INHIBITION OF HUMAN HAEMOGLOBIN GLYCOSYLATION BY FLAVONOIDS CONTAINING METHANOLIC EXTRACTS OF THE LEAF OF *Cnestis ferruginea*

By

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ABSTRACT

Protein glycosylation by a sugar adduction, an oxidative process is exemplified by the glycosylation of haemoglobin A. The objective of this study, therefore, is to assess the influence of the leaf extracts of *Cnestis ferruginea* on human haemoglobin glycosylation. The methanolic extracts of the leaf of *Cnestis ferruginea* were prepared and tested for the presence of flavonoids and the total phenolic content obtained was 0.0175mg/ml Catechin.

Haemoglobin was partially purified from the erythrocytes of normal and diabetic individuals according to the method of Asgary *et al.*, (1999). An assessment of the effect of glucose on the time-dependent glycosylation of haemoglobin shows that the degree of human haemoglobin glycosylation increased with the period of incubation in a concentration-dependent manner up to 20mg glucose/ml. A study of the effect of varying concentrations (10-30μg/ml) of the flavonoid-containing methanolic extracts of *Cnestis ferruginea* (Cn.f) and quercetin (Q), a known antioxidant, reduced the extent of haemoglobin glycosylation at all the concentrations used irrespective of the incubation period. Glycosylation in the presence of 20mg glucose/ml was about 72% and varying concentrations (10, 20, 30μg/ml) of Cn.f reduced the glycosylation to 41, 31, and 29% respectively. Similar effects were obtained for quercetin. The observed inhibition on glycosylation was concentration and time dependent. Results obtained from the investigations carried out on the possible effect of the extracts of Cn.f on haemoglobin glycosylation in the presence of physiological concentrations of glucose (≤ 1mg/ml) indicated that there was no significant glycosylation ($P \geq 0.05$) at the varied concentration of glucose up to 1mg/ml. 4% inhibition of glycosylation was observed after 24hr in the

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presence of 10µg/ml extract of Cn f. The extent of inhibition increased from 5.5 to 7% after 48 and 72hr respectively. Using 20 and 30µg/ml methanolic extracts of Cn f and quercetin, the inhibition was total even after prolonged hours of incubation. Statistical analysis of these results showed that there was no significant difference ($P \geq 0.05$) in the inhibitory effects of methanolic extracts of Cn f and quercetin on haemoglobin glycosylation at the concentrations of glucose used in this study.

On the contrary, quercetin and α-tocopherol, a naturally occurring antioxidant, which has been widely used as dietary supplement, and extracts of Cn f, did not show any significant effect (<14%) on glycosylated haemoglobin (HbA_{1c}) in diabetic subjects. Although, the mechanism of inhibition of haemoglobin glycosylation is not yet clear, it seems likely that quercetin and leaf extracts of Cn f being very rich in flavonoids could be converted into glycoside adducts for there to be a reduction in haemoglobin glycosylation. In view of the fact that methanolic extracts of Cn f inhibited haemoglobin glycosylation, leaves of *Cnestis ferruginea* may be used as food supplement in order to prevent or inhibit haemoglobin glycosylation in diabetics.

In conclusion, leaf extracts of *Cnestis ferruginea* inhibited haemoglobin glycosylation possibly by preventing the condensation of glucose with the N- terminal amino groups of β- chains of HbA.

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Adisa, Rahmat Adetutu.

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DEDICATION

This work is dedicated to my daughters
Mariam and Rasheedat ADISA.

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CERTIFICATION

I certify that this work was carried out by Rahmat Adetutu ADISA at the Department of Biochemistry, University of Ibadan, Nigeria.

Professor O.O. Olorunsogo, Ph.D
SUPERVISOR. 17.10.02

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CHAPTER ONE

INTRODUCTION

1.1 HISTORICAL BACKGROUND OF THE DISCOVERY OF HAEMOGLOBIN

The term haemoglobin (Hb) came into being in the eighteenth century when Hoppeseyler (1864) first used it in referring to the pigments of the blood. It is now a widely accepted term used to include the respiratory oxygen carrying protein of the vertebrates. About a century later, studies showed that haemoglobin and myoglobin have very similar structures and that it was probably also true for the erythrocruorins (from larva of the fly *Chironomus tummi)* and Hb in the root nodules of leguminous plants. Therefore, it was suggested that a phylogenetic relationship could exist between these biomolecules and the transport and storage of oxygen and that it also appeared that these molecules evolved from a common precursor (Braunitzer *et al.*, 1964). The first investigation on the structure of the intact haemoglobin molecule dated back to the nineteenth century. There is no doubt that the advanced state of our knowledge today evolved from those earlier findings. Using measurements of sedimentation, diffusion, electrophoresis, results compiled from the work of several independent studies revealed that haemoglobin is a molecule of molecular weight 68,000 daltons with four heme groups.

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Rhinesmith *et al*., (1958) and Ingram (1958) in their independent studies using trypsin hydrolysis of haemoglobin and amino acid sequence determination of the peptides produced, found that haemoglobins are composed of four polypeptide chains two of which are identical. They suggested further that the two dissimilar chains of human haemoglobins should be designated α and β chains and this has been extended to the hemoglobins of other species. In this regard, the haemoglobin molecule is being chemically defined as $\alpha_2\beta_2$, each attached to the heme prosthetic group (Braunitzer *et al*., 1964).

1.2 STRUCTURE OF HAEMOGLOBIN

The haemoglobins are essential to the life of all vertebrates, they also occur in some invertebrates and in the root nodules of leguminous plants. They all carry the same prosthetic heme group, Iron (II) protoporphyrin (IX), associated with a polypeptide chain of between 136 and 153 residues. In all of them, the ferrous iron of the heme is linked to N_ε of a histidine, the porphyrin is wedged into its pocket by a phenylalanine and non-polar residues occupy about 35 other specific sites along the polypeptide chain. The amino acid sequences of the haemoglobin for the rest of the molecule are variable, the number of amino acid differences between any two species rising with their distance of separation of the evolutionary tree (Dayhoff, 1972).

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Specifically, the normal human adult haemoglobin (HbA), the oxygen carrier within red blood cells, is a protein with a molecular weight of about 64,500 daltons (Dickerson and Geis, 1983). It consists of four subunits, i.e. two identical α-chains of 141 amino acids each and two identical β-chains of 146 amino acids each. HbA is probably the most studied protein and has served as a model or paradigm for the structure-function relationship in multimeric, allosteric proteins. The oxygenation process of Hb is cooperative i.e. the binding of the first O_2 molecule enhances the binding of the second, third and fourth oxygen molecules. The oxygenation process is also regulated by interactions between individual amino acid residues and various solutes known as heterotropic allosteric effectors. These effectors include ions or molecules such as hydrogen ion, chloride ion (Perutz *et al.*, 1994), inorganic phosphate, carbondioxide, and organic polyanions, such as 2,3-bisphosphoglycerate (2,3-BPG) and inositol hexa phosphate (IHP) (Dickerson and Geis, 1983). It is noteworthy that not all the haemoglobin in the blood of normal adults is haemoglobin A. About 2.5% of the haemoglobin is haemoglobin A_2, in which β-chains are replaced by δ chains ($\alpha_2 \delta_2$). The δ chains also contain 146 amino acid residues, but ten (10) individual residues differ from those in the β-chains (Ganong, 1993). Another type of haemoglobin is haemoglobin F (HbF). It is normally found in the blood of human foetus. Its structure is similar to that of haemoglobin A except that the β chains are replaced by γ-chains (i.e. HbF is $\alpha_2\gamma_2$). The γ chains also contain 146 amino acid

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residues but have 37 that differ from those in the β-chains. In certain individuals, it fails to disappear and persists throughout life.

Perutz and colleagues (1970) carried out a comparative study of the detailed structural features of HbA in deoxy (T) and oxy (R) or CO forms. It was shown that during the transition from the deoxy to the oxy state, the $\alpha_1\beta_2$ subunit interface undergoes a sliding movement while the $\alpha_1\beta_1$ subunit interface remains essentially unchanged. Specific hydrogen bonds, salt bridges and noncovalent interactions characterize both subunit interfaces. Human Hbs with mutations in the $\alpha_1\beta_2$ subunits interface are known to have altered oxygen affinity and cooperativity. It is also known that the Hb molecule has a lower O_2 affinity in the deoxy quaternary structure (T structure) than in the oxy quaternary structure (R-structure) (see Perutz, 1970; Dickerson and Geis, 1983).

On this basis, focus is now being directed towards designing novel recombinant (r) Hbs that could serve as a safe, reliable blood substitute since infections through blood transfusions with the human immunodeficiency virus that causes AIDS were reported in the mid 1980s. Also, haemoglobin-based oxygen carriers are potential sources of blood substitutes during emergency medical situations (Winslow, 1992). The designed recombinant Hbs are required to have,

(i) low affinity because allosteric effectors such as 2,3-BPG which lower the oxygen affinity of Hb within the red blood cell are absent in the extracellular environment and

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(ii) high cooperativity for efficient delivery of oxygen (Winslow, 1992).

Sequel to this, an expression system was developed to produce authentic human normal adult haemoglobin (HbA) (Shen *et al.*, 1993) in good yield in *Escherichia coli* (Shen *et al.*, 1993, 1997). The expression system has been used to design and express mutant Hbs with low oxygen affinity and high cooperativity (Kim *et al.*, 1995; Tsai *et al.*, 1999). A unique feature of this class of rHbs is that their R (ligated) quaternary structure can be switched to the T (unligated) structure without changing the ligation state of Hb molecule, by lowering the ambient temperature and/or by adding an allosteric effector such as inositol hexaphosphate (IHP). rHb (α V96W) (Kim *et al.*, 1995) is the first low-oxygen affinity mutant rHb with high cooperativity developed. Several other rHbs with improved properties over rHb (α V96W), which is easily autoxidized, have been developed (Tsai *et al.*, 1999; Frischknecht *et al.*, 1999; Jeong *et al.*, 1999). And this factor makes it less desirable as an Hb-based oxygen carrier in a blood subsitute system (Dickerson and Geis, 1983). Most recent is the construction of novel recombinant haemoglobin, rHb (βN108Q) with the amino acid substitution located in the $\alpha_1\beta_1$ subunit interface and in the central cavity of the Hb molecule. rHb (βN108Q) exhibits low oxygen affinity, high cooperativity and stability against autoxidation, therefore it is being considered a potential candidate for the Hb-based oxygen carrier in a blood substitute system (Tsai *et al.*, 2000).

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1.3 GLYCOSYLATION OF PROTEINS

Glycosylation reactions are common and occur naturally in the body. Normally, though, they take place in a controlled environment, regulated by enzymes, the resulting molecules are classically called glycoproteins comprising of serum and cell membrane glycoproteins of approximate molecular weight range 20-2000 kda (Hounsell, 1993). Glycosylation causes a large diversity of oligosaccharide sequence to become linked to proteins at the OH group of serine, threonine, hydroxylysine or asparagine residues i.e. O- or N- linked respectively. Glycoproteins cover mucins which are traditionally defined as larger molecular weight glycoproteins of 10 kda and upwards having >60% oligosaccharides mainly O- linked via GalNAC-containing oligosaccharide cores (Poole, 1986) and proteoglycans or glucosaminoglycans which also have a high carbohydrate/protein ratio but classically have disaccharide repeating units with an alternate uronic acid residue and a large degree of sulfation (King *et al.*, 1989).

The classical mucin and proteoglycan sequences were reported to occur on cell membrane – attached proteins of relatively low molecular weight (Poole, 1986). They are in forms attached to the membrane by lipid-linked anchors called Glycosyl phosphatidyl inositol anchors (Thomas *et al.*, 1990, Ferguson, 1991) and cytoplasmic glycoproteins having GlcNAC linked to serine and threonine are known too (Torres and Hart, 1984, Holt and Hart, 1986). Thereafter, additional oligosaccharide to- protein linkages were found in proteoglycans (Poole, 1986)

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and bacterial glycoproteins (Paul *et al.*, 1986; 1987). These glycoproteins on the cell surface play an important role of cellular recognition (Hounsell 1993). Most membrane-associated and secreted glycoproteins are intercalated into or sequestered within the rough endoplasmic reticulum during synthesis. As part of this process, the nascent polypeptide are cleared and modified by the attachment of oligosaccharide side chains (Sabatini *et al.*, 1982). As these proteins transit from the rough endoplasmic reticulum to final cellular locations, the oligosaccharide side chains and in some cases the polypeptide backbones can be processed by an array of compartment specific reactions. The oligosaccharide side chains or structural elements within the side chains function as recognition sites for specific cellular components that are responsible for the selective transport of glycoproteins into various intracellular compartments as they transit in vesicles from the rough endoplasmic reticulum through intracellular membranes on their way to the cell surface and/or extracellular environment (Firestone, 1983). Moreover, several other studies have suggested that carbohydrate moieties on glycoproteins can play a role in polypeptide backbone folding (Polonoff *et al.*, 1982; Chu *et al.*, 1978; Wang *et al.*, 1977). However, while glycosylation of some proteins is essential for their compartmentalization and processing, it does not appear to be correlated with proper maturation of others. It is noteworthy that protein glycosylation is particularly important in

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maintenance of the integrity of plasma membranes and in facilitating the secretion of proteins into the extracellular space.

1.4 HAEMOGLOBIN GLYCOSYLATION

Haemoglobin takes part in some reactions such as haemoglobin glycosylation or glycation, which is the non-enzymatic condensation of glucose and haemoglobin A (HbA). Glycated haemoglobins (GHb) were first recognized and called "fast haemoglobins" (A_{1a}, A_{1b} and A_{1c}) by Allen *et al*., (1958) because they showed less positive charge at neutral pH and migrated more rapidly than HbA when placed in an electric field (Kunkel *et al*., 1955; Rahbar, 1968). They are formed by a direct reaction between reducing sugars and primary amino groups in the protein. The resulting schiff base adduct-aldimine is in equilibrium with sugar concentration. If this concentration falls, uncoupling will occur and the consequent level of aldimine will fall. These are also is equilibrium with the local medium. They dissociate if the surrounding sugar level falls sufficiently, and the equation moves back towards the left (Brownlee *et al*., 1988). Certain longer lived macromolecules such as collagen and DNA are also glycated, and having undergone stabilization to become Amadori products will then transform further to irreversibly arranged forms known as 'advanced glycation end products' (AGE's)(Brownlee *et al*., 1988).

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Haemoglobin may become glycosylated at sites other than amino terminal ends of β-chains e.g. at α as well as certain ε-amino groups (Bunn *et al.*, 1979) of lysine residues (Bookchin and Gallop, 1968).

Glycosylation of haemoglobin is slow and nearly irreversible during the 120-day life span of the red cells (Bunn *et al.*, 1976; Goldstein, 1995). The human erythrocyte is freely permeable to glucose, and within each erythrocyte glycated haemoglobin (GHb) is formed from HbA at a rate dependent on the ambient concentration of glucose (Bunn *et al.*, 1978). Thus, the level of GHb in a blood sample provides a glycemic history of the previous 120 days, the average erythrocyte life span (Bunn *et al.*, 1978). In this regard, the eldest cells will be most glycosylated while the youngest will be least glycosylated (Fitzgibbons *et al.*, 1976). However, all ages of cells will have been exposed to recent levels of glycaemia while only the eldest of the cells will have been exposed to glucose levels from 4 months previously. Therefore, the more recent the period of glycaemia, the larger its influence will be on the glycated haemoglobin value. Consequently, Tahara and Shima (1993) suggested that half of an HbA_{1c} value is attributable to changes in glycaemia over the preceding month, a further quarter is due to the month prior to that, with the remaining quarter being a reflection of months 3 and 4. The most important of the GHbs with respect to diabetes is HbA_{1c} in which glucose is attached to the NH_2-terminal valine residue of one or

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both chains of HbA (Holmquist and Schroeder, 1966). It is this modification that imparts enough negative charge to the haemoglobin molecule to allow separation of the respective haemoglobins by charge dependent techniques. In contrast, glycosylation at sites other than the β-chain amino terminus results in a compound with a charge not dissimilar to non-glycosylated haemoglobin and so is indistinguishable by these methods (Kilpatrick, 1997). However, this portion which accounts for about half of all haemoglobin glycosylations can be detected if glycation-specific "total glycated haemoglobin" methods such as boronate affinity chromatography (Mallia *et al.*, 1981) is used.

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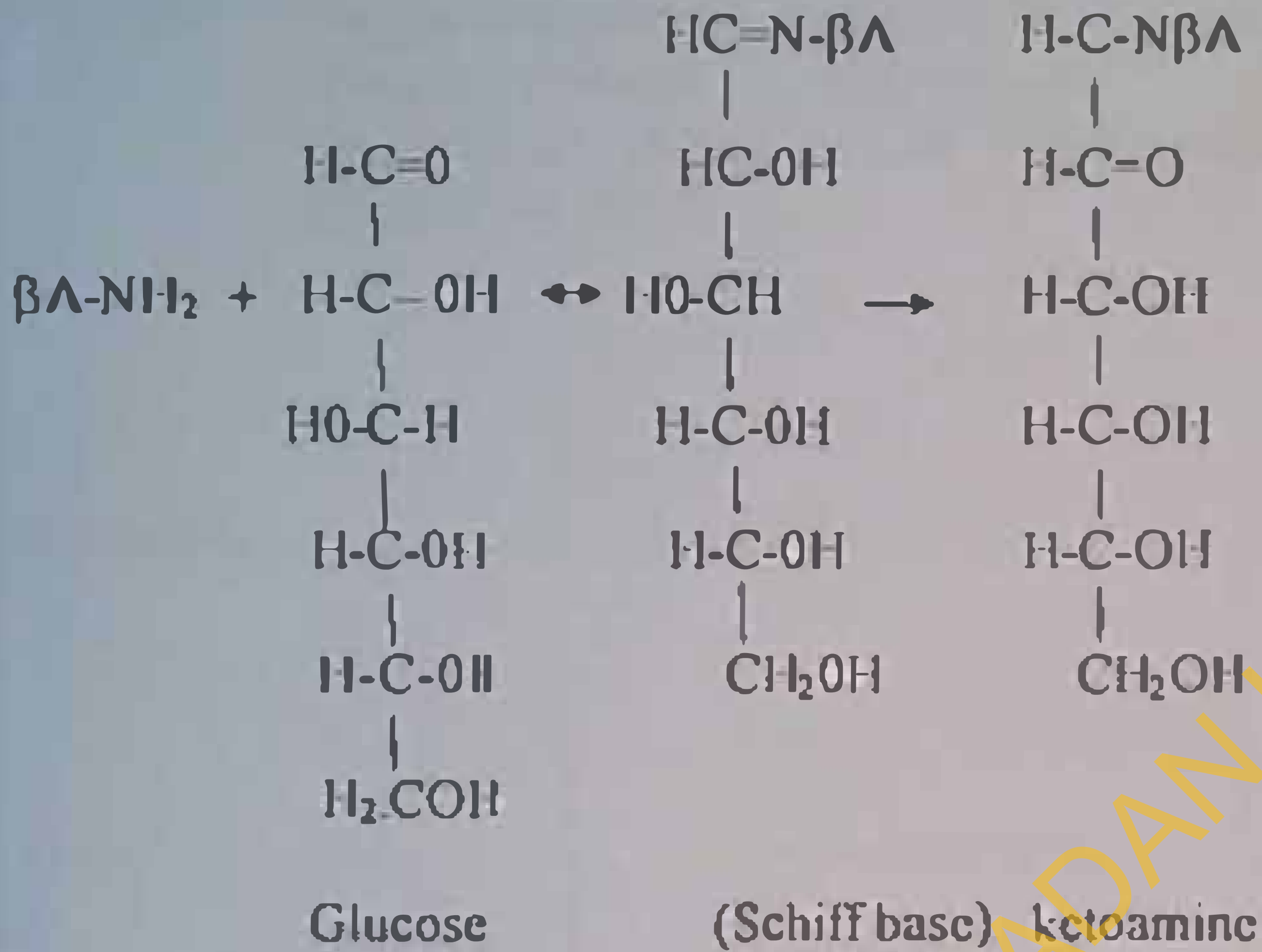

Fig.1: Diagramatic representation of the reaction process of haemoglobin glycosylation (Bunn *et al.*, 1979)

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Table 1: The carbohydrates involved in the modification of charge-separated hemoglobins with their approximate abundance in non-diabetic individuals (Kilpatrick, 1997)

Hemoglobin		Modification	Abundance (%)
	Ao		95
HbA_1	HbA_{1a1}	Fructose 1,6-diphosphate	0.2
HbA_1	HbA_{1a2}	glucose-6-phosphate	0.5
HbA_1	HbA_{1b}	Carbohydrate	
	HbA_{1c}	glucose	4

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1.5 EFFECT OF GLYCOSYLATION ON HEMOGLOBIN FUNCTION

As earlier discussed, Hb's A_{1a}, A_{1a2} and A_{1c} are modified at the NH_2 terminal amino group of the β-chains, a side normally involved in the binding of organic phosphates (Arnone 1972). Within the mammalian red cells, 2, 3-diphosphoglycerate (2,3-DPG), an important regulator of intracellular haemoglobin function, is present in equimolar concentration with the haemoglobin tetramer. The 2,3-DPG polyanion binds more strongly to deoxy-hemoglobin than to oxyhemoglobin thereby causing a marked reduction in the affinity of hemoglobin for oxygen (Arnone, 1972). Its negatively charged groups form salt bonds with positively charged residues on the two β-chains at the entrance to the central cavity of the haemoglobin molecule, including the NH_2-terminal amino groups. If this site is blocked by a covalent attachment such as hexose (Bunn and Briehl, 1970), or an acetyl or carbamyl group (Kilmartin *et al.*, 1973), the reactivity of haemoglobin with 2,3-DPG is markedly reduced. In comparison to HbA, the oxygen affinity of HbA is much less responsive to the addition of 2,3-DPG (Bunn and Briehl, 1970). In like manner, synthetic Glucose-6-phosphate (G-6-P)-hemoglobin has a similarly decreased reactivity with 2,3 DPG (Haney and Bunn, 1976).

McDonald *et al.*, (1979) examined oxygen equilibra of haemoglobins A, A_{1a1}, A_{1a2}, A_{1b} and A_{1c} as well as the kinetics of the binding of carbon monoxide

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with deoxy haemoglobin and found that Hb's A_{1a_1} and A_{1a_2} have a low affinity for heme ligands and decreased cooperatively between subunits. Like HbA_{1c} they show decreased interaction with organic phosphates. It is likely that the covalently bound phosphates on Hb's A_{1a_1} and A_{1a_2} enhance the stability of the deoxy or T conformation, thereby lowering oxygen affinity. Since Hb_{A1c} has decreased reactivity with 2,3-DPG, red cells of diabetic patients might be expected to bind oxygen abnormally. As predicted by oxygen equilibria in dilute solution, the oxygen affinity of diabetic red cells is slightly greater than that of normal red cells having a comparable amount of 2,3-DPG (normal individuals have a P_{50} of about 26mm-Hg, whereas diabetics may have a P_{50} of 24 to 26mm-Hg. Obviously, there is considerably overlap between the two groups (Bunn *et al.*, 1978).

1.6 BIOCHEMICAL AND CLINICAL IMPLICATION OF PROTEIN GLYCOSYLATION IN DIABETES MELLITUS

Huisman and Dozy in 1962 were the first to relate glycosylated haemoglobin to diabetes mellitus, when they observed a two- three fold increase in haemoglobin A_{1a-c} in tolbutamide-treated diabetics. In a remarkable independent study, Rahbar (1968) detected an abnormal pattern on agar gel electrophoresis in some diabetic patients. Trivelli *et al.*, (1971) eventually found that the haemoglobin component present in the diabetic subjects had the same

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chromatographic and electrophoretic properties as haemoglobin A_{1c} with a two fold increase over values found in normal subjects. These increased levels were not related to the age of the patients, duration of the disease, type of therapy, or the presence of the complications of diabetes mellitus. Hyperglycemia has been reported by Spiro (1971, 1976), to stimulate the attachment of glucose molecule to intracellular proteins found in organs and tissues (Kidney, retina, lens, peripheral nerves, and blood vessels) that are not insulin-dependent for glucose entry. Increased intracellular glucose levels have been demonstrated to be responsible for the formation of some diabetic complications such as cataract, neuropathy by increased shunting of glucose metabolism into accessory pathways [such as sorbitol pathway, (Javid *et al.*, 1977)] that are normally quiescent during periods of normoglycemia. Hyperglycemia consequently results in gradual accumulation of 'advanced glycation end-products' (AGEs) in certain tissues. For instance, high levels of AGE formed from glycated collagen in the subintima can bind covalently to low density lipoproteins (LDL), especially if the apolipoprotein of the LDL is also heavily glycated (Brownlee *et al.*, 1988). This is believed to accelerate atherosclerosis even in the presence of normal serum cholesterol concentrations (Witztum *et al.*, 1982). Also, local adverse effects on the clotting cascade via a reduction in thrombomodulin activity and activation of clotting factors IX and X can induce thrombosis (Brownlee *et al.*, 1981, Vlassara *et al.*, 1989). This is enhanced by local vasoconstriction from an excess of

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endothelin-1 and the aforementioned reduction in vasodilation secondary to the'quenching' and inactivation of nitric oxide in vessel walls (Bucala *et al.*, 1991). These combined abnormalities are related undoubtedly to the predisposition of diabetic subject to atherosclerosis and widespread microvascular disease. (Stanaway and Gill, 2000).

In a similar way, the formation of crosslinks in the protein matrix of the basement membrane of the micro circulation due to heavy irreversible glycation of the macromolecules, tends to trap excess amounts of albumin and immunoglobulins which cannot be removed (Michael and Brown, 1981; Graham and Johson, 1985). These become glycated themselves and attract further accumulation of proteins from the plasma. There is disruption of the micro-architecture of the basement membrane, resulting in 'leakier' structure, allowing the extravasation of plasma proteins and disrupted binding of the membrane to adjacent cells. Extravasation of plasma protein is accelerated in the face of hypertension. The adverse effects of diabetes on peripheral nerves are believed to be at least partially microvascular in aetiology though there is also a direct effect of intraneuronal cytoplasmic protein glycation that has a direct effect on nerve conduction velocity (Stanaway & Gill, 2000).

Macrophages, endothelial cells and mesangial cells, all express high affinity receptors for AGEs that can trigger the release of local growth factors (Vlassara *et al.*, 1985; Esposito *et al.*, 1992). These include interleukin 1 (IL-1)

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and insulin-like growth factor 1 (IGF-1) and can cause mesangial proliferation in the renal glomerulus with consequent effects on renal function (Doi *et al.*, 1992). This is in conjunction with basement membranes disruption allowing leakage of an excess of plasma proteins into the urine by ultra filtration. The long term histological effects of this process are seen histologically in the kidney as the classical 'glomerulosclerosis' lesion.

Furthermore, several disorders of the musculoskeletal system found more commonly in diabetic subjects are believed to be due to AGE accumulation causing excessive cross-linking of collagen molecules. There is reduced remodeling and accelerated ageing of the skin, which becomes thicker and can take a 'yellow' appearance particularly on the palms and soles. Localised ischaemia and fibrosis due to microvascular AGE effects exacerbate this process and there is associated tendon sheath sclerosis. Joints underlying the affected area become increasingly stiff due to this thickening. This is known as 'cheiroarthropathy'. It occurs in 8-50% of type I diabetic subjects (Clark *et al.*, 1991). Trigger finger is another commonly associated symptom. Together this collection of phenomena is known as the 'diabetic hand syndrome'. Cheiroarthropathy is best demonstrated as the 'prayer sign'.

Frozen shoulder (periarthritis, adhesive capsulitis) is another common musculoskeletal disorder associated with diabetes. It is believed to be due to excessive crosslinking of collagen molecules in the shoulder capsule. It is

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characterized clinically by pain and restricted active and passive movement at the glenohumeral joint. The most affected movements are external rotation and abduction and pain is often worst at night and during the first six months when there is active inflammation followed by a progressive capsular fibrosis resulting in stiffness. Albumin can be glycated by exactly the same principles as all other proteins. As its life span is shorter (aprox. 2-3 weeks) measurements of plasma glycated albumin levels gives an impression of glycaemic control over this reduced time period. (Johnson *et al.*, 1982; Ashby and Frier, 1988). This is known as the 'fructosamine' assay. The fructosamine level correlates better with actual average glucose levels, as there is a more rapid turnover in the glycated albumin due to its shorter half-life.

1.7 FREE RADICALS

Free radicals as described by Halliwell in (1994), are specie(s) capable of an independent existence that contains one or more unpaired electrons. They could be produced by oxygen involving biochemical redox reaction occurring in normal cell metabolism or by phagocytes as part of a controlled inflammatory reaction or in response to occasional exposure to Ionizing radiation, u.v. light, pollution, cigarette, smoke, ischaemia etc. These radicals are collectively referred to as "Reactive oxygen species" (ROS) and comprises of superoxide ($\dot{O}_2^-$), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), singlet oxygen, hypochlorous

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acid (HOCl), ozone (O_3) and perhydroxyl radical (Harber and Weisis, 1934). In addition, peroxyl radical (ROO$^-$), alkoxyl radical (RO$^-$) and ferryl radical belong to this group.

1.8 FREE RADICAL AND PATHOLOGICAL CONDITIONS

Free radical pathology is consequent to uncontrolled, abnormal radical reaction occurring in cells and probably initiated by physiologically occurring radicals (Tolin and Fox, 1967) already dislocated or by exogenous agents such as chemical which are already radicals or metabolized into radicals or may induce radical states in normal chemical constitutents of cells (Thomas *et al.*, 1968). This pathological state may also arise as a result of imbalance between the production of free radicals and the body antioxidant system. Free radicals have been associated with not less than 100 human and animal diseases such as malaria, rheumatoid, arthritis, Parkinson's disease, inflammation, asthma, neuro-degeneration, diabetes and ageing (Muller 1992). Cerutu in (1994) suggested that membrane damage resulting from free radical mediated process of lipid peroxidation might contribute to oxidative DNA damage implicated in carcinogenesis. Recent study has shown that oxygen radicals and lipid peroxidation are involved in the course of aging, age related disease and cancer (Ames, 1995). Further study by En-Huacao (1995) was evident that there is a link

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or relationship between lipid peroxidation process, mutagenesis and carcinogenesis.

1.9 PATHOLOGICAL EFFECTS OF FREE RADICALS IN DIABETES MELITUS

Early suspicion of the involvement of reactive oxygen species (ROS) in the pathophysiology of diabetes mellitus emanated from studies on alloxan-induced diabetes in rats which supported the generation of reactive oxygen species in this disease condition.

Alloxan injected into rats accumulates only in the liver and the islet of Langerhams where it generates superoxide radical from oxygen during the oxidation of its unstable reduction product – dialuric acid. This oxidation step depends on the presence of trace amounts of transition metal and also leads to the generation of hydrogen peroxide and hydroxyl radicals by Fenton – type reactions (Halliwell and Gutteridge, 1984).

Thornalley *et al.,* (1984) also reported that monosaccharides can undergo oxidation to generate reactive-oxygen species in the presence of catalytic amounts of iron or copper ions. This further fortified the belief that reactive oxygen species could play an important role in the pathway of diabetic complications. Recent studies on the level of enzymic antioxidants – cardiac Cu – Zn superoxide dismutase and catalase show increased activities in diabetic patients. This may be

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a resultant compensatory response to an increased level of free radical generation. Previously, a higher catalase activity reported by Godin and Wohacib, (1988) was thought to be in response to increased peroxisomal production of hydrogen peroxide in diabetics (Horie *et al.*, 1981), however, this observation may also be partly attributed to the production of hydrogen peroxide by the dismutation of superoxide radical. Furthermore, De Bono (1994), in an independent study showed that the non-enzymic glycation of proteins, particularly under conditions of high glucose concentration such as in diabetes mellitus, could generate free radicals and then cause cell damage.

Indeed, these pathways suggest that oxidative stress could be an important feature of diabetes mellitus. Reactive oxygens species (ROS) are normally formed in aerobic organisms in the course of different cellular functions (Halliwell and Gutteridge 1989). They can be very toxic, leading to cellular damage and even apoptosis (cell death), if there is an imbalance between their production and the body defence mechanism against free radicals, called antioxidant system.

During recent years, the molecular mechanisms and potential treatment of acute and chronic neurologic disorders have become a research area of paramount importance (Krieglstein and Oberpichler 1990; Dunn 1995, 1996). Trauma, spinal cord injury and stroke fall into the category of acute neurologic disorders while chronic neurologic disorders include sclerosis, Alzheimers and Parkinson's

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disease. Also, increasing evidence suggests that free radicals and specifically reactive oxygen species (ROS) play an important role in the aetiology of these neurologic diseases (Gutteridge, 1993; Jenner, 1994; and Simonian and Coyle, 1996).

1.10 ANTIOXIDANTS IN BIOLOGICAL SYSTEM

Antioxidant is any substance which when present at low concentration compared to those of oxidizable substrate, such as DNA, lipids, proteins, and carbohydrates significantly delays or prevents substrate oxidation (Halliwell and Gutteridge, 1989). They are naturally existing body defence mechanism for protecting tissues from free radical effect. Most times free radical reactions are deleterious to cellular life, hence the body has evolved many overlapping defence mechanism to protect against oxidative stress. These include both enzymes and small molecules that act as scavengers of reactive oxygen species. The enzymatic defence system comprises of superoxide dismutase (SOD), glutathione peroxidase (GSG-PX), catalase, and DNA repair enzymes. The non-enzymatic defence mechanism includes metal binding proteins (Ferritin and Ceruloplasmin), vitamins E, C, β-carotene, albumin and minerals such as selenium, copper, zinc (Diplock, 1991).

Antioxidants whether naturally present or added [Butylated hydroxytoluene (BHT), Butylated hydroxyanisole, (BHA), propylgallate (PG),

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ascorbic acid and α-tocopherol) retard but may not completely inhibit lipid peroxidation. Antioxidants with radical scavenging activity react directly with free radicals formed during oxidation reactions and convert them to less reactive compounds by donating a hydrogen atom rather than blocking the initial free radical generation reaction (Mahoney and Graf, 1986). These antioxidants have been further grouped into three classes according to their mode of operation.

(i) **Primary antioxidants:** These prevent the formation of new free radical species either by converting existing free radicals into less harmful molecules before they have a chance to react or by preventing formation of free radicals from other molecules. Examples are SOD, glutathione peroxidase, catalase, metal-binding proteins (Ferritin and Ceruloplasmin).

(ii) **Secondary antioxidants:** They scavenge radicals by preventing chain reactions.
Examples include vitamin C, E, β-carotene, uric acid, bilirubin, and albumin.

(iii) **Tertiary antioxidant:** These repair free radicals damaged biomolecules. They include DNA repair enzymes, and methionine sulphoxide reductase.

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1.11 ENZYMIC ANTIOXIDANTS: These are cellular enzymes that provide the first line of antioxidant defences in cells. Examples are:

1.11.1 Superoxide dismutase (SOD):

SOD is an enzymic antioxidant having electron configuration of 1:15:1:1. It scavenges superoxide radical by having its dismutation to hydrogen peroxide and oxygen as shown below:

$$O_2^- + O_2 \xrightarrow[SOD]{2H+} H_2O_2 + O_2$$

SODs are essential for the survival of aerobic cells (Burton 1991). The erythrocyte SOD is known as erythroscuprien (McCord and Fridovich, 1969) while that of bovine is known as hemocuprein (McCord and Fridovich, 1969). SODs are unique metalloproteins, for example hemocuprein, first isolated from Ox blood in (1938) as a blue-green protein containing 0.38% copper with a molecular weight of approximately 35,000 daltons (McCord and Fridovich, 1969). The importance of superoxide in oxygen toxicity (Oberley, 1982) led to the following presumptions regarding SODs, all of which have proved correct.

1. Only organisms existing as obligate anaerobes would not have superoxide dismutases.
2. An increased exposure to oxygen should result in the increased synthesis of SOD.

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3. Increased exposure to oxygen through the application of compounds such as methyl viologen (paraquat) or streptonigrin should increase superoxide dismutase synthesis.
4. Metabolism changes that increase dependence on aerobic metabolism should increase superoxide dismutase synthesis.
5. The resistance to hyperbaric oxygen should correlate with SOD levels and
6. Mutant defective in SOD should exhibit a decreased resistance to oxygen.

There are different types of superoxide dismutases, which catalyse the same reaction and appear to have comparable deficiencies. They have been classified according to the type of metal at their active site.

(a) Copper-zinc containing superoxide dismutase.

It has a molecular weight of 32,000 daltons. This dimeric molecule contains an atom of copper and an atom of zinc tightly but reversibly bound per 16,000 molecular weight (Steinman, 1983).

The active site of the enzyme consists of a copper ion surrounded by four histidine residues in a distorted square planar configuration. The histidines are at sites 44, 46, 61 and 118. The copper and zinc are in close proximity with the nitrogen of the imidazole ring of His 61 bound to the copper and the other nitrogen bound to the zinc. The copper and zinc ions within each subunit are

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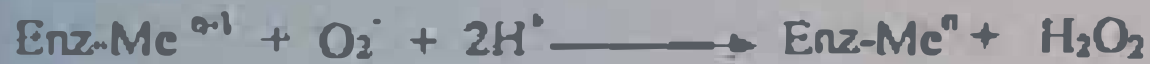
approximately 6A° apart. According to the proposed mechanism shown below, the copper ions which are relatively exposed are catalytically active and are alternately reduced and reoxidized by superoxide as follows

$$Enz\text{-}Me^{n} + O_2^{-} \longrightarrow Enz\text{-}Me^{n-1} + O_2$$



$$Enz\text{-}Me^{n-1} + O_2^{-} + 2H^{+} \longrightarrow Enz\text{-}Me^{n} + H_2O_2$$

where Enz is enzymes and Me is metal.

Net reaction: $O_2^{-} + O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2$

(b) Manganese – containing superoxide dismutase

The manganese superoxide dismutase is a dimer containing one atom of manganese per sub unit. It has a molecular weight of approximately 40,000 daltons and was first isolated from *Escherichia. coli* (Steinman, 1983). Its mechanism of catalysis is thought to be an alternate reduction and reoxidation similar to that of the copper in SOD upon reaction with superoxide.

The eukaryotic manganese enzyme was first obtained from chicken liver mitochondria and contains four subunits instead of two. It has a molecular weight of approximately 80,000 daltons and is very similar to the bacterial SOD leading to the same speculation that indeed, mitochondria evolved from a prokaryote source through its entry into an endocellular symbiosis with a proto-eukaryote (Steinman, 1983).

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(c) Iron – containing super oxide dismutase.

The iron containing SOD is similar in amino acid sequence to the manganese- containing enzyme and was first isolated from *Escherichia coli*. But about two decades ago, they were isolated from bacteria and some plants. The mechanism of the enzyme is similar to that of the Cu-Zn and manganese containing enzymes but appears to involve the sequential reduction and reoxidation of the active metal.

1.11.2 Catalase

Catalase an enzymic antioxidant with electronic configuration of 1.11.1.6 which functions mainly by dismutating hydrogen peroxide formed by superoxide dismutase or by the uncatalized reaction of hydroxyl peroxyl radical into water and molecular oxygen.

$$H_2O_2 + H_2O_2 \xrightarrow{CATALASE} H_2O_2 + O_2$$

Catalase is present in major animal body organs especially concentrated in the liver and erythrocytes (Marklund, 1982). Most aerobic cells contain catalase activity, however, a few such as bacterium *Bacillus popilliae* and *Mycoplasma pneumoniae* do not. Also, a few anaerobic bacteria contain catalase, an example is *propionibacterium shermanii* but most do not (Marklund, 1982). Four protein subunits have been shown in most purified catalases, and each of them contains a haem (Fe (III) – protoporphyrin) group bound to its active site. Turrens *et al.*,

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(1984) reported that the dissociation of the molecule into its subunits, which easily occurs on storage, freeze-drying or exposure to acid or alkali, causes loss of catalase activity.

In animals and plant tissues, catalase activity is largely located in peroxisomes, which are a membrane bound subcellular organelles. Although, studies carried out on catalase activity in tissue homogenates of plant and animal indicated that a significant proportion of this activity is not bound to organelles. This could partly or wholly be due to rupture of fragile peroxisomes during homogenization. At least, the liver mitochondria, chloroplast and the endoplasmic reticulum contain little, if any, catalase activity therefore, any hydrogen peroxide generated by them *in vivo* cannot be disposed of in this way (Sinet *et al.*, 1980). The mechanism of catalase reaction is as follows:

$$Fe(III) + H_2O_2 \xrightarrow{K_1} \text{compound I}$$

$$\text{Compound I} + H_2O_2 \xrightarrow{K_2} \text{Catalase} - Fe(III) + 2H_2O + O_2$$

The two second-order rate constant K_1, and K_2 for rat liver catalase have values of $1.7 \times 10^7\ M^{-1}\ S^{-1}$ and $2.6 \times 10^7\ M^{-1}\ S^{-1}$ respectively (Chance *et al.*, 1979).

From the above equation, the complete removal of hydrogen peroxide requires the impact of two meolecules of hydrogen peroxide upon a singlet active site. This becomes less likely as hydrogen peroxide concentration falls. The amount of compound 1 present in a mixture of catalase and hydrogen peroxide

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depends on the concentrations of catalase and hydrogen peroxide and on the rate constants K_1 and K_3

If the concentration of hydrogen peroxide is fixed, the initial rate of removal of it will be proportional to the concentration of catalase present and hence will be higher in liver than in say, brain or heart (Sinet *et al.*, 1980). Similarly for a given concentration of catalase, the initial rate of hydrogen peroxide removal will be proportional to the hydrogen peroxide concentrations.

1.11.3 Glutathione peroxidase

Glutathione peroxidase (GSG-PX EC 1.11.1.9) is a widely dispersed animal enzyme, first discovered, partially purified and characterized by Mills in (1959). Although, it has been reported in some algae and fungi, it is not present in higher plants or bacteria. This membrane bound enzyme scavenges hydrogen peroxide, fatty acid and hydroperoxides by reducing them to water and molecular oxygen and also forming the oxidized glutathione (GSSG) from its reduced form.

$$H_2O_2 + 2GSH \longrightarrow 2GSSG + 2H_2O$$

The enzyme consists of four protein sub-units, each of which contains one atom of the selenium element in its active site. It is probably present at the active site as selenocysteine, which reduces the peroxide to an alcohol and is oxidized to selenic acid (E – Se – OH) (Gutteridge, 1988).

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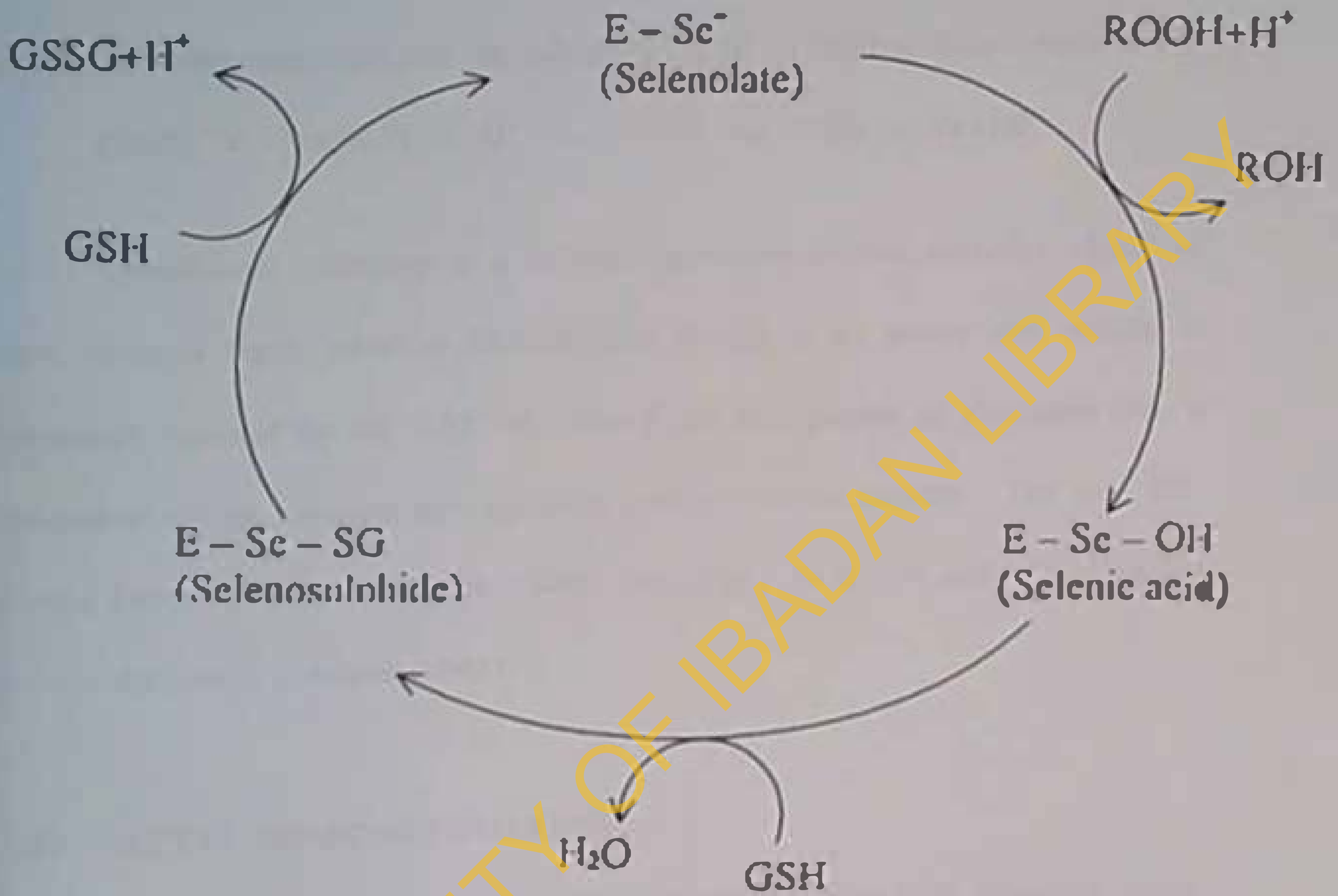

Fig. 2 Proposed Catalytic Mechanism of Glutathione Peroxidase.

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In normal cells, the ratio of GSH/GSSG is usually kept high, hence glutathione reductase catalyzes the reduction GSSG to GSH as shown below:

$$GSSG + NADPH + H^+ \longrightarrow GSH + NADP^+$$

Glutathione reductase is a protein consisting of two subunits. Each of them contains flavin adenine dinulcleotide (FAD) at its active site, which, is apparently reduced by the NADPH. The FAD then passes its electrons onto a disulphide bridge between two cysteine residues in the protein. The two SH-groups formed then interact with GSSG, reducing it to 2GSH and reforming the protein disulphide (Meister, 1983).

1.12 METAL BINDING PROTEINS

A major antioxidant defence of human plasma is to bind transition metal ions in forms that will not stimulate free-radical reactions, or to otherwise prevent the metal ions from participating in such reactions (Halliwell and Gutteridge 1986).

(i) **Transferrin**. This is an iron binding protein present in plasma of humans. Iron bound to transferrin will not participate in OH radical formation or lipid peroxidation (Aruoma and Halliwell, 1987).

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provided that the iron present does not overwhelm the iron-binding capacity of the transferrin.

(ii) Lactoferrin. This is a protein present in human secretions and released by neutrophils. It also acts as an antioxidant by binding iron (Gutteridge, Paterson *et al.*, 1981). The physiological importance of this sequestration of iron ions into "safe" forms is clearly illustrated by an inspection of the multi-organ damage seen in patients with iron-overload disease in whom low molecular mass iron ion complexes (principlally involving citrate ligands) (Grootveld *et al.*, 1989) capable of stimulating lipid peroxidation and OH generation (Gutteridge *et al.*, 1985) circulate in the plasma.

(iii) Haptoglobins and Hemopexin. These are plasma proteins that diminish the effectiveness of iron compounds in stimulating lipid peroxidation. Haptoglobulin is a plasma protein binding haemoglobin liberated into plasma and other body fluids after tissue injury. The hemopexin is also a protein binding heme released from damaged heme proteins that are usually powerful stimulator of peroxidation (Tappel, 1955).

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There are two mechanisms for haemoglobin accelerated lipid peroxidation.

(a) The reaction of the heme ring with equimolar concentrations of H_2O_2 produces an oxo-iron species such as ferryl, that can lead to stimulation of lipid peroxidation by interacting with free fatty acids or fatty acyl side chains in membranes while still remaining bound to the proteins.

(b) Excess H_2O_2 can cause degradation of the heme rings of myoglobin and haemoglobin, releasing from the protein iron ions that are capable of stimulating $OH^{\cdot}$ production and lipid peroxidation (Gutteridge, 1986, Puppo and Halliwell, 1988).

The haemoglobin-haptoglobin or heme-hemopexin complexes are rapidly cleaved from the circulations (Oshiro and Nakajima, 1988) since failure to remove haemoglobin in this way results in brain damage (Panter *et al.*, 1985).

(iv) Ceruloplasmin. This is a plasma copper containing protein with antioxidant activity (Gutteridge and Stocks, 1981). It exhibits ferroxidase activity by oxidizing Fe^{2+} to Fe^{3+} while reducing oxygen to water.

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

The ferroxidase activity of ceruloplasmin allows it to inhibit iron-ion dependent lipid peroxidation (Gutteridge 1985). Similarly, ceruloplasmin non-specifically binds copper ions and can thus inhibit copper ion-

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stimulated formation of reactive oxidants e.g. (OH) and lipid peroxidation (Gutteridge *et al.*, 1984; Lovstad, 1987). In addition, ceruloplasmin reacts with H_2O_2 and with superoxide (O_2) non-significantly, hence, the enzymatic protein has little or no "SOD-like" or catalase-like activity (Calabrese and Carbonaro, 1986).

(v) **Albumin**. This also binds copper ions and usually inhibits copper ion-dependent lipid peroxidation and OH radical formation (Gutteridge and Wilkins, 1983; Halliwell, 1988). The binding of copper ions to albumin may lead to albumin damage if O_2 and H_2O_2 are generated in plasma, however, it prevents the copper ions from attaching to more important targets such as key –SH groups on the membranes of endothelial cells or erythrocytes where binding of Cu^{2+} can lead to oxidative damage (Hochstein *et al.*, 1980). Furthermore, it prevents Cu^{2+} from accelerating the peroxidation of low density lipoproteins and promoting atherosclerosis (Esterbauer *et al.*, 1989).

Albumin transports fatty acids in the blood and the bile pigments-bilirubin is bound to it. It was shown by Stocker *et al.*, (1987) that bilirubin acts as an antioxidant inhibitor of lipid peroxidation *in-vitro*. Perhaps, therefore, it protects albumin-bound fatty acids against peroxidation *in-vivo*. However, the prevention of copper ion-mediated damage by albumin is probably a property of the protein itself. Albumin

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is also a powerful scavenger of hypochloric acid (HOCl) in plasma (Wasil *et al.*, 1987) and bilirubin contributes partly to this activity (Stocker and Peterhans, 1989).

Other metal chelating agents that reduce lipid peroxidation are citrate, diethylene tramine, pentaacetic acid (DTPA), malic acid and tartaric acid (Lemon *et al.*, 1950; Mahoney and Graf 1986). Such agents either chelate metal ions or suppress reactivity by occupying cell co-ordination sites on the metal ion and therefore, may be effective agents in retarding metal-catalysed lipid peroxidation.

1.13 NON-ENZYMIC ANTIOXIDANTS

1.13.1 Ascorbic acid. This is a vitamin necessary for the functioning of humans, other primates, guinea pigs and certain fruit eating birds. It is a white crystalline solid that is very soluble in water when pure. It can be synthesized from glucose by plants and most animals but humans and other primates have lost one of the necessary synthetic enzymes during their evolution hence they require vitamin C from their diets. The *in-vivo* biosynthesis of collagen involving proline hydroxylase and lysine hydroxylase (enzymes having Fe at their active sites) require vitamin C as a cofactor. In the absence of vitamin C, insufficiently hydroxylated collagen which does not form fibrous property is synthesized. Thus,

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resulting in poor wound healing and fragility of blood vessels (Seib and Tolbert, 1982).

Ascorbate is an electron donor i.e a reducing agent having the chemical ability to reduce Fe (III) to Fe (II) thereby promoting iron uptake in the gut. The importance of its reducing nature was shown by Hodges (1982) that dietary ascorbate inhibits the carcinogenic action of several nitroso-compounds fed into animals because it converts them to inactive forms. Indeed ascorbate required by the prolyl hydroxylase enzymes probably maintains the copper (Cu) and Iron (Fe) at its active site in the reduced active form necessary for the hydroxylation reaction during collagen synthesis (Prockop *et al.*, 1979).

As an antioxidant with hydrophilic nature, it directly reacts with O_2^-, OH radicals and lipid hydroperoxides on the hydrophilic layer of the membrane but not with the lipophilic radicals within the interior of the membranes. Although tocopherol and ubiquinol are the primary scavengers of radicals within the membrane, tocopherol acts in concert with ascorbate which causes the regeneration of tocopherol from its α-tocopheryl radicals at the surface of membranes. And the tocopherol then continues to scavenge free radicals within the membrane (Hansen *et al.*, 1991).

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For vitamin C to exert its total antioxidant activity, there is need for the careful sequestration of free transition metal ions that is generated during tissue injury from the plasma (Halliwell and Gutteridge, 1984). The sequestration was highly essential because the administration of ascorbic acid to patients with iron-overload can lead to serious consequences, however, unless desferrioxamine is given simultaneously. By contrast, the overall effect is that depending on the concentration of the ascorbate, free radical reactions may also be stimulated.

1.13.2 Uric acid. The antioxidant activity of uric acid involves:

(i) binding of copper and iron ions in forms that do not accelerate free radical reactions and

(ii) directly scavenging oxidizing species such as singlet oxygen, HOCl, and peroxyl radicals (Ames *et al.*, 1983; Davies *et al.*, 1986). Grootveld (1989) in collaboration with other researchers observed an increased concentration of breakdown products of uric acid in the body fluids taken from humans with rheumatoid, arthritis or iron-overload disease. These workers therefore, suggested that uric acid does react with some oxidants '*in vivo*'. The reaction of uric acid with certain oxidizing species such as OH radical produced a uric acid radical capable of reacting with oxygen to form a peroxyl radical ($R^{\cdot}O_2$) which however, is less reactive than hydroxyl radical but can also

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damage/inactivate certain enzymes (Kittridge and Willson, 1984). But, fortunately, ascorbic acid can reduce these uric acid derived radicals (Maples and Mason, 1988).

1.14 POLYPHENOLIC COMPOUNDS

Polyphenolic compounds are antioxidants usually from natural origin such as in fruits, vegetables, nuts, seeds, flowers, barks of trees, and marine organisms. They are of diverse chemical structure and characteristics. They are majorly classified into flavonoids, tannins, coumarins, lignans etc. (Cook and Samman, 1996). These polyphenolic compounds have been found to be involved in the alleviation of some ROS degenerative diseases such as aging, arthritis, cancer and diabetes (etc).

1.14.1 Flavonoids. Flavonoids are a ubiquitous group of low molecular weight naturally occurring polyphenolic compounds widely distributed as secondary metabolites in plant kingdom (Middleton, 1993).

The major classes of flavonoids include: flavones, flavonones, catechins, anthocyanidins, Isoflavone, dihydro-flavonols and challiones (Kuhnau, 1970). Flavonoids are absorbed from the gastrointestinal tracts of humans and animals and excreted either unchanged or as flavonoid metabolites in the urine and faeces. The extent of absorption of flavonoids being an important unsolved problem in

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adjudging their many alleged health effects was considered by Hollman and Katan, (1997). Flavonoids present in foods bound to sugars as beta-glycosides were thought to be non-absorbable whereas aglycones-sugar free flavonoids are able to pass through the gut walls. Hydrolysis only occurs in the colon by microorganisms, which at the same time degrade flavonoids. On this base, Hollman and Katan, (1997) in a study quantified absorption of various dietary forms of quercetin. Surprisingly, the quercetin glycosides from onions were absorbed far better than the pure aglycone. Subsequent pharmacokinetic studies with dietary quercetin glycosides showed marked differences in absorption rate and bioavailability. Absorbed quercetin was eliminated only slowly from the blood. It was also found that two major sites of flavonoid metabolism are the liver and the colonic flora.

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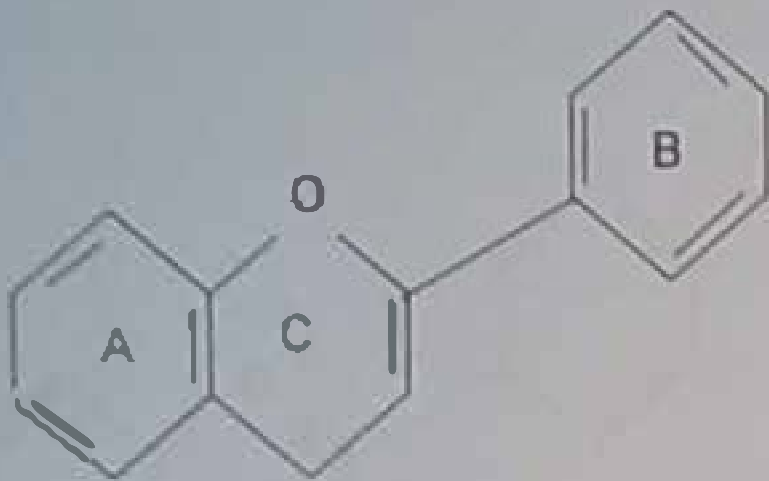
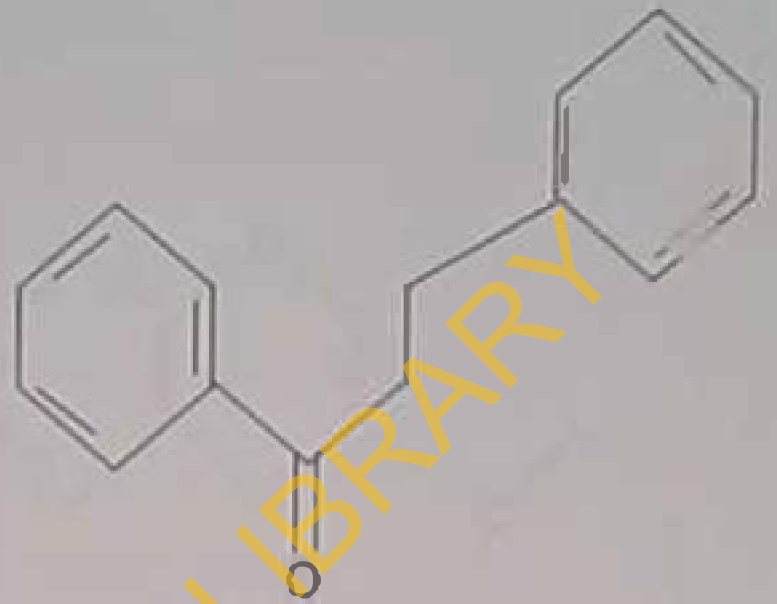
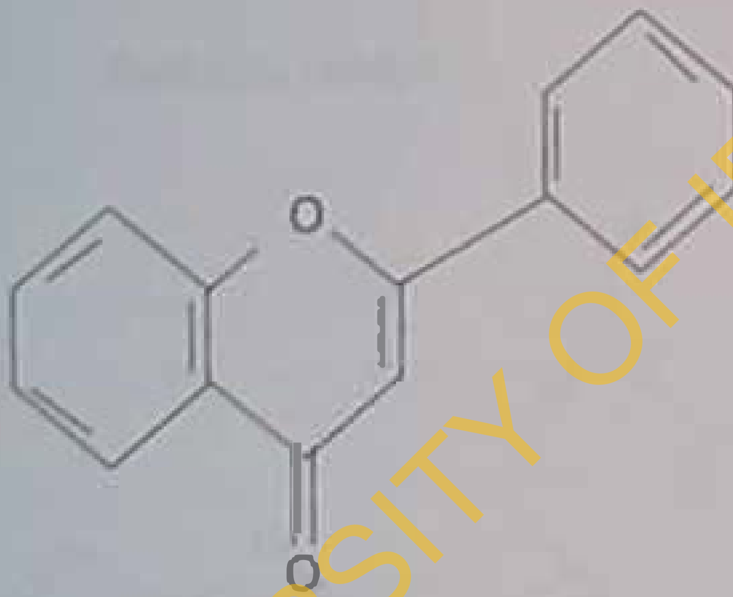


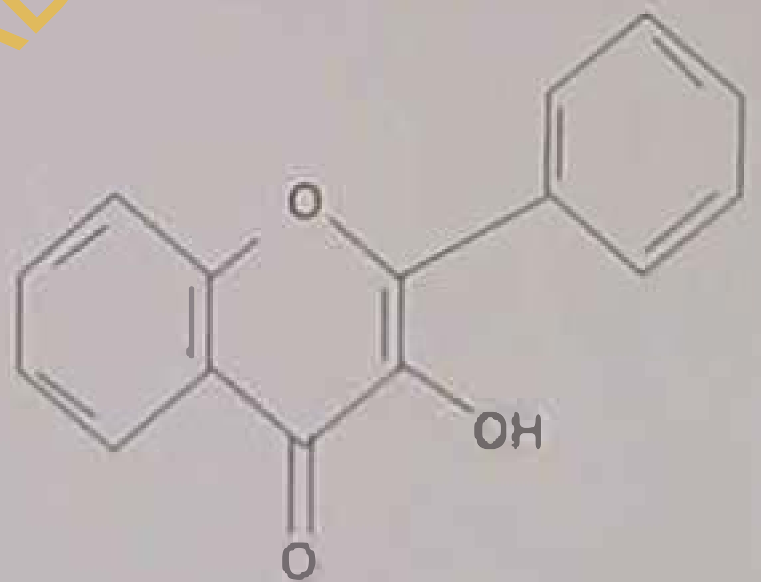
Flavonoid structure



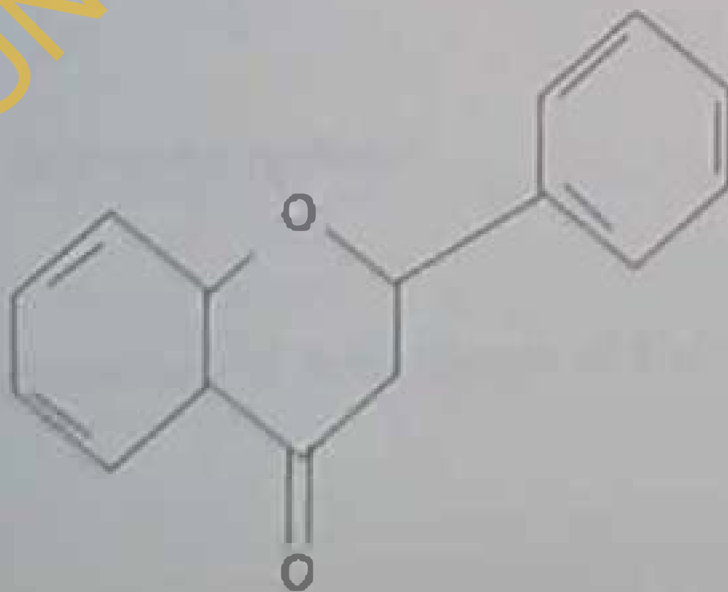
Chalcone



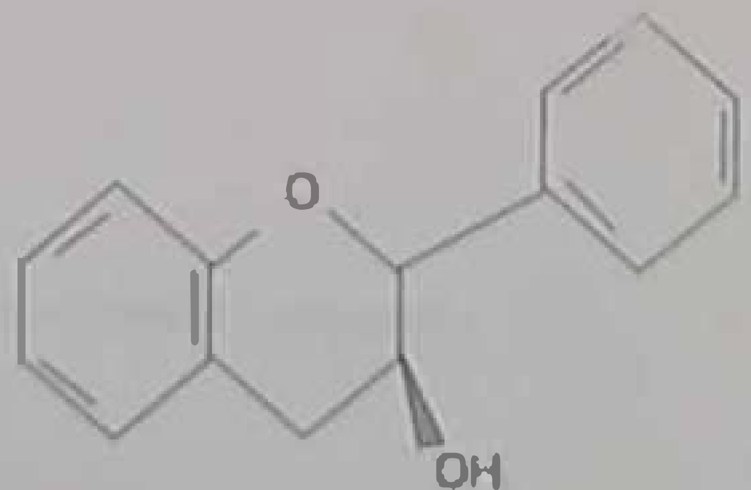
Flavone



Flavonol



Flavonone



Flavanal

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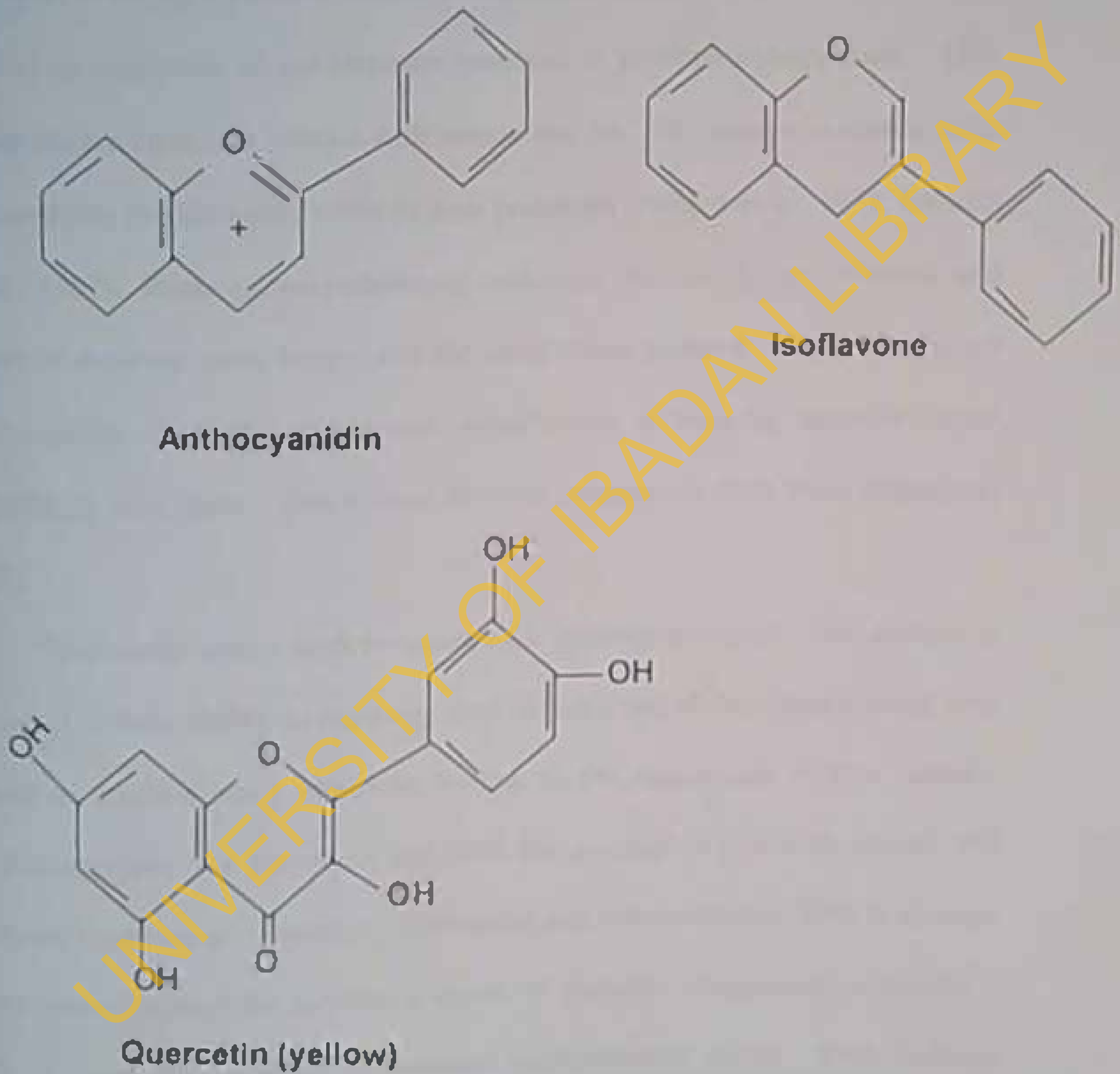

Fig 3. The structure of some classes of Flavonoid and related compounds

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1.15 BIOLOGICAL PROPERTIES OF FLAVONOIDS

Flavonoids are found in the thylakoid membranes of plant. They probably participate in the light phase of photosynthesis as catalyst of the electron transport and (or) as regulators of ion channels involved in photophosphorylation. Also, flavonoids are known to interact with proton and Na^+ - K^+ pumps in animal cells, thus implying that they play a role in such processes (Pollard *et al.*, 1979; Ravanal *et al.*, 1981). When photosynthesizing cells die, flavonoids are released and appear in the plant juice, honey, and the resin. Bees gather fluids which are rich in flavonoids from the plants and manufacture a resinous material called PROPOLIS from them. This is used to close openings in their hives (Hausteen, 1983).

Flavonoids have a high propensity for electron transport. This priority is exhibited in their ability to scavenge free radicals and chelate heavy metal ions known to catalyse many processes leading to the appearance of free radicals. Furthermore they are known to influence the permeability of both natural and synthetic membranes. Therefore, Roufogalis and collaborators (1999) in a recent investigation showed the inhibitory action of phenolic compound, on the Ca^{2+}, Na^+ - K^+ and Mg^{2+} ATPase as potential cardiovascular agents. Their findings indicated that flavonoids inhibition of the calcium pump is independent of calmodulin stimulation and that bisphenolic compounds in particular may be

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useful in studying the role of plasma membrane Ca^{2+} ATPase and regulation of intracellular Ca^{2+}. It was further suggested that they may as well have potential cardiovascular activities.

Furthermore, wine especially, the red wine has been reported to contain a range of polyphenols such as {(p-coumaric, cinnamic, vanillic acid, trihydroxystilbenes and flavonoids (catechin, epicatechin and quercetin} that have desirable biological properties such as promotion of nitric oxide production by vascular endothelium, inhibiting the synthesis of thromboxane in platelets and leukotriene in neutrophils, modulating the synthesis and secretion of lipoproteins in whole animals and human cell lines, arresting tumour growth as well as inhibiting carcinogenesis in different experimental models. These effects were accounted for through target mechanisms which include inhibition of Phospholipase A_2 and cyclooxygenase inhibition of phosphodiesterase with increase in cyclic nucleotide concentrations and inhibition of several protein kinases involved in cell signaling (Soleas *et al.*, 1997).

1.16 PHARMACOLOGICAL EFFECTS OF FLAVONOIDS

Flavonoids have been reported to have a wide range of medicinal effects including antibacterial, antiviral, anti-inflammatory, anti-allergic (Hope *et al.*, 1983), vasodilatory (Duarte *et al.*, 1993, and just recently cardiovascular actions (Roufogalis *et al.*, 1999). Moreover, they have been reported to inhibit lipid

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peroxidation (Salvayre, *et al.*, 1988) platelets aggregation, capillary permeability and fragility as well as the activity of enzymes like cyclo-oxygenase and lipo-oxygenase. Flavonoids also exhibit activities such as antioxidants, free-radical scavenging and divalent cation chelators (Afanasev, 1989; Fraga 1987). Natural concentrates of flavonoids such as Propolis (a resinous substance obtained by bees from plants for use as glue in their hives) have been used for years to treat a wide range of human disease conditions including gastroduodenal ulcers and cancer (Hausteen, 1983, Hertog *et al.*, 1993).

In order to clarify whether the antioxidant properties of flavonoids are dependent on radical scavenging or iron-chelating activities, Shim *et al.*, (1999) in an investigation used an experimental approach based on the notion that iron chelators suppress DNA scission and cytotoxicity caused by tert-butyl hydroperoxide, whereas radical scavenging antioxidants prevent only the latter response. It was clearly evident in their experiment that the activity of the flavonoid, quercetin resides in its iron chelating ability. Moreover it was suggested that their experimental approach could be utilized for the assessment of iron chelation in the biological activity of flavonoids or other antioxidants.

1.17 MODE OF ACTION OF FLAVONOIDS

Reactivity of flavonoids have been studied under several experimental conditions and such investigations have shown that the antioxidant properties of

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flavonoids are due to radical scavenging properties directed towards superoxide, hydroxyl, peroxyl and alkoxyl radicals as well as a concomitant capability of chelating iron, a metal known to increase the rate of appearance of free radicals (Potterat, 1997). For instance, the inhibitory action of quercetin and rutin (Muller, 1992) in lipid peroxidation result from scavenging superoxide anion, inhibition of hydroxyl formation by chelating iron ions and reaction with lipid radical (Afana'sev, 1989).

Within the last decade, focus has been directed towards elucidating the structure – activity relationship of flavonoids. Bors and co-workers in particular found that their structural groups were important determinants for radical scavenging and antioxidative potentials. They are the O-dihydroxy B (catechol) structure in the B-ring, the 2,3-double bond in conjugation with a 4-oxo function and the additional presence of 3 and 5-hydroxyl groups for maximal radical-scavenging potential (Bors *et al*., 1987, 1990). Also, qualitative structure-activity relationship (QSAR) has revealed that the presence of 1, 4 and 1, 2-hydroquinones in the A and /or B ring and the hydrophobicity of the molecules are responsible for the *in vitro* inhibition of lipid peroxidation (Miyahara *et al*., 1993).

Somewhat recently, a structure – activity study of 14 different chemical classes of flavonoids on phosphatidyl inositol-3-kinase alpha revealed that the position, number, and substitution of the hydroxyl group of the B ring and

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saturation of C2-C3 are important factors affecting flavonoid inhibition of phosphatidyl inositol – 3 kinase (Agullo *et al.*, 1997), an enzyme recently indicated to play an important role in signal transduction and cell transformation. Also, reactivity of flavonoids with the superoxide radical was shown to depend on the redox properties which are highly sensitive to the hydroxyl substitution on the B-ring. The reaction mechanism appeared to be electron transfer with concerted proton transfer (Jovanovic *et al.*, 1994).

1.18 *Cnestis ferruginea*

Cnestis ferruginea with local names 'Oko – Aja' or Gboyin – Gboyin (in Yoruba) belongs to a family of *connaraceae*. It is found in deciduous and secondary forests as well as secondary scrub e.g. Udi-Plateau. They are widely distributed with imparipinnate leaves, ten inches long, eight pairs of leaflets, all parts ferruginously pubescent. The flowers are white with ferruginous sepals. Their fruits are red, velvety, curved pod, containing black seeds, one of each capsule.

Cnestis ferruginea is used in traditional medicine for a variety of purposes. The leaf decoction is used by Yorubas of South West Nigeria as a laxative (Dalziel, 1937). It can further be used as an enema for dysentery and gonorrhoea. The powdered roots of *Cnestis ferruginea* in ripe papaw is usually used in

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forklore medicine to treat madness caused by ill-fortune and for some other superstitious purposes (Kerharo and Bouquet, 1950).

In Nigeria, the Ibos use the roots for tooth -ache and tooth-caries while the powdered bark is rubbed on the gums for pyorrhea. The fruit is juicy and the taste acid and bitter. It is widely used in West-Africa for cleaning the teeth because it has a very refreshing and cleaning influence upon the mouth and teeth. The boiled or fresh fruits are crushed with rum or palm-wine as a remedy for snake-bite and/or applied locally (Dalziel, 1937). Moreover, the fruit juice is used for treating wounds (Flore du Congo Belge, 1952). Rubbing with the whole pulped plant is good for every kind of pain.

1.19 CHEMICAL CONSTITUENTS OF *Cnestis ferruginea*

It has been shown that petroleum ether fraction of *Cnestis ferruginea* fruit contains among other constitutents, octacosanyl sterarate and 1-myristo-2-stearo-3- palmitin (Ogbechie *et al.*, 1987). Phytochemical screening of this also revealed the presence of flavonoids, combined anthraquinones saponins, tannins, steroidal glycoside (Ogbechie *et al.*, 1987) and a novel isoflavone glycoside, afrormosin - 7.0-beta-D-galactoside in the fruit (Parvez *et al.*, 1992). Furthermore, its biological activities were investigated against different organisms and it was observed that this compound has antimicrobial activity against *staphylococcus*

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aureus and *Escherichia coli*, but did not inhibit the growth of *Aspergillus niger*. The compound also had antifungal effects against *Candida albicans* (Parvez *et al.*, 1992). Over a decade ago, extracts of roots, stems and leaves of *Cnestis ferruginea* have been reported to have anti-bacterial activity (Boakye and Konning, 1975) and anticonvulsant activity (Declume *et al.*, 1984).

Recent phytochemical studies on the leaves and roots of *Cnestis ferruginea* shows the presence of flavonoid and flavonoidal compounds, however, their exact structures are yet to be determined (Oke *et al.*, 1999).

1.20 QUERCETIN

Quercetin like morin, myricetin and kaempferol belongs to a family of polyhydroxyl flavones which are a subgroup of well-known natural antioxidant molecules. Worldwide, it occurs in conjugated or free forms in many edible plant foods (Hermann, 1976; Harborn and Williams, 1975; Mabry and Ulubelen, 1980). Quercetin has been reported very widely as a non-carcinogenic naturally occurring flavone (De Eds, 1968; Sugimura, 1979). Some workers, however, have demonstrated the mutagenicity and carcinogenicity of Bracken fern (*Pteridium aquilinum*), a component of which is quercetin (Bryan and Pamukou, 1979, Pamukou *et al.*, 1980). Quercetin has been shown to affect the growth of transplanted sarcoma (Bohm, 1968). About a decade ago, Yoshida *et al.*, (1990) reported that quercetin markedly inhibits the growth of human gastric cancer cells

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and blocks cells progression from the G_1 to the S phase. In addition results obtained from the study on mouse epidermis by the two-stage model of carcinogenesis promoted by 12-0-tetradecanoyl Phorbol-13 acetate and telocidine suggests that the inhibitory effects of quercetin on such cell growth may be mediated by a blockade of protein kinase C (PKC), a known cellular receptor for such tumour growth promoters. This inhibition was also found to be independent of Ca^{2+} and phospholipids (Feri *et al.*, 1989).

Studies carried out on the anti- free radical effects of quercetin suggest that this action of quercetin may be related to a reduction in superoxide anion formation and/or with a direct scavenging of superoxide anions radical (Xie *et al.*, 1989; 1992). Also, the protective action of quercetin in induced-cardiac arrhythmias and acute cerebral ischema have been associated with the inhibition of oxygen-free radical formation, the direct scavenging of such radicals and the inhibition of SOD inactivation (Xie *et al.*, 1991; Feng *et al.*, 1994). Futhennore, quercetin has been implicated in the inhibition of platelet aggregation and thrombin-induced increase in Ca^{2+} concentration in blood platelets. These workers found that the inhibitory effects on such aggregation could be reduced by adding calcium to the medium, whereas quercetin had no effect on thrombin-induced intracellular Ca^{2+} release. The results suggest that the inhibitory action of quercetin on aggregation and the increase in Ca^{2+} levels in platelets are mainly due to an inhibition of Ca^{2+} influx (Xiao and Gu, 1995).

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Similarly, Quercetin inhibits the activities of miscellaneous enzymes including the ion-motive ATPases (Lang and Racker, 1974, Gu *et al.*, 1994) and glutathione S-transferase in a solitary bee, *Megachile rotundata* (Frohlich *et al.*, 1989). Specifically, quercetin inhibited plasma membrane Ca^{2+}-ATPase of human erythrocyte inside-out vesicles, although it inhibited the Ca^{2+}-ATPase of plasma membrane "ghosts" with lower potency. Quercetin has been shown to bind directly to calmodulin in a Ca^{2+}-dependent manner (Wuthrich and Schatzmann, 1980).

1.21 EFFECT OF QUERCETIN ON HYPERGLYCEMIA

Quercetin was shown in a study with aldose reductase to possess enzyme inhibitory properties with an IC_{50} of 0.344 Umol/L (Mao and Zhang, 1993). Therefore, it may have future value in the prophylaxis and treatment of the complications of diabetes.

Another enzyme study investigated the effects of quercetin on Na^{+}/K^{+}-ATPase activity in the Sciatic nerve of diabetic rats. It was found that sorbitol levels in sciatic nerve were markedly reduced by quercetin treatment. When compared with controls, Na^{+}/K^{+}-ATPase in sciatic nerve was significantly increased but there was no significant change in the inositol levels, thus

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suggesting that the response to quercetin treatment is independent of the inositol levels in sciatic nerve (Mao and Zhang, 1995).

Of recent, Asgary *et al.*, (1999) in a study compared the relationship between structure-activity of quercetin, rutin and kaempferol (flavonoids) and their inhibitory effect on haemoglobin glycosylation. Quercetin was found to possess the highest inhibitory potential on haemoglobin glycosylation, thus supporting their probable use in the treatment of diabetic complications (Asgary *et al.*, 1999).

1.22 OBJECTIVE OF THE STUDY

Human haemoglobin demonstrates marked heterogeneity mainly as a consequence of post-translational changes due to the non-enzymatic binding of various carbohydrates in a process called Glycosylation. Glycosylation occurs via a carbohydrate such as glucose reacting in its free aldehyde form with a haemoglobin molecule to form the schiff base compound aldimine (Koenig *et al.*, 1977) which may undergo an amadori rearrangement to form a stable glycated ketoamine product (Acharya and Manning, 1980). The extent of the reaction is increased during diabetic hyperglycemia (Bunn *et al.*, 1981). A variety of proteins are subject to non-enzymatic glycation and this is thought to contribute to the long-term complication of the disease (Robins and Bailey, 1974; Renold *et al.*, 1978; West 1978; Valssarn *et al.*, 1981). Measurement of glycated haemoglobin

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has proven to be particularly useful in monitoring the effectiveness of therapy in diabetes (Goldstein, 1995). However, since the glycosylation of proteins is an oxidative reaction, antioxidants should be able to prevent this reaction. Antioxidants such as flavonoids are found in fruits, vegetables, nuts, seeds, stems, flowers as well as tea, and wine and are important constituents of human diet (Harborn, 1994, Soleas *et al.*, 1997). These compounds have been demonstrated to

(i) affect a large variety of enzymes

(ii) possess free radical scavenging activity

(iii) chelate certain metal cations

(iv) have antioxidant properties

(v) increase resistance of LDL to oxidation

(vi) protect biomembranes against peroxidative damage

(vii) affect cellular protein phosphorylation (Robak and Gryglewski, 1988; Liu *et al.*, 1992; Harborn, 1994; Gliterog *et al.*, 1997; Ishikawa *et al.*, 1997; Fuhrman *et al.*, 1997).

Cnestis ferruginea is a medicinal plant used extensively in African traditional medicine for a variety of purposes. Extracts of *Cnestis ferruginea* have been shown to possess antibacterial (Boakye and Konning, 1975), antimicrobial (Parvez *et al.*, 1992), anticonvulsant properties (Declume *et al.*,

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1984). Recently, phytochemical screening of this plant showed that it contains maorly polyphenolic compounds and metabolites such as alkaloids (Oke and Hamburger, 2002). It is against this background that this study focuses on:

(i) determining the presence of flavonoids in the leaf extracts of *Cnestis ferruginea*

(ii) assessing the probable mechanism of inhibitory action of *Cnestis ferruginea* on haemoglobin glycosylation.

Thus, considering the fast growing acceptability of glycated haemoglobin (GHb) by the Diabetes Control and Complications Trial (DCCT) as a simple blood test that is highly predictive of risk for development and/or progression of microvascular and neuropathic complication in diabetes, the discovery of a new drug that could have direct arrest on the formation of GHb would provide a breakthrough in the therapy and management of the long term complications of the disease.

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CHAPTER TWO

MATERIALS AND METHODS

2.1 COLLECTION OF BLOOD SAMPLES

30ml of blood was collected from 10 commercial donors of the University College Hospital, Ibadan. The samples were collected in vernoject bottles containing EDTA as anticoagulant.

2.2 PREPARATION OF HAEMOGLOBIN

REAGENTS

(i) **0.14M NaCl**

8.18g of sodium chloride (NaCl) (Hopkins and Williams, Essex, England) was dissolved in a little quantity of distilled water and made up to $1dm^3$ with more distilled water.

(ii) **0.01M Phosphate buffer pH 7.4:** To make up this buffer, the following solutions were prepared.

(a) **0.01M $Na_2HPO_4.12H_2O$**

0.90g of disodium monohydrogen orthophosphate dodecahydrate (Hopkins & Williams, Essex, England) was dissolved in little quantity

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of distilled water and made up to 250ml with more distilled water in a 250ml volumetric flask.

(b) **$0.01M\ KH_2PO_4$**

0.34g of potassium dihydrogen orthophosphate (Hopkins and Williams, Essex, England) was dissolved in a little quantity of distilled water and made up to 250ml in a volumetric flask with more distilled water.

(c) Solution (b) was gradually added to solution (a) with continuous stirring and the pH was adjusted to 7.4 with 0.1M NaOH solution.

PROCEDURE

Red blood cells haemolysate was prepared by the procedure of Asgary *et al*., 1999 based on the principle of hypotonic lysis. The red blood cells were washed thrice with 0.14M NaCl solution. 1 volume of red blood cells suspension was lysed with 2 volumes of 0.01M phosphate buffer, pH 7.4 and 0.5 volume of carbon tetrachloride. The haemolysate was then freed from the debris by centrifugation at 1500g for 15 mins at room temperature. The haemoglobin rich fraction (upper layer) was separated and dispensed into sample bottles for storage at $-10^{\circ}C$ until required for use.

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2.3 ESTIMATION OF HAEMOGLOBIN CONCENTRATION

PRINCIPLE

The haemoglobin concentration (Hb) of a solution may be estimated by any of several methods by measurement of its

(i) colour haemoglobin

(ii) power of combining with oxygen or carbon monoxide or

(iii) iron content

In this study, the haemoglobin content of blood samples were estimated using the procedure of Drabkin and Austin (1932) based on the dilution of blood in a solution containing potassium cyanide and potassium ferricyanide. Haemoglobin and inert pigments such as methaemoglobin (Hi) and carboxyhaemoglobin (HbCO) (but not sulphaemoglobin (SHb), were converted to haemoglobin cyanide (HiCN). The absorbance of the resulting solution was then measured in a spectrophotometer at a wavelength of 540nm or a photoelectric colorimeter with a yellow-green filter (e.g. Ilford 625).

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The Diluent

This is the Drabkin's cyanide – ferricyanide solution or reagent (pH 8.6). It consists of 200mg potassium ferricyanide, 50mg potassium cyanide in 1L of distilled water. The diluent was clear and pale yellow in colour. When measured against water as blank in a photoelectric colorimeter at a wavelength of 540nm, absorbance must be zero. It was stored at room temperature in a brown borosilicate glass bottle to keep for several months.

REAGENTS

DRABKIN REAGENT: 200mg of potassium ferricyanide (J.T Baker Chemical, Co. Phillipsburg, N.J.) and 50mg of potassium cyanide (J.T Baker Chemical Co. Phillipsburg, N.J.) were dissolved in 1 litre of distilled water. The diluent, a clear and pale yellow solution was stored at room temperature in a brown borosilicate glass bottle.

PROCEDURE

20µl of blood was added to 4ml of diluent. After stirring the solution was allowed to stand at room temperature for 30 mins to ensure completion of the

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reaction. The resultant solution of HiCN was then compared with a standard blank and a reagent blank in a spectrophotometer (Spec-Metertek – Sp-850) at 540nm.

Concentration of standard/reference HiCN = y

The haemoglobin concentration was calculated using the formula below:

$$Hb\,(g/L) = \frac{A^{'}540 \text{ of test sample}}{A_{540} \text{ of standard}} \times y \times \frac{\text{Dil. factor}}{1000}$$

2.4 PREPARATION OF METHANOLIC EXTRACTS OF *Cnestis ferruginea*

Collection and Authentication of Plant Material

The leaves of *Cnestis ferruginea* were obtained from a forest in Mamu, a village in Oyo State. The samples were authenticated and identified by Mr. T.K. Odewo of the Herbarium, Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State.

The fresh leaves of the plant were thoroughly rinsed and spread on trays and allowed to air-dry for one week at room temperature. The dry leaves were blended to powder and weighed. 200g of the powdered leaves of *Cnestis ferruginea* was soaked in 2L of methanol (95% v/v) Sigma. Chemical Co.,

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London) in a covered glass fluted container and kept in a dark room for 4 days. The soaked material was then decanted and the filterate evaporated on water bath at 40°C until a dark greenish brown sticky substance was obtained. The weight of the extract was calculated as follows:

Weight of extract + crucible = x

Weight of crucible = y

Weight of extract = x − y.

The extract was stored in glass container and left in a dessicator.

2.5 TEST FOR THE PRESENCE OF FLAVONOIDS IN METHANOLIC EXTRACTS OF *Cnestis ferruginea*

PRINCIPLES

Flavonoids are polyphenolic compounds which have free radical scavenging properties. The radical 2,2 Diphenyl-picryl hydrazine (DPPH) was first used by Takao *et al.*,(1994) to demonstrate the presence of flavonoid components of marine bacteria present in fish and fruits of the sea. This test for flavonoids is based on the presence of phenolic hydroxyl groups which act as electron or hydrogen donors to quench electron mobility in DPPH and thus, interrupting the free-radical chain reaction (Shahidi *et al.*, (1992) and forming complexes that show the observed yellow coloration of DPPH on a violet background.

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PROCEDURE

1mg each of quercetin and methanolic extracts of *Cnestis ferruginea,* (Cn f) were suspended in 10mls of methanol. The mixture was shaken vigorously following which a capillary tube was used to apply aliquots of the mixture as spots on the aluminum coated plate about, 10mm away from the bottom of the plate. The site of the spot was carefully labeled and the plate was allowed to dry in air. The plate was immersed in a tank containing the mobile phase (Ethylacetate: formic acid: water; 85: 10: 5) and allowed to develop until the solvent front had moved to about 1mm to opposite the edge of the aluminum coated plate.

The plate was then allowed to dry and viewed under UV light at 365 and 254nm. The fluorescent bands were marked at those wavelengths and the plate was sprayed with Diphenyl-picryl-hydrazyl reagent (10mg/10ml methanol) using a spraying gun. The plate was left to dry and later scanned in a computer. Quercetin was used as the standard flavonoid (Cuendet *et al.*, 1997).

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2.6 QUANTIFICATION OF TOTAL PHENOLIC COMPOUNDS IN METHANOLIC EXTRACTS OF *Cnestis ferruginea*

REAGENTS

FOLIN DENIS REAGENT

Folin Denis reagent was prepared by a slight modification of the methods of AOAC (1970).

20g sodium tungstate (Hopkins & Williams Ltd, Essex, England), 4g phosphomolybdic acid (B.D.H., Laboratory Chemicals Division, England) and 10ml Orthophosphoric acid (Hopkin & Williams Ltd, Essex, England) were added to 150ml distilled water in a redox reaction flask. The mixture was refluxed for 2 hrs in the presence of anti-bumping granules (BDH Chemicals, Poole, England), cooled and diluted to 200ml with distilled water.

Catechin: (0.2mg/ml)

2mg catechin (Sigma Chemical Co USA) was mixed with 4ml of distilled water and the mixture was warmed to facilitate complete dissolution. The solution was cooled and made up to 10ml with distilled water and used fresh

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10g/100ml sodium Carbonate (Na_2CO_3)

5g sodium carbonate (Hopkin and Williams, Essex, England) was dissolved in about 45ml distilled water and shaken until completely dissolved. The solution was made up to 50ml with distilled water.

PROCEDURE:

The total phenolic compounds in methanolic extract of *Cnestis ferruginea* was quantified by using a modification of the method described by Gow-chin Yen and Pin-Der Duh (1994). 0.1ml of methanolic extract of *Cnestis ferruginea* (1mg/ml) was diluted with distilled water (3.25ml) and 0.25ml of Folin Denis reagent added.

The mixture was thoroughly shaken, 0.5ml Na_2CO_3 was added 3 minutes later and finally made up to 5ml with distilled water. The reaction medium was allowed to stand for 30 minutes with intermittent shaking while the blue colour was developing. The absorbance readings were taken with a spectrophotometer (photomech 300.D$^+$) at 390nm. Catechin was used as standard. The concentration of total phenolic compounds in the extract was extrapolated from the standard catechin curve.

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Protocol for Standard Catechin Curve.

Vol of Catechin (ml)	Conc. of (mg/ml)	Conc. of Extract (mg/ml)	A390nm
0.0	0.000		
0.2	0.008	–	
0.4	0.016	–	
0.6	0.024	–	
0.8	0.032	–	
1.0	0.040	–	
–	–	0.02	

Each experiment was run in triplicates.

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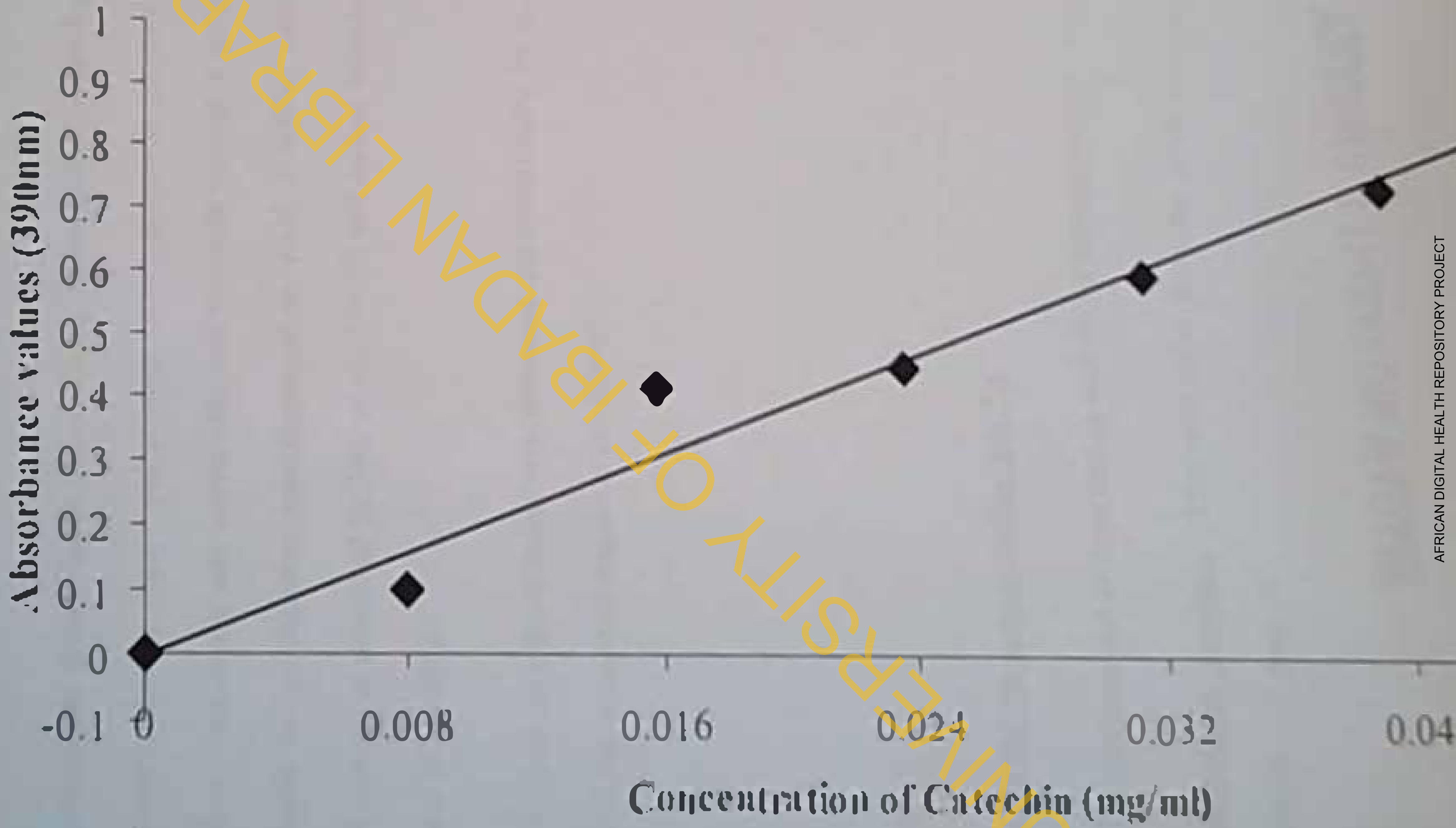

Fig. 4 Standard Curve for Catechin

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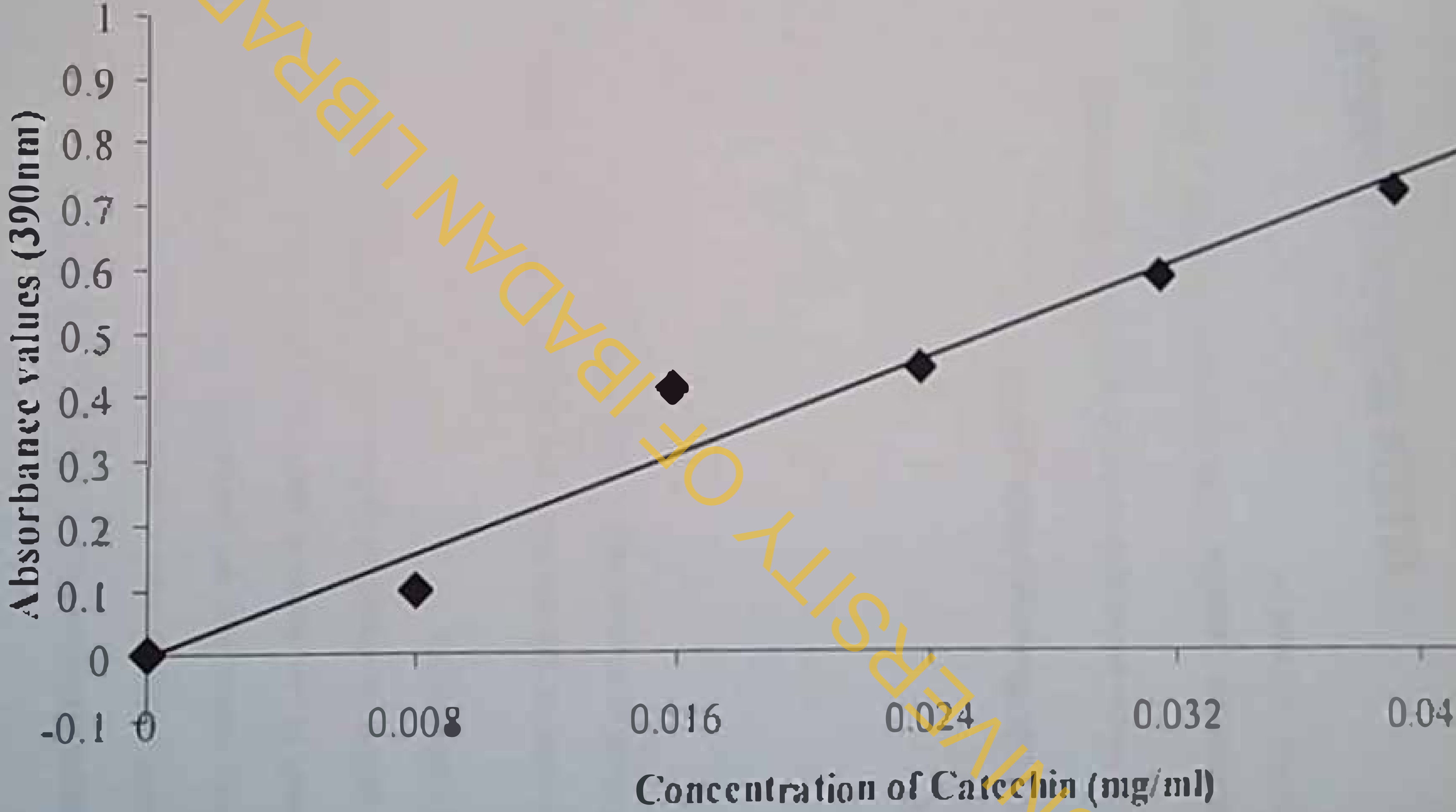

Fig. 4 Standard Curve for Catechin

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2.7 ESTIMATION OF HAEMOGLOBIN GLYCOSYLATION

PRINCIPLE

Non-enzymatic glycosylation of protein e.g. haemoglobin occurs by direct reaction between reducing sugars and primary amino groups in proteins. The reaction is slow, continuous, and irreversible and proceeds through a schiff base adduct followed by an amadori rearrangement to yield a stable ketoamine derivative of protein as described by Day *et al.*, (1979) and further substantiated by Goldstein *et al.*, (1995)

The amount of glycosylation was measured colorimetrically by a slight modification of the method of Asgary *et al.*, (1999).

REAGENTS

(i) <u>**0.01M Phosphate buffer pH 7.4**</u>

This was prepared as described in section 2.2 Subsection (ii)

(ii) <u>**Haemoglobin solution:**</u> This was taken from the stock prepared in Section 2.2 page 54.

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(iii) Glucose solution: Different concentrations of glucose were prepared by dissolving 0.2g, 1g and 2g of D-glucose (Hopkins & Williams Ltd Essex England) in 100ml of 0.01M phosphate buffer pH 7.4 and diluting the stock to the desired concentrations

PROCEDURE

1ml of haemoglobin fraction and 1ml of the solution containing different concentrations of glucose (0.2g, 1g, and 2g) in 100ml of 0.01M phosphate buffer pH 7.4 were incubated at room temperature for 72 hrs.

A test control was also prepared without glucose. The haemoglobin concentrations of the test and control tubes were estimated at different incubation periods (0, 24hr, 48hr, and 72hr) as described in section 2.3 page 56. This estimation was used as the index of measurement of haemoglobin glycosylation. Sample and blanks were prepared in duplicates.

2.8 ASSAY OF THE EFFECT OF METHANOLIC EXTRACT OF *Cnestis ferruginea* ON HAEMOGLOBIN GLYCOSYLATION

PRINCIPLE

The degree of haemoglobin glycosylation was measured colorimetrically by a slight modification of the methods of Asgary *et al.*, (1999). It is based on the

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condensation reaction believed to occur between proteins e.g. haemoglobin and sugars e.g. glucose. A schiff base, adduct that undergoes an amadori rearrangement to yield a stable ketoamine derivative is formed. The experiment was carried out *in vitro* and the residual haemoglobin level estimated colorimetrically by the method of Drabkin and Austin, (1932).

REAGENTS

(a) Haemoglobin solution: This was prepared as earlier described in section 2.2 page 54

(b) Glucose (2g/100ml): This was also prepared as earlier discussed in section 2.7 page 65

(c) Quercetin (Q): 0.1g of quercetin (Sigma Chemical Co. USA) was dissolved in 100ml of methanol and different concentrations prepared from this stock.

(d) Methanolic extract of *Cnestis ferruginea* (C.nf): 0.1g of methanolic extract of C.nf was dissolved in 100ml of methanol and desired concentrations dispensed from it.

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PROCEDURE

1ml of haemoglobin solution, 1ml of (2g/100ml) glucose solution and 5μl of gentamycin in 0.01M phosphate buffer pH 7.4 were mixed and incubated in the dark at room temperature in the presence or absence of varying concentration (10μg, 20μg or 30μg/ml) of quercetin or C.nf respectively. The haemoglobin concentrations at the incubation periods (0, 24, 48 and 72 hrs) were estimated colorimetrically at 540nm by the method of Drabkin and Austin (1932) as described in section 2.3 page 56. The assay was carried out in duplicates.

2.9 DETERMINATION OF THE EFFECT OF METHANOLIC EXTRACT OF *Cnestis ferruginea* ON HAEMOGLOBIN GLYCOSYLATION IN THE PRESENCE OF PHYSIOLOGICAL CONCENTRATION OF (≤ 1mg/ml) GLUCOSE

REAGENTS

(a) Haemoglobin solution: This was prepared as earlier described in Section 2.2 page 54.

(b) Quercetin: This was prepared as earlier described in Section 2.8 page 66.

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(c) Methanolic extract of *Cnestis ferruginea*: This was also prepared according to Section 2.8 page 66.

(d) Glucose: Different concentrations of glucose were prepared by dissolving the following amounts of glucose (1mg, 2mg 4mg, 6mg, 8mg, 10mg, 15mg and 20mg) (Hopkin & Williams Ltd, Essex, London) in 20ml each of 0.01M phosphate buffer at pH 7.4.

PROCEDURE

1ml of haemoglobin solution, 1ml of glucose solution and 5μl of gentamycin in 0.01M phosphate buffer pH 7.4 were mixed and incubated in the dark at room temperature in the presence or absence of 10μg, 20μg or 30μg/ml of quercetin or *Cnestis ferruginea* respectively. Different concentrations of glucose 1mg, 2mg, 4mg, 6mg, 8mg, 10mg, 15mg and 20mg in 20mls each of 0.01M phosphate buffer, pH 7.4 were used. Haemoglobin concentrations were estimated 24hrly throughout the incubation period (72hrs) as an index for measuring the degree of haemoglobin glycosylation. Assay was carried out in duplicates. And the absorbance read at 540nm colorimetrically according to the procedure of Drabkin and Austin (1932) as earlier described in section 2.3 page 56.

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2.10 ASSAY OF THE EFFECT OF *Cnestis ferruginea*, QUERCETIN AND VIT. E ON HAEMOGLOBIN GLYCOSYLATION IN CONTROLLED AND UNTREATED DIABETIC PATIENTS.

Collection of Blood Sample

(a) 10ml of blood was collected from 20 patients at the Medical Outpatient Department (MOP) of University College Hospital. The patients were selected based on whether they are newly diagnosed, untreated or well managed, treated patients. Their fasting blood sugar (FBS) and 2 hour postprandial blood glucose levels were used to assign them into 2 groups labeled, Diabetic (FBS = 163.5mg/dl, 2 Hrpp = 298.5mg/dl) and Diabetic control (FBS = 83mg/dl, 2 Hrpp = 120mg/dl). Verbal consent was sought from the patients before collecting their blood samples in EDTA – containing vernoject bottles. Haemoglobin solutions were prepared from these blood samples as described in section 2.2 page 54.

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REAGENTS

(a) Quercetin (Q) This was prepared as described in Section 2.8 page 66

(b) Methanolic extract of *Cnestis ferruginea* (C.nf). This was also prepared as described in section 2.8 in page 66.

(c) DL-α-Tocopherol (Vit. E): This was purchased from Wako pure chemical Ltd. Japan (98% purity; Sp. Gr = (0.948 - 0.960).

PROCEDURE

To 1ml each of haemoglobin solution prepared from blood samples collected from the 2 groups of patients was added 5µl of gentamicin in 0.01M phosphate buffer, pH 7.4. The resulting mixture was stirred and incubated in the dark at room temperature in the presence of different concentrations (10µg, 20µg or 30µg/ml) of quercetin or extract of C.nf or α-tocopherol. The incubation period was 72 hours and haemoglobin concentration was estimated 24 hourly as an index for measuring the degree of hemoglobin glycosylation using the method of Drabkin and Austin (1932) as described in section 2.3 page 56. The experiment was carried out in duplicates.

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STATISTICAL ANALYSIS

Data are expressed as mean absorbance ± SD of at least three separate experiments. Statistical analysis was performed by a one- way analysis of variance (ANOVA using the SPSS software version (SPSS Inc. Chicago, IL.)

Graphs were plotted with Microsoft excel on windows version

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CHAPTER THREE

EXPERIMENTS AND RESULTS

EXPERIMENT I: Test for the presence of flavonoids and estimation of total phenolic compounds in methanolic extracts of *Cnestis feruginea*

INTRODUCTION

Flavonoids are polyphenolic compounds which have free radical scavenging properties. The radical 2,2 Diphenyl-2-picryl hydrazine (DPPH) was first used by Takao *et al*., (1994) to demonstrate the presence of flavonoid components of marine bacteria present in fish and fruits of the sea. The DPPH test provided information on the reactivity of test compounds with a stable free radical. 2,2 diphenyl-picryl-hydrazyl radical (DPPH) gives a strong absorption band at 517nm in visible spectroscopy (deep violet colour) because of its odd electron. As the electron becomes paired off in the presence of a free radical scavenger e.g flavonoid, the absorption vanishes and the resulting decolourisation is stoichiometric with respect to the number of electrons taken up (Cotelle *et al*., 1996).

PROCEDURE

200g of dried, powdered leaves of *Cnestis ferruginea* were soaked in 2L of methanol (95%v/v) for 4 days in the dark room. The filtrate obtained was

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evaporated on water bath at $40^{0}C$. And the percentage yield of the extract was calculated as described on page 59. The chromatographic plate was also prepared as described on the same page.

RESULT

Table 2 shows that 10.16g (5.08%) of methanolic extracts of Cn.f was obtained from 200g of the powdered leaves. Table 3 further showed that 10.16g of the extract contains 8.79g catechin.The chromatogram obtained shows (Fig. 5) the pattern of separation of the constituents of the methanolic extracts of ***Cnestis ferruginea***. After spraying with DPPH reagent, yellow colouration on a violet background was observed. This confirmed the presence of flavonoids in the methanolic extracts of *Cnestis ferruginea*.In fact, the whole length of the distance travelled by methanolic extracts of ***Cnestis ferruginea*** showed evidence for the presence of flavonoids thus indicating that Cn.f is very rich in different kinds of flavonoids.

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Table 2: **DETERMINATION OF PERCENTAGE YIELD OF METHANOLIC EXTRACTS OF *Cnestis ferruginea***

Plant	Weight of powdered leaves	Weight of Extract (g)	Solvent (ml)	Yield %)
Cold Extraction	200g	10.16	2000	5.08

Table 3: **ESTIMATION OF TOTAL PHENOLIC COMPOUNDS IN METHANOLIC EXTRACTS OF *Cnestis ferruginea***

Concentration of *Cnestis ferruginea* *(mg/ml)*	Absorbance values (390nm)	Concentrations of phenolic compound in Extract of *Cnestis ferruginea* (mg/ml Catechin)
0.02	0.337 ± 0.0007	0.0175

Each value is the mean ± standard deviation, n = 5

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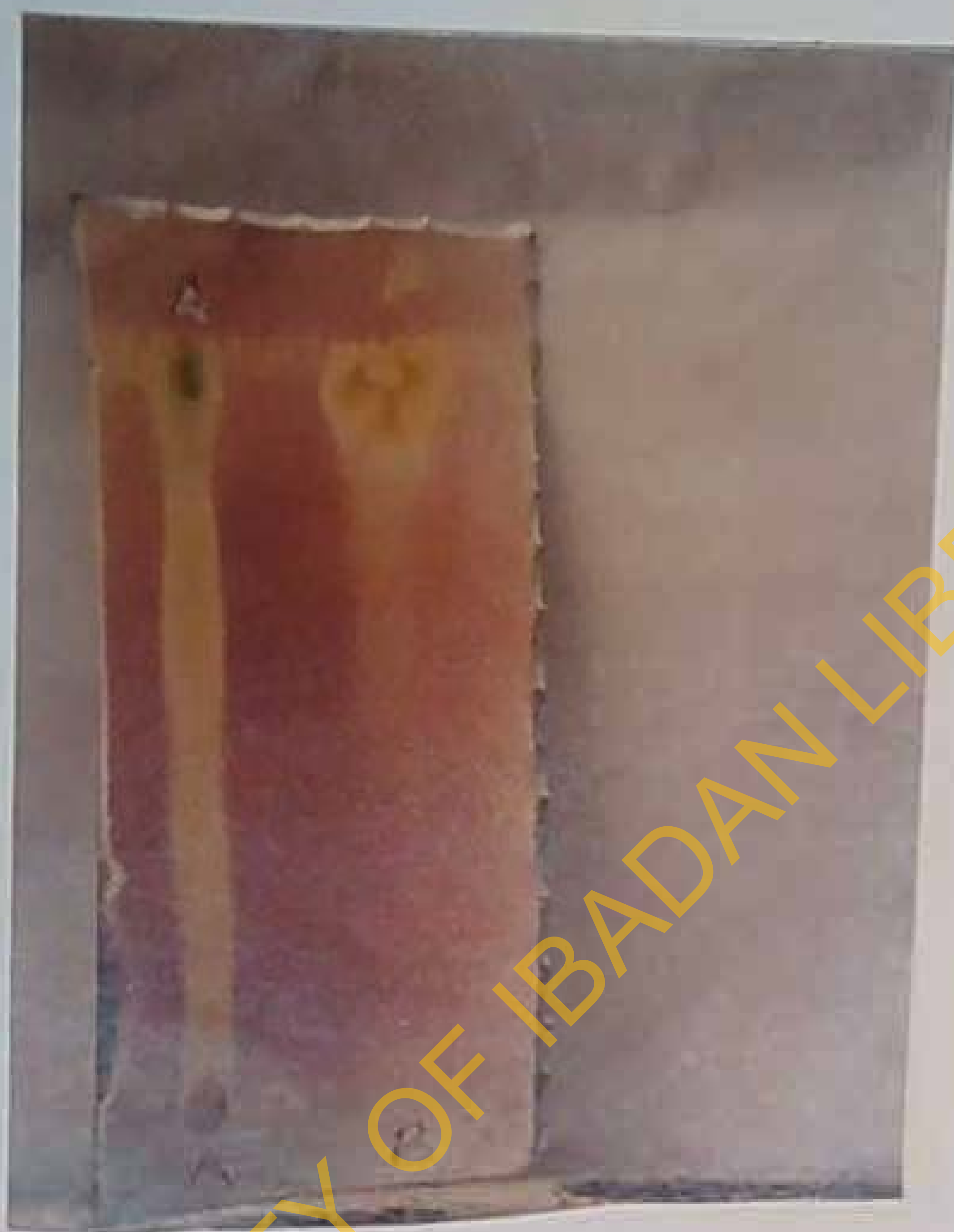

Fig 5: Thin layer chromatogram of methanolic extract of the leaf of *Cnestis ferruginea* in (Ethylacetate : formic acid: water; 85: 10: 5) A = *Cnestis ferruginea*, B = Quercetin (standard).

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EXPERIMENT 2: ASSESSMENT OF THE TIME-DEPENDENCE OF HAEMOGLOBIN GLYCOSYLATION

INTRODUCTION

Enzyme-catalysed protein glycosylation is particularly important in the maintenance of plasma membrane integrity and in facilitating the secretion of proteins into the extracellular spaces (Gallop *et al.*, 1975; Uy and Wold, 1977). On the contrary, certain proteins e.g. haemoglobin undergo a non-enzymatic glycosylation which depends on the presence of a high concentration of free sugar and quite often requires non-physiologic incubation conditions (Bunn *et al.*, 1978) and resulting in formation of compounds such as glycosylated or glycated haemoglobin (GHb). (Allen *et al.*, 1958, Rahbar 1968, 1969).

Almost two decades ago, two groups in their independent studies on the red cell age-related change in Hb_{A1a-b} and Hb_{A1c} in normal and diabetic subjects revealed that glycosylation of haemoglobin occurs slowly, continuously and nearly irreversibly throughout the 120-day life time of the red cell. Therefore, the more recent the period of glycemia, the larger its influence on glycated haemoglobin (GHb) value (Fitzgibbons *et al.*, 1976; Tahara and Shima, 1993). These findings formed the basis of this preliminary study which was designed to

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assess haemoglobin glycosylation in the relation to glucose concentration and period of incubation. Haemoglobin glycosylation is measured by determining the amount of free haemoglobin following the exposure of the haem protein to glucose thereby encouraging non-enzymatic glycosylation of the protein. In this study the level of the haemoglobin concentration is used as an index of haemoglobin glycosylation. In this instance, the lower the amount of free haemoglobin, the higher the extent of haemoglobin glycosylation.

PROCEDURE

Blood samples were collected from commercial donors in the University College Hospital, Ibadan. Haemoglobin rich-fraction was prepared from the samples as described on page 54. Haemoglobin concentration of the fraction was also estimated as contained on page 56. 1ml of the haemoglobin fraction was incubated with 1ml each of solution containing varying glucose concentrations (0.2g, 1g and 2g per 100ml of 0.01 M phosphate buffer pH 7.4) in the dark at room temperature for an incubation period of 72 hrs. The extent of glycosylation was estimated at varying incubation periods (0, 24, 48 and 72hrs) as described on page 65. Each experiment was carried out in duplicates.

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RESULTS

Table 4 shows the data obtained from experiments on the determination of the effect of increasing glucose concentrations on haemoglobin glycosylation at varying periods of incubation. As can be seen from the Table, exposure of haemoglobin to varying concentrations of glucose (2, 10, 20mg/ml) reduced free haemoglobin to varying extents throughout the incubation period of 72hrs. For example, at 2mg glucose/ml haemoglobin concentration was reduced from 15.14 ± 1.765g/dl to 8.00 ± 0.750 by the end of the incubation period of 72hrs. Similarly, haemoglobin concentration was reduced to 11.25 ± 1.103g/dl within the first 24hrs of incubation with 10mg glucose/ml. By the end of 48 and 72hrs incubation period, the haemoglobin concentration had been reduced to 6.75 ± 0.702 and 5.10 ± 0.602g/dl respectively. On increasing the glucose concentration two fold (20mg glucose/ml) haemoglobin concentration was lowered to 9.65 ± 0.953g/dl within the first 24hrs of incubation. Furthermore, the concentration of haemoglobin reduced to 6.10 ± 0.652 and 4.20 ± 0.453g/dl at incubation periods at 48 and 72hrs respectively at 20mg glucose/ml. Fig. 6 is a graphical representation of the relationship between haemoglobin glycosylation and the incubation time in the presence of varying concentrations of glucose. The figure shows that haemoglobin glycosylation increases linearly with the ambient glucose concentration.

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CONCLUSION

The degree of haemoglobin glycosylation increases linearly with the period of incubation in a concentration dependent manner up to 20 mg glucose/ml where it appeared to be saturated.

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Table 4: Time Dependent Glycosylation of Human Haemoglobin

Period of Incubation (hrs)	HAEMOGLOBIN CONCENTRATION (g/dl)			
	Glucose concentration (mg/ml)			
	0	2	10	20
24	15.14 ± 1.765[a]	13.56 ± 1.461[b]	11.25 ± 1.103[e]	9.650 ± 0.953[h]
48	15.10 ± 1.765[a]	10.26 ± 0.992[c]	6.75 ± 0.702[f]	6.10 ± 0.652[i]
72	15.11 ± 1.578[a]	8.00 ± 0.750[d]	5.10 ± 0.602[g]	4.20 ± 0.453[j]

Each value is a mean of 10 different estimations ± standard deviation.

a-j Means in the same row carrying the same superscript are not significantly different ($P \geq 0.05$)

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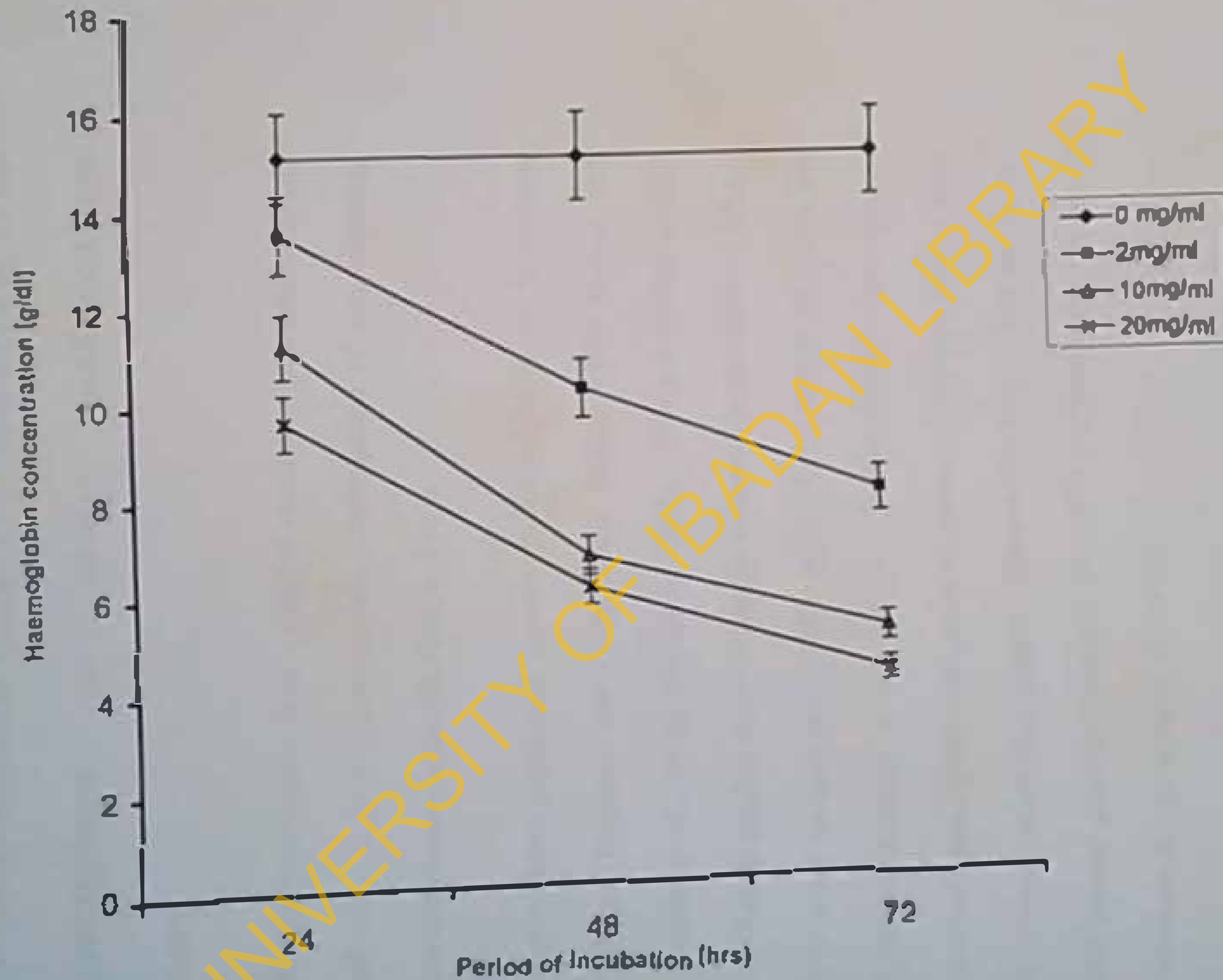

Fig. 6. Time dependent glycosylation of human haemoglobin at varying concentrations of glucose.

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EXPERIMENT 3: INFLUENCE OF QUERCETIN AND EXTRACTS OF *Cnestis ferruginea* ON HUMAN HAEMOGLOBIN GLYCOSYLATION

Haemoglobin glycosylation is an oxidative reaction which occurs slowly in circulating red cells. It is a non-enzymatic condensation of two abundant reactants – glucose and haemoglobin (Goldstein, 1995).

Several studies have implicated increase in haemoglobin A_{1c} in patients with diabetes mellitus (Huisman and Dozy, 1962; Rahbar 1968; Koenig and Cerami, 1975; Koenig *et al.*, 1976; Goldstein, 1995). Nearly a decade passed before a flurry of clinical studies showed that the increased proportions of HbA_{1c} in diabetic patients could be used as a reliable index of glycemic control over the preceding 6-8 weeks (Gabbay *et al.*, 1977). Further studies demonstrated correlation of glycated haemoglobin with other indicators of diabetic control such as 24hr urinary glucose excretion, plasma "glucose brackets", (Koenig *et al.*, 1976), daily mean plasma glucose (Gonen *et al.*, 1977) and area under the curve of the glucose tolerance test (Koenig *et al.*, 1976).

Inspite of the inconsiderable methodological difficulties in measurement, the use of glycated haemoglobin was rapidly accepted by diabetologists and other

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healthcare workers because, for the first time, they had an apparently simple tool which could give a completely objective assessment of a patient's glucose control (Goldstein *et al.*, 1986; Kilpartrick 1997). Therefore, measurement of glycated proteins, especially haemoglobins is now a routine procedure for monitoring previous glycemic control in diabetic patients in the developed world (Rahlenbeck, 1998).

Quercetin is a known flavonoid with anticancer, analgesic properties, anti-free radical, cardiovascular activity and effects on the complications of hyperglycemia (Ranelletti *et al.*, 1992; Yan *et al.*, 1996; Mao and Zhang, 1995; Roufogalis *et al.*, 1999). Just recently, the antioxidant activity of quercetin was implicated in the treatment or prevention of complication of diabetes because it inhibited haemoglobin glycosylation (Asgary *et al.*, 1999).

Nowadays, a great deal of attention is being directed towards the bioactivity of flavonoids as dietary sources of antioxidants. The daily western diet averagely contains approximately 1g of mixed flavonoid, (Kuhnau, 1970) a quantity that could produce pharmacologically of significant concentrations in body fluids and tissues. Meanwhile, flavonoids have been reported to have a myriads of biological effects on enzymes such as possession of free radical scavenging activity, chelation of certain metal cations, increased resistance of LDL to oxidation, protection against peroxidative damage to biomembrane and

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cellular protein phosphorylation (Robak and Gryglewski, 1988; Liu *et al.*, 1992; Harborn, 1994; Gliteriog *et al.*, 1997; Ishikawa *et al.*, 1997; Fuhrman *et al.*, 1997). These properties may elicit pharmacological responses resulting in the elucidation of novel drugs. For this reason, extracts of *Cnestis ferruginea* with little or no documented pharmacological information was chosen in this study in order to assess its possible inhibitory effect on haemoglobin glycosylation. In the same vein, quercetin was used as the control drug.

PROCEDURE

This experiment was designed to compare the effect of quercetin and flavonoid - containing methanolic extracts of *Cnestis ferruginea* on haemoglobin glycosylation over an incubation period of (24-72) hrs. 1ml of haemoglobin fractions prepared as described in the section 2.2 on page 54 were incubated with 1ml of the best concentration of glucose (20mg/ml in 0.01M phosphate buffer pH 7.4) that gave the highest level of haemoglobin glycosylation as reported in experiment 2. The mixture was kept in the dark at 27°C ± 1 in the presence or absence of (10, 20 and 30) μg/ml of quercetin or methanolic extracts of C.nf. The amount of residual haemoglobin (Hb) measured in g/dl at the different incubation periods was used as an index of glycosylation by a modification of the method of

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Asgary *et al.*, 1999 and as described in section 2.7 under Materials and Methods on page 65.

RESULTS

Fig.7 and Table 5 show the effects of varying concentration of quercetin and methanolic extracts of C.nf on human haemoglobin glycosylation after 24, 48 and 72 hrs of incubation respectively. The results obtained indicated that haemoglobin levels increased on incubation with these antioxidants. Furthermore, the results showed that the increase was observed at all the concentrations of the quercetin and extracts of Cn.f (10, 20, 30) μg/ml used irrespective of the incubation period. For example, while incubation with 20mg/ml glucose caused glycosylation of haemoglobin by about 72% after 72 hrs. Increasing concentrations of quercetin resulted in an inhibition of glycosylation in a concentration dependent manner. In this regard, the longer the incubation period, the greater the effect of the antioxidant on the process of glycosylation. Also the higher the concentration of the antioxidant the greater the extent of prevention of glycosylation. In general the effect of quercetin was highest after 72 hrs of incubation. The degree of inhibition using 10, 20, and 30 μg/ml quercetin was 40, 33, and 25% respectively. Similarly, methanolic extract of Cn.f

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prevented glycosylation to the extent that 30 μg/ml of the extract gave the maximum effect after 72 hrs of incubation. Although, the degree of prevention of glycosylation by quercetin and extracts of Cn.f are similar over the varying periods of incubation, quercetin appears to be somewhat more effective given the fact that Cn.f is a mixture of flavonoids and other components

CONCLUSION

The inhibitory activity of quercetin and extracts of C.nf on human haemoglobin glycosylation are both concentration and time-dependent. Quercetin and C.nf inhibited haemoglobin glycosylation to about the same extent

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Table 5 : Comparative effects of Methanolic extracts of Cnestis ferruginea and Quercetin on Human Haemoglobin Glycosylation at varying Periods of Incubation

Incub. Period (hr)	Control	+Gluc 20mg/ml	Haemoglobin concentration(g/dl) — Concentration of flavonoids (μg/ml): Quercetin			*Cnestis ferruginea*		
			10	20	30	10	20	30
24	15.14±1.765*	9.63±1.070 (36.4%)	11.20±1.22^{a} (26%)	12.60±1.3^{b} (17%)	13.50±1.40^{c} (11%)	11.10±1.14^{a} (27%)	12.40±1.3^{b} (18%)	12.80±1.40^{a} (16%)
48	15.10±1.755*	6.00±0.786 (60%)	10.60±1.1^{d} (29%)	11.10±1.12^{e} (27%)	11.60±1.30^{f} (23%)	10.40±1.1^{d} (31%)	11.00±1.20^{e} (27%)	11.30±1.20^{f} (25%)
72	15.11±1.752*	4.19±0.520 (72%)	9.14±101^{g} (40%)	10.20±1.0^{h} (33%)	11.30±1.25^{i} (25%)	8.95±0.95^{g} (41%)	10.40±1.1^{h} (31%)	10.70±1.12^{i} (29%)

Each value is a mean of 10 different estimations ± standard deviation. Gluc = Glucose

( ) Values in brackets represent the % of haemoglobin glycosylated in the presence of 20mg/ml glucose.

a-i Means in the same row carrying the same superscript are not significantly different ($P \geq 0.05$)

* Not significantly different ($P \geq 0.05$)

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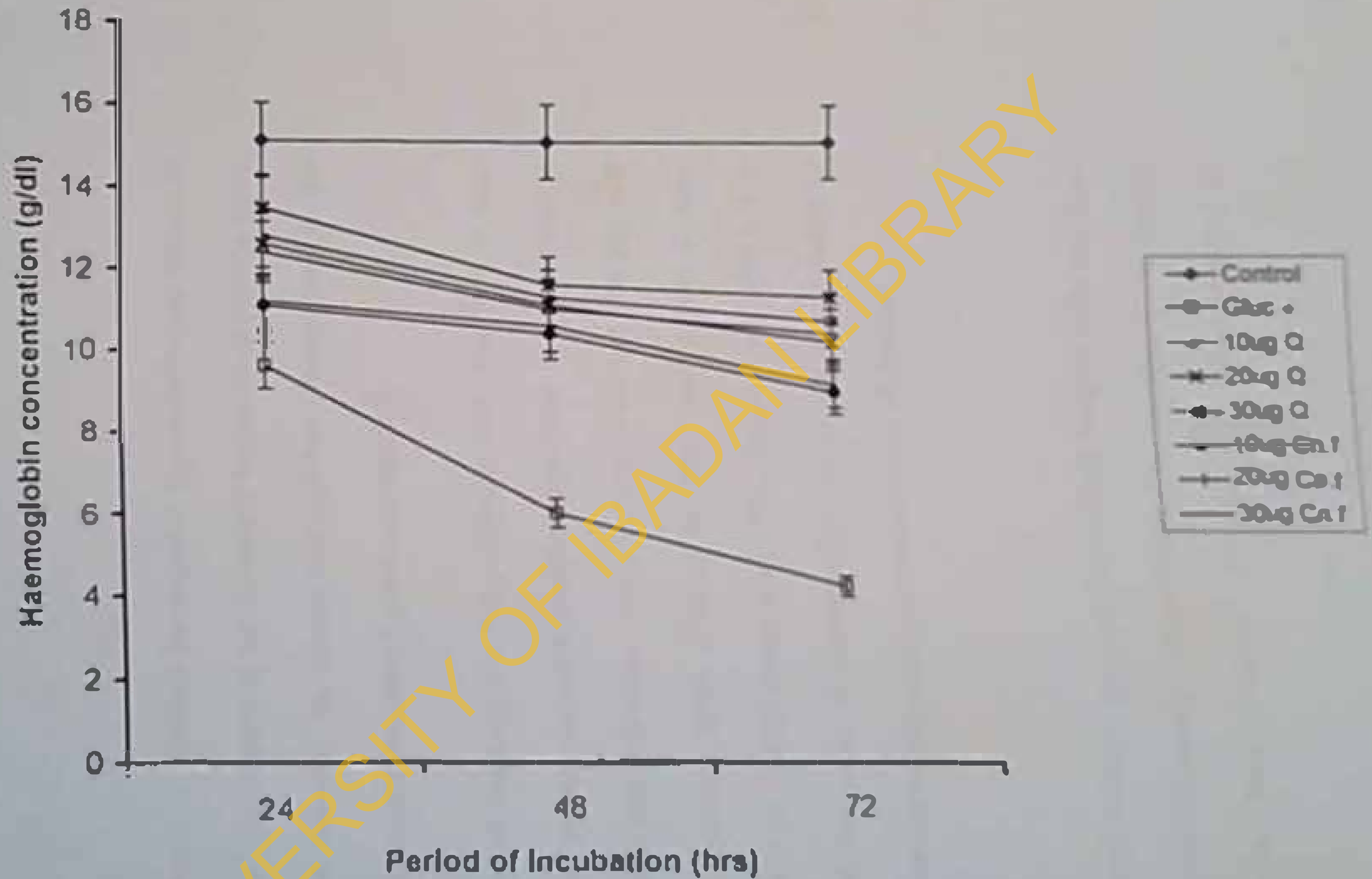

Fig. 7. Comparative effects of varying concentrations of quercetin and methanolic extract of Cn.f on human haemoglobin glycosylation

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EXPERIMENT 4: EFFECT OF QUERCETIN AND EXTRACT OF C.nf ON HUMAN HAEMOGLOBIN GLYCOSYLATION IN THE PRESENCE OF PHYSIOLOGICAL CONCENTRATIONS OF GLUCOSE (≤ 1mg/ml).

INTRODUCTION

The findings that C.nf inhibited haemoglobin glycosylation and mimicked diabetic state *"in vitro"* (experiment 3) has stimulated further studies on the effect of this flavonoid-rich extracts on human haemoglobin glycosylation in the presence of physiological concentration of glucose (≤ 1 mg/ml).

Although glycated haemoglobin had previously been described in non diabetic adults, it was present in much smaller proportions in comparison to diabetic patients (Schnck and Schroeder, 1961). In view of the fact that glycated haemoglobin species represent about 5.7% of the total haemoglobin in non-diabetic adults (Bunn *et al.*, 1978; James *et al.*, 1981, Little *et al.*, 1991), it is pertinent to determine the extent of inhibition by C.nf of the glycosylation of haemoglobin in the presence of physiological concentrations of glucose.

PROCEDURE

1ml haemoglobin fraction, prepared from blood samples collected from commercial donors of the University College Hospital was incubated with 1 ml of glucose solution (ranging from 50-1000µl) taken from the stocks prepared as

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described page 68. The mixture was incubated in the presence or absence of varying concentrations (10-30) µg/ml of quercetin and extracts of C.nf as well as 5µl of gentamicin to prevent microbial growth. Incubation took place in the dark at 27° + 1°C over a period of 72hrs. Haemoglobin level was estimated as described in section 2.3 page 56 at the incubation periods of 24, 48 and 72hrs.

RESULT

The effects of varying concentrations of quercetin and methanolic extracts of *Cnestis ferruginea* on haemoglobin glycosylation were investigated in the presence of physiological concentrations of glucose (≤ 1mg/ml). Table 6 shows the effect of 10µg quercetin and 10µg/ml extracts of C.nf. From the table, it can be seen that there is no significant glycosylation of haemoglobin at varying concentrations of glucose up to 1mg/ml. Indeed, 4% inhibition of glycosylation was observed after 24hr of incubation with 1mg/ml glucose, while the extent of inhibition increased from 5.5 to 7% after 48 and 72hr of incubation respectively. Conversely, 10µg/ml quercetin did not allow haemoglobin glycosylation even after 72hrs of incubation. Similar results were observed with *Cnestis ferruginea*. Using 20µg/ml quercetin and 30µg/ml quercetin and *Cnestis ferruginea* respectively, the extent of prevention of glycosylation was total. These substances did not allow glycosylation to take place even after prolonged hours of glycosylation (Tables 6, 7 and 8).

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CONCLUSION

Quercetin and extracts of C.nf inhibited human haemoglobin glycosylation in the presence of physiological concentrations of glucose in a concentration dependent manner.

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Table 6

Effect of 10 µg/ml methanolic extract of *Cnestis ferruginea* and 10 µg/ml quercetin on haemoglobin glycosylation in the presence of physiological glucose concentration (≤ 1 mg/ml)

Gluc.conc (g)	Haemoglobin Concentration (g/dl)								
	Control			+quercetin (µg/ml)			+C. ferruginea (µg/ml)		
	24(hrs)	48(hrs)	72(hrs)	24(hrs)	48(hrs)	72(hrs)	24(hrs)	48(hrs)	72(hrs)
50	15.15±1.610	15.13±1.550	15.092±1.510	15.21±1.530	15.16±1.518	15.19±1.521	15.20±1.521	15.15±1.520	15.18±1.522
100	15.10±1.498	14.96±1.499	14.76±1.485	15.20±1.529	15.19±1.520	15.20±1.522	15.19±1.522	15.18±1.519	15.19±1.524
200	14.92±1.510	14.85±1.490	14.60±1.502	15.19±1.525	15.19±1.520	15.18±1.519	15.18±1.520	15.19±1.524	15.17±1.522
300	14.84±1.500	14.77±1.485	14.51±1.493	15.16±1.526	15.17±1.522	15.17±1.520	15.17±1.521	15.18±1.522	15.18±1.522
400	14.376±1.520	14.69±1.472	14.42±1.505	15.17±1.524	15.16±1.529	15.17±1.522	15.16±1.524	15.17±1.520	15.17±1.520
500	14.69±1.471	14.57±1.605	14.31±1.452	15.15±1.518	15.15±1.522	15.15±1.519	15.14±1.520	15.16±1.519	15.16±1.520
750	14.62±1.471	14.46±1.465	14.20±1.445	14.98±1.520	15.10±1.521	15.10±1.515	14.97±1.499	15.11±1.516	15.10±1.513
1000	14.54±1.601	14.36±1.443	14.16±1.393	14.95±0.502	15.00±1.520	15.10±1.516	14.94±1.501	15.00±1.515	15.11±1.515

Each value is a mean of 10 different estimations ± standard deviation. Gluc = Glucose

Values are not significantly different at (P ≥ 0.05)

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Table 7

Effect of 20µg/ml methanolic extract of Cnestis ferruginea and 20µg/ml quercetin on haemoglobin glycosylation in the presence of physiological glucose concentration (≤ 1 mg/ml)

Haemoglobin Concentration (g/dl)

	Control			+quercetin (µg/ml)			+C. ferruginea (µg/ml)		
Glug. (µg/ml)	24(hr)	48(hr)	72(hr)	24(hr)	48(hr)	72(hr)	24(hr)	48(hr)	72(hr)
50	15.16±1.521	15.14±1.522	15.10±1.521	15.22±1.552	15.15±1.522	15.18±1.532	15.21±1.530	15.16±1.523	15.19±1.192
100	15.11±1.512	14.97±1.503	14.77±1.483	15.21±1.541	15.18±1.531	15.21±1.545	15.20±1.541	15.19±1.531	15.18±1.532
200	14.95±1.501	14.88±1.491	14.61±1.473	15.19±1.532	15.17±1.540	15.19±1.521	15.19±1.549	15.18±1.521	15.10±1.520
300	14.87±1.492	14.80±1.501	14.52±1.463	15.18±1.543	15.16±1.532	15.17±1.532	15.18±1.532	15.17±1.513	15.16±1.531
400	14.79±1.493	14.72±1.482	14.41±1.450	15.17±1.534	15.15±1.530	15.18±1.534	15.17±1.522	15.15±1.521	15.15±1.532
500	14.71±1.471	14.64±1.473	14.30±1.431	15.15±1.521	15.14±1.520	15.16±1.522	15.15±1.520	15.14±1.520	15.13±1.521
750	14.64±1.462	14.55±1.464	14.91±1.420	15.00±1.522	15.10±1.520	15.00±1.512	14.97±1.501	15.10±1.511	15.11±1.522
1000	14.56±1.501	14.45±1.452	14.09±1.413	14.96±1.502	15.00±1.511	15.10±1.521	14.95±1.500	15.00±1.510	15.10±1.521

Each value is a mean of 10 different estimations ± standard deviation. Gluc = Glucose.

Values are not significantly different at ($P \geq 0.05$)

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Table 6

Effect of 30µg/ml methanolic extract of *Cnestis ferruginea* and 30µg/ml quercetin on haemoglobin glycosylation in the presence of physiological glucose concentration (≤ 1 mg)

Haemoglobin Concentration (g/dl)

Gluc (µg/m)	Control			+quercetin (µg/ml)			+C. ferruginea (µg/ml)		
	24(hr)	48(hr)	72(hr)	24(hr)	48(hr)	72(hr)	24(hr)	48(hr)	72(hr)
50	15.17±1.521	15.15±1.502	15.10±1.551	15.22±1.542	15.16±1.523	15.18±1.532	15.21±1.521	15.14±1.521	15.18±1.533
100	15.17±1.512	14.98±1.492	14.97±1.512	15.20±1.511	15.17±1.532	15.19±1.521	15.20±1.541	15.17±1.520	15.20±1.542
200	14.93±1.501	14.89±1.487	14.60±1.482	15.18±1.521	15.18±1.521	15.18±1.531	15.19±1.502	15.18±1.531	15.19±1.541
300	14.85±1.492	14.80±1.481	14.51±1.521	15.19±1.520	15.17±1.512	15.16±1.512	15.18±1.524	15.16±1.523	15.18±1.531
400	14.77±1.481	14.73±1.475	14.40±1.452	15.18±1.521	15.15±1.523	15.16±1.523	15.17±1.521	15.15±1.512	15.17±1.522
500	14.70±1.482	14.63±1.465	14.31±1.443	15.16±1.522	15.16±1.531	15.15±1.513	15.16±1.523	15.14±1.511	15.16±1.520
750	14.63±1.472	14.54±1.60	14.18±1.421	15.10±1.514	15.11±1.521	15.12±1.502	15.10±1.502	15.10±1.501	15.11±1.512
1000	14.55±1.603	14.44±1.503	14.08±1.413	14.98±1.502	15.10±1.512	15.12±1.510	15.10±1.501	15.00±1.512	15.10±1.511

Each value is a mean of 10 different estimations ± standard deviation. Gluc =Glucose

Values are not significantly different at (P ≥ 0.05)

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EXPERIMENT 5: A COMPARISON OF THE ACTIONS OF *Cnestis ferruginea*, QUERCETIN AND VIT. E ON HAEMOGLOBIN GLYCOSYLATION IN DIABETIC PATIENTS

INTRODUCTION

Detection of glycated haemoglobin (GHb) in blood is fast becoming the internationally established method of assessing long-term glycaemic control in individuals with diabetes even though test results have to be standardized among laboratories (Santiago, 1993). In view of the fact that accumulation of advanced glycation end products (AGEs) have been implicated as a major pathogenic process leading to diabetic complications, an increasing number of drug candidates have recently been developed as potential inhibitors of AGEs formation (Rahbar, 1999). For instance, aminoguanidine, a hydrazine – like molecule is the first drug extensively studied both "*in vitro*" and "*in vivo*" as an inhibitor of AGE formation and it is currently undergoing clinical trial. It works by blocking the reactive carbonyl groups in molecules that can form AGEs. It does not interfere with normal enzyme mediated collagen cross-linking and appears to be largely free of other side effects (Brownlee *et al.*, 1988). Several other agents have been shown to be effective in the inhibition of the detrimental effects of excessive protein crosslinking, e.g aspirin (Yue *et al.*, 1984), carnosine

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(Hipkiss *et al.*, 1995), antioxidants such as vitamin E and flavonoids (Ceriello *et al.*, 1988). However, since, aminoguanidine was later found to act as an inhibitor of nitric oxide synthase, the effect of a novel inhibitor of advanced glycation end-products formation, that does not inhibit nitric oxide synthase, known as 2,3 diaminophenazine (2,3DAP) was evaluated by Soulis *et al.*, (1999). It was revealed in their study that both aminoguanidine and 2,3 DAP reduced the formation of advanced glycation end-products as measured by radioimmunoassay and as assessed immunohistochemically in mesenteric vessels. Furthermore, it offers a means to differentiate between inhibitors of the early and late stages of glycation and provides a rapid method of screening large numbers of potential inhibitors of glycation or glycosylation (Rahbar, 1999).

The findings in the present study that quercetin and extracts of C.nf are potential inhibitors of haemoglobin glycosylation and the fact that the ingestion of large amount of vitamin C or E (e.g. > 1g day) can lower glycated haemoglobin levels, perhaps by blocking glycation (Ceriello *et al.*, 1991; Davie *et al.*, 1992) suggest that there is need to ascertain whether C.nf and quercetin have any effect on glycated haemoglobin in the diabetic state. Therefore, it seems pertinent in the present study, to investigate the effects of quercetin and extracts of C.nf on AGEs in order to ascertain whether these flavonoids would facilitate the breakdown of AGEs. This will definitely be of tremendous benefit to diabetic individuals where

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there is an unusual accumulation of AGEs prior to the development of diabetic complications.

PROCEDURE

Blood samples from controlled and untreated diabetic patients at the Medical OutPatient, Department of the University College Hospital, Ibadan were collected in Na-EDTA treated bottles. Blood glucose levels were determined in these blood samples in order to ascertain their status with regards to hyperglycemia. Haemoglobin fractions were prepared from the blood samples within 24hrs of collection as described in the section 2.3 page 56. The haemoglobin fractions were then incubated in the dark at 27°C ± 1 for 3 days with or without varying concentrations (10-30µg) quercetin, or C.nf or Vitamin E. The haemoglobin concentrations were estimated on a daily basis (i.e. 24 hourly) by a slight modification of the method of Asgary *et al.*, (1999) as described on page 70.

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RESULT

Tables 9 and 10 show the effect of varying concentrations methanolic extracts of C.nf, quercetin and Vit. E on glycated haemoglobin in blood samples from controlled and untreated diabetic patients. A comparison of the haemoglobin levels obtained after incubation of the haemoglobin sample of controlled and untreated diabetic patients separate with C.nf and quercetin indicated a slight insignificant increase in the haemoglobin level (14%) across board. Although the addition of 30µg of any of the three substances (quercetin, C.nf and Vit. E) increased the Hb level by 13% in the untreated diabetics however, there was no significant increase recorded in controlled diabetics after 2 days of exposure to quercetin, C.nf and Vit. E.

CONCLUSION

Although the levels of glycosylation were reduced by varying concentrations of extracts of C.nf quercetin and Vit E, these antioxidants somewhat had no significant effect on the glycated haemoglobin. The potency of the three compounds were almost the same in untreated diabetic patient (Q = 13 %, C.n.f = 13 %, Vit E = 14%)

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Table 8

Effect of methanolic extract of *Cnestis ferruginea*, quercetin and Vit. E on haemoglobin glycosylation in controlled diabetic patients.

HAEMOGLOBIN CONCENTRATION (g/dl)

Incubation Period (hrs)	Control	Quercetin (µg/ml)			*Cnestis ferruginea* (µg/ml)			Vit. E (µg/ml)		
		10	20	30	10	20	30	10	20	30
24	15.10±1.01	15.11±1.23	15.12±1.23	15.15±1.24	15.01±1.22	15.11±1.23	15.16±1.23	15.02±1.23	15.12±1.18	15.15±1.25
48	15.11±0.95	15.12±1.21	15.14±1.21	15.16±1.23	15.13±1.21	15.13±1.24	15.18±1.24	15.12±1.24	15.15±1.25	15.18±1.22
72	15.11±1.[illegible]	15.20±1.23	15.18±1.22	15.20±1.21	15.19±1.20	15.18±1.21	15.21±1.23	15.16±1.25	15.19±1.24	15.21±1.21

Each value is a mean of 10 determination ± Standard deviation.

Mean values are insignificantly different ($P \geq 0.05$)

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Table 10

Effect of methanolic extract of *Cnestis ferruginea*, quercetin and Vit. E on haemoglobin glycosylation in diabetic patients.

HAEMOGLOBIN CONCENTRATION (g/dl)

Incubation Period (hrs)	Control	Quercetin (μg/ml)			*Cnestis ferruginea* (μg/ml)			Vit. E (μg/ml)		
		10	20	30	10	20	30	10	20	30
24	13.44±0.90	13.60±0.98	13.81±1.01	14.05±1.23	13.70±1.04	13.91±1.08	14.70±1.14	13.75±1.04	13.85±1.06	14.80±1.25
48	13.42±0.94	13.80±0.99	14.20±1.24	15.10±1.24	13.90±1.10	14.32±1.15	15.01±1.18	13.90±1.10	14.25±1.14	15.13±1.21
72	13.43±0.90	14.00±1.01	14.25±1.30	15.22±1.31	14.10±1.14	15.00±1.20	15.20±1.31	14.00±1.14	15.01±1.21	15.30±1.24

Each value is a mean of 10 different estimations ± standard deviation.

Mean values are not significantly different ($P \geq 0.05$)

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CHAPTER FOUR

DISCUSSION

Proteins are subject to an extensive and complex series of co-translational and post-translational reactions that include site-specific cleavage, covalent modification, and compartmentalization to particular intracellular or extracellular locations (Sabatini *et al*., 1982). For example the enzyme catalyzed glycosylation of proteins which plays a vital role in intracellular transport, processing and polypeptide back-bone folding (Firestone, 1983). In addition, protein molecules can in general bind non-enzymatically with glucose or other sugars to form initially unstable aldimine and ketamine adducts of the original protein (Maillard reaction) and then later, more stable structures called advanced glycated end-products (AGEs) (Brownlee *et al*., 1988). The degree to which this happens is proportional to the concentration of the sugar in the surrounding medium of the protein molecule, and to the duration of the exposure of the proteins in this medium (Bunn *et al*., 1978).

Red blood cells have insulin independent glucose transporters on their surface and therefore do not require insulin for the uptake of glucose, therefore, if

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glucose levels in the blood plasma are high, then the glucose levels inside the red blood cells will also be high. In this state, cytoplasmic proteins such as albumin, haemoglobin, calmodulin and ribonuclease become glycated, and they no longer perform the same function they were originally slated to carry out (Garlick and Mazer, 1983; Watkins *et al.*, 1985). In fact most protein which are enzymes for example Na^+ - K^+ ATPase, Ca^{2+}-ATPase, and Calmodulin are unable to perform their catalytic role when they become glycated (Gonzalez-flecha, 1993; Swammy-mruthinti, 2001). It has been shown that non-enzymatic glycation takes place at the ε-amino groups of lysine or hydroxylysine residues as well as at α-amino groups of amino terminal residues proteins (Thorpe and Baynes, 1982). In the case of the transport protein, haemoglobin, a high cytoplasmic glucose concentration causes the protein to become glycated at any of the α amino groups of amino terminal residues of the β- chains as well as certain ε- amino groups of lysine residues. Thus, producing a glycated haemoglobin (HbA_{1c}) (Bunn *et al.*, 1979). HbA_{1c} is the best known example of a glycated protein and is being used to monitor overall glycenemic control in diabetes in the preceeding 2-3 months because this time period corresponds to the natural average lifespan of the HbA molecule in red blood cells (Goldstein *et al.*, 1995).

In addition, it has been reported that glucose may auto oxidize in the presence of catalytic amounts of iron or copper ions generating free radicals

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(Thornalley *et al*., 1984). An excess of oxidative stress can occur through an increase in the generation of these radicals and their metabolites and thus overwhelming the protective capacity of the normal defence mechanism of the body and/or through a decrease in the protective ability of the body to withstand normal oxidative stress. Free radicals may also delay proliferation, modify mobility of membrane components and membrane integrity, influence platelet – derived growth factors and other secretory protein production in a variety of cell systems (Curcio *et al*., 1995). In this condition of suppressed antioxidant level, it seems likely that antioxidant supplements (such as vitamin E, C, carotenes, flavonoid etc.) would normally neutralize the effects of free radicals (Davie *et al*., 1992). It is in this regard, that the present study was designed to assess the inhibitory effect of flavonoid-containing methanolic extracts of *Cnestis ferruginea* (Materials and Methods Section 2.8 page 67) on the process of haemoglobin glycosylation. Quercetin, a flavonoid and antioxidant was used as a control. Although quercetin is found in onions, tea and apples which are part of the human diet, it was shown in one study to have mutagenic or carcinogen properties (Pamukcu *et al*., 1980). In an earlier study by Bohm (1968), quercetin was reported to affect the growth of transplanted sarcoma. All these contradictory reports makes it imperative to direct attention to a search for a rich, edible and non-toxic source of antioxidants that could also inhibit haemoglobin glycosylation.

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This study that was designed to determine the effect of methanolic extracts of *Cnestis ferruginea* on the process of haemoglobin glycosylation. As a preliminary to the main study, a suitable period of incubation for non-limiting concentration of glucose (≤20mg/ml) was first determined as indicated in experiment 1. The results presented in Table 2 showed that the degree of haemoglobin glycosylation increases with the period of incubation (24-72hr) in a concentration dependent manner up to 20mg/ml where glycosylation appeared to be saturated. This finding is in agreement with earlier reports that the amount of glycosylation increases linearly up to the concentration of 2g/100ml of glucose (Asgary *et al.*, 1999). These findings are in agreement with earlier reports that the amount of HbA_{1c} formed depends on the concentration of and period of exposure to glucose (Bunn *et al.*, 1976).

Having therefore, determined the period of incubation required for haemoglobin to be saturated with glucose, the effect of the flavonoids -containing leaf extracts of Cn.f on haemoglobin glycosylation was investigated. In this study a decrease in the haemoglobin level was used as a criterion for increase in haemoglobin glycosylation at varying periods of incubation of 24, 48 and 72 hrs in the presence of varying concentrations (10-30 µg/ml) extract. Table 5 shows the effects of increasing glucose concentration on haemoglobin glycosylation at varying periods of incubation and in the presence of varying amounts of extracts

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increase in the generation of these radicals and their metabolites and thus overwhelming the protective capacity of the normal defence mechanism of the body and/or through a decrease in the protective ability of the body to withstand normal oxidative stress. Free radicals may also delay proliferation, modify mobility of membrane components and membrane integrity, influence platelet – derived growth factors and other secretory protein production in a variety of cell systems (Curcio *et al.*, 1995). In this condition of suppressed antioxidant level, it seems likely that antioxidant supplements (such as vitamin E, C, carotenes, flavonoid etc.) would normally neutralize the effects of free radicals (Davie *et al.*, 1992). It is in this regard, that the present study was designed to assess the inhibitory effect of flavonoid-containing methanolic extracts of *Cnestis ferruginea* (Materials and Methods Section 2.8 page 67) on the process of haemoglobin glycosylation. Quercetin, a flavonoid and antioxidant was used as a control. Although quercetin is found in onions, tea and apples which are part of the human diet, it was shown in one study to have mutagenic or carcinogen properties (Pamukou *et al.*, 1980). In an earlier study by Bohm (1968), quercetin was reported to affect the growth of transplanted sarcoma. All these contradictory reports makes it imperative to direct attention to a search for a rich, edible and non-toxic source of antioxid[illegible] that could also inhibit haemoglobin glycosylation.

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Having therefore, determined the period of incubation required for haemoglobin to be saturated with glucose, the effect of the flavonoids -containing leaf extracts of C.n.f on haemoglobin glycosylation was investigated. In this study a decrease in the haemoglobin level was used as a criterion for increase in haemoglobin glycosylation at varying periods of incubation of 24, 48 and 72 hrs in the presence of varying concentrations (10-30 µg/ml) extract. Table 5 shows the effects of increasing glucose concentration on haemoglobin glycosylation at varying periods of incubation and in the presence of varying amounts of extracts

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of Cn.f. The data obtained showed that the inhibitory effects of *Cnestis ferruginea* and quercetin on human haemoglobin glycosylation are concentration and time dependent. The data also revealed further that quercetin and Cn.f inhibited haemoglobin glycosylation to about the same extent. Maximum inhibitory percentage of 75% and 71% were obtained for quercetin and Cn.f at a concentration of 30µg/ml (Table 5). It seems likely from these results that the flavonoids in the methanolic extracts of Cn.f and quercetin are acting as antioxidants by interfering with the oxidative process of glycosylation. This finding is in agreement with the reports of Odetti *et al.* (1990) that haemoglobin glycosylation was significantly reduced in diabetic rats treated with rutin, a flavonoid. However, since the leaf extract used in this study is crude, there is the likelihood that other components in the crude extract may interfere with the potency or otherwise of the constituent flavonoid. It therefore, becomes pertinent to purify, characterize and re-evaluate the inhibitory action of the pure forms of the various flavonoids in the leaf extracts of Cn.f.

Although, the mechanism of action by which quercetin and extracts of Cn.f inhibit haemoglobin glycosylation is still obscure, it is probably logical to ascribe their actions to the formation of a semi-acetal link between the hydroxy (OH) groups of the compound and the available glucose molecule thus forming an aglycoside adduct of quercetin and *Cnestis ferruginea* which accumulates. This

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notion is based on the fact that they have been detected to undergo such a reaction (Goodwin and Mercer, 1974). Consequently, the potency of the antioxidants fades off as they are being depleted from the reaction medium by conversion to the aglycoside adduct in form of monosides, biosides, dimonosides, trimonosides or dibroside etc (Goodwin and Mercer, 1974). This may account for the decrease in haemoglobin concentration and the apparent saturation of the flavonoid by glucose.

Since, it has been well documented that depending on the assay method, the proportion of total haemoglobin that exists as HbA_{1c} is approximately 5 – 7% in non-diabetic adults (Bunn *et al.*, 1978, Goldstein, 1995), experiments were carried out in this study to determine the effect of methanolic extract of C nf on haemoglobin glycosylation in the presence of physiological concentration of glucose (≤1 mg/ml). As shown on Tables (6, 7, 8), quercetin and extracts of C nf inhibited human haemoglobin glycosylation insignificantly (7%) in the presence of physiological concentrations of glucose (≤1mg/ml). Even though, the long term effect of HbA_{1c} component in non-diabetics is not yet clear this result could be interpreted to mean that if C n f is ingested by non-diabetic individuals, the formation of the small proportion of HbA_{1c} that accumulates over a long period of years could be inhibited.

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It is well known that if untreated, diabetic patients are prone to developing several long term complications which could lead to increased morbidity and early mortality. For example, they have an increased risk of premature atherosclerosis (Kannel *et al.*, 1979) which is partly due to an increased oxidizability of their low density lipoprotein fraction, reduced remodeling, and accelerated ageing of the skin, localized ischeamia and fibrosis (Clark *et al.*, 1991). Indeed, diabetic patients have an increased oxidative stress which is accompanied by a decreased antioxidant capacity and an increased oxidation of cellular components and lipoprotein (Baynes, 1991). Non-enzymatic glycation of proteins which leads to accumulation of advanced glycation end products (AGEs), is now known to be one of the sources of free radicals contributing to this increased oxidative stress (Brownlee, 1994) and it is also strongly linked to the presence of complications in diabetes (McCance *et al.*, 1993). In this connection inhibition of glycation and oxidation processes by co-adjuvant therapy could therefore prevent or at least delay the onset and/or the progression of these complications. Since diabetic patients are to follow this therapy from the onset of the disease the drug must be effective, safe, inexpensive and readily available. This has stimulated focus being directed towards current search for drugs with such properties. An example of such a drug is Daflon (t) 500, a flavonoid preparation consisting of 90% diosmin and 10% hesperidin (Manuel *et al.*, 1999). Aminoguanidine was the first potent inhibitor of formation of advanced glycation

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end products (AGEs) to be discovered (Brownlee, 1986) but it was also found to act as an inhibitor of nitric oxide synthase and this opened a new wave in the discovery of a novel inhibitor of AGEs formation that does not inhibit nitric oxide synthase known as 2,3 diaminophenazine (2, 3 DAP) (Soulis *et al.*, 1999).

The management of diabetes complications is difficult for both health-care providers and patients. Maintaining blood glucose levels near normal, an important care goal requires the correct balanced food, exercise and medications. Recent advances in monitoring have simplified the tesk considerably. Self Monitoring of Blood Glucose (SMBG) requires patients to perform SMBG frequently and adjust insulin dosage or meal plans as needed to keep blood glucose levels as close as possible to target levels (Singer *et al.*, 1989). The fact that glycated haemoglobin involves a simple blood test taken at any time of the day without regard to food intake or blood glucose over an extended period of time (Nathan *et al.*, 1984; Goldstein, 1986) is indicative that the use of drugs that would act directly on HbA_{1c} levels would be applauded and encourage easier and simpler monitoring therapy in diabetes.

Therefore, having demonstrated in the present study that methanolic extracts of Cn f inhibited haemoglobin glycosylation, an attempt was further made to determine its mechanism of action. In this regard, its effect was determined on glycated haemoglobin HbA_{1c} present in blood samples of diabetic patients.

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Haemoglobin was partially purified from blood samples collected from 2 groups of diabetic patients (untreated and controlled) attending the Medical Outpatient Department of University College Hospital, Ibadan. The haemoglobin solution was incubated separately with varying concentrations (10-30μg of quercetin, Vitamin E or extract of Cn f at different incubation periods (24-72hrs) Tables 9 and 10 show the effect of quercetin Cn f and Vit E on glycated haemoglobin in samples from controlled and untreated diabetic patients. The result obtained showed that quercetin and the extracts of Cn f used in this study have insignificant effect on the hydrolysis of HbA_{1c} to yield Hb and glucose. It appears here that the glycated haemoglobin could be decomposed to give free haemoglobin. This indeed is understandable because the process of glycation is oxidation and it involves formation of covalent bonds.

Glycated haemoglobin (HbA_{1c}) when compared to HbA in oxygen affinity is less responsive to the additions of 2,3-diphosphoglycerate (2, DAG) (Bunn *et al.*, 1976). This is because, within the mammalian red cells, 2,3-DPG polyanion binds more strongly to deoxy haemoglobin (T state) than to oxy haemoglobin (R state). Its negatively charged groups form salt bridges with positively charged groups in both β - chains of the haemoglobin via their amino terminal amino acids (Val NA_1, Lys EF_6 and His H_{21}). Should this site be blocked by a hexose, an acetyl (Bunn *et al.*, 1978) or a carbamyl (Kilmartin *et al.*,

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1973) groups, the reactivity of haemoglobin with 2,3-DPG will be markedly reduced leading to the degenerative complications associated with diabetes as well as the supply of oxygen to peripheral tissues.

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CONCLUSION

The present study revealed that

1. Methanolic extracts of *Cnestis ferruginea* is rich in flavonoids.

2. The degree of haemoglobin increases linearly with the period of incubation in a concentration dependent manner up to 20mg glucose/ml where it appeared to be saturated.

3. The inhibitory activity of quercetin and Methanolic extracts of *Cnestis ferruginea* on human haemoglobin glycosylation is both concentration and time dependent. Quercetin and extracts of *Cnestis ferruginea* inhibited haemoglobin to about the same extent.

4. Quercetin and extracts of *Cnestis ferruginea* inhibited human haemoglobin glycosylation in the presence of physiological concentration of glucose in a concentration dependent manner.

5. Quercetin and extracts of *Cnestis ferruginea* and Vit. E had no significant effect on glycated haemoglobin.

6. Extracts of Cn.f. like quercetin inhibits glycosylation of haemoglobin possibly by preventing the condensation of glucose with the N-terminal amino groups of β-chains of HbA. This may cause terminal amino

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groups of HbA, β -chains to form salt bridges with 2, 3-DPG within the peripheral tissues so that HbA can proceed with its normal oxygen transport function.

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