg/ml.
[c] mg/ml.

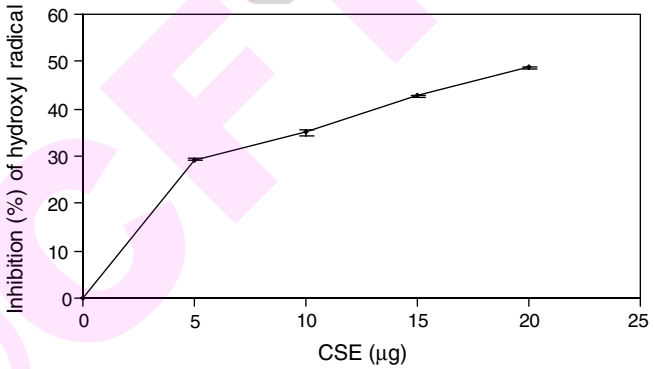

Fig. 3. Inhibitory effect of CSE on deoxyribose oxidative damage. Values are mean ± SE of three determinations.

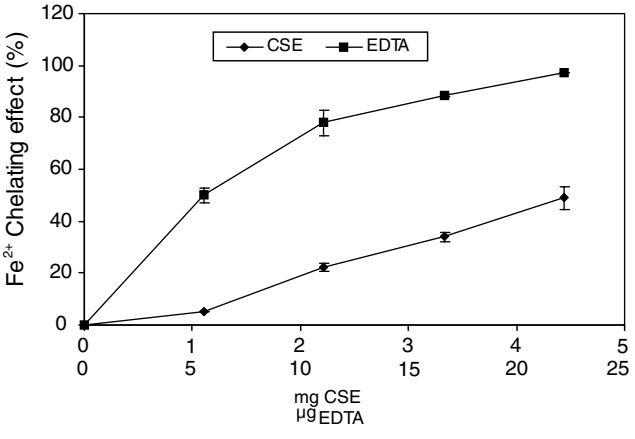

Fig. 4. Chelating effect of CSE on Fe^{2+} ion. Values are mean ± SE of three determinations.

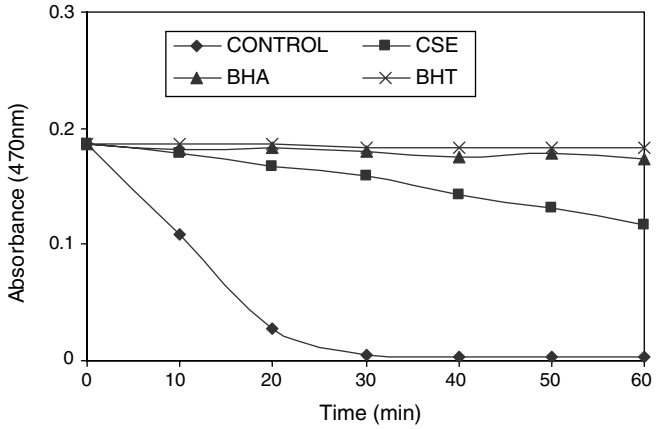

Fig. 5. Antioxidant activity of Cashew nut skin extract (CSE) in β-carotene-linoleic acid system.

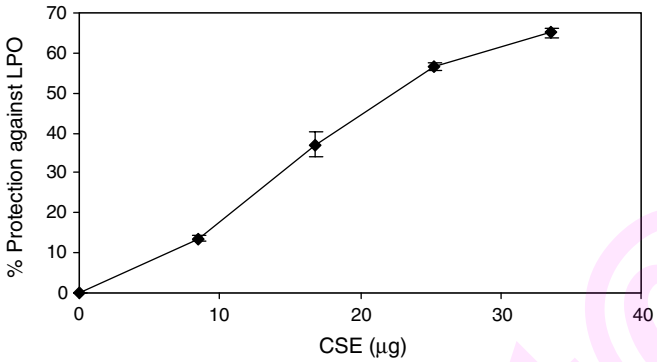

Fig. 6. Inhibition of Fe^{2+}–H_2O_2 induced lipid peroxidation by CSE in rat brain homogenate. Values are mean ± SE of three determinations.

can hinder the extent of β-carotene destruction by "neutralizing" the linoleate free radical and any other free radicals formed within the system. In the present study, CSE exhibited marked antioxidant activity, nearly equal to that of BHA as shown in Fig. 5. However, the greatest antioxidative efficacy was from the synthetic antioxidants (i.e. BHA and BHT) which practically inhibited β-carotene bleaching throughout the incubation period.

The inhibition of LPO may be considered as one of the test to determine the antioxidant activity of a compound (Cos, Calomme, Pieters, Vlietinck, & Vanden Berghe, 2000). Results have demonstrated that treatment with CSE successfully inhibited TBARS formation in the rat brain homogenate. Addition of CSE to the Fe^{2+}–H_2O_2 system resulted in a concentration-dependent decrease in the formation of tissue oxidation levels (Fig. 6) confirming that CSE was also a scavenger of $^{\cdot}OH$.

From the data obtained from the various assay systems, it is clear that the order of effectiveness of CSE in the antioxidant assays was as follows: ABTS > superoxide > deoxyribose > LPO in rat brain homogenate > iron chelation (Table 1). Hence CSE appears to be a more powerful radical scavenger than a metal chelator. The cashew nut skin antioxidant activity may principally be attributed to its phenolic composition, due to the ability of these compounds as free radical scavengers. Earlier studies have shown that cashew nut testa is a good source of hydrolysable tannins (Pillai et al., 1963). More than 40% of the total polyphenol in the testa is reported to be constituted by (+) catechin and (−) epicatechin (Mathew & Parpia, 1970). An attempt was made to elucidate the molecule responsible for the strong antioxidant characteristics of CSE observed in the present study. Ascending TLC of CSE on silica gel plates developed in chloroform–methanol (8:2, v/v) yielded a single spot which gave strong reaction with β-carotene-linoleate spray. The spot on extraction and HPLC analysis yielded a peak similar to standard epicatechin (data not shown). This finding prompted us to speculate that probably the epicatechin present in CSE may be largely responsible for the antioxidant activity of CSE. However these findings have to be confirmed by LC-MS.

It is clearly evident that ethanolic extract of cashew nut skin has significant antioxidant activity in various antioxidant assay systems. The polyphenols, in general present in the skin appears to significantly contribute to the antioxidant activity of CSE. According to the findings in our study, cashew nut skins are a natural source of phenolic compounds. Further, the presence of the potent bioactive phenolic compounds in the skins of cashew nut could be of interest to both food and pharmaceutical industries, where it could be employed as an economical source of natural antioxidants.

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