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IN VITRO STUDY ON INHIBITION OF GLYCOSYLATION OF METHANOLIC LEAF EXTRACT OF *Hibiscus cannabinus.*

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ABSTRACT

The inhibitory properties of Methanolic leaf extract of *Hibiscus cannabinus* (Malvaceae family) on glycosylation formation, was investigated in haemoglobin using Gallic acid as Standard. The periodic glycosylation of haemoglobin at varying concentration of glucose shows a decrease in haemoglobin concentration indicating the glycosylation of haemoglobin. While the subsequent administration of *Hibiscus cannabinus* Methanolic leaf extract inhibit haemoglobin glycosylation, where a concentration of 20 mg/ml of the extract gave a significant inhibition by yielding haemoglobin concentration of 1.877±0.40 µg/ml for test extract as against 0.032±0.013 µg/ml for the standard. This suggests that the plant extract inhibits the binding of glucose to hemoglobin, since at higher concentration of glucose the concentration was found to be high.

Key words: Glycosylation, Haemoglobin, Gallic acid, *Hibiscus cannabinus*, inhibition.

INTRODUCTION

Glucose reacts nonenzymatically with proteins in vivo, chemically forming covalently attached glucose-addition products and cross-links between proteins. The excessive accumulation of rearranged late-glucose-addition products, or advanced glycosylation end products (AGEs), is believed to contribute to the chronic complications of diabetes mellitus (Makita *et al.*, 1991).

Apart from protein (enzymes) that enhances glycosylation, nonenzymatic glycosylation also occur in nucleic acids. In the later reaction, reversible Schiff base and Amadori product are form in proportion to glucose concentration. Equilibrium is reached after several weeks and further accumulation of these nonenzymatic glycosylation products ceases. Subsequent reactions of the Amadori product slowly give rise to nonequilibrium advanced glycosylation end-products which continue to accumulate indefinitely on longer-lived molecules. Excessive formation of both types of nonenzymatic glycosylation product appears to be the common biochemical link between chronic hyperglycemia and a number of pathophysiologic processes potentially involved in the development of long-term diabetic complications (that affect the eyes, kidneys, and nerves). The major biological effects of excessive nonenzymatic glycosylation include: inactivation of enzymes; inhibition of regulatory molecule binding; crosslinking of glycosylated proteins and trapping of soluble proteins by glycosylated extracellular matrix (both may progress in the absence of glucose); decreased susceptibility to proteolysis; abnormalities of nucleic acid function; altered macromolecular recognition and endocytosis; and increased immunogenicity (Brownlee *et al*., 1984).

The normal level for glycosylated hemoglobin is less than 7%. Diabetics rarely achieve such levels, but tight control aims to come close to it. Levels above 9% show poor control, and levels above 12% show very poor control. It is commonly recommended that glycosylated hemoglobin should be measured every 3 to 6 months in persons suffering from diabetes. Since the glucose stays attached to hemoglobin for the life of the red blood cell (normally about 120 days), the level of glycosylated hemoglobin reflects the average blood glucose level over the past 3 months (Medicine Net. Com, 2010), hence the inhibition of the glycosylation processes may abate the complication seen diabetic individuals.

Natural plant product have shown great potential in the management of various ailment and *Hibiscus cannabinus* L. (Malvaceae) is one of such plant that has long been used as folk medicine in India and Africa for the treatment of various disease conditions (Duke, 1983). The plant is commonly known as Rama in northern Nigeria, where it is used as vegetables in the preparation of local dish called 'pate' (i.e. maize portage) and the preparation of a local salad. Audu (1989) indicated that *H. cannabinus* in combination with *Leptadenia hastata* is used as an anti-diabetic remedy in Bauchi State, Nigeria.

Since glycosylation reaction involves the formation of reactive oxygen species, natural plant products may likely inhibit advanced glycosylation end products (AGEs) (Adisa *et al*., 2004). Studies conducted by James *et al*., (2010), Agbor *et al*., (2005) and Ibrahim *et al*., (2005) all implicated the plant *H. cannabinus* to have glycosides such as flavonoids and Anthroquinone in addition to possessing antioxidant activity. This study is aimed at ascertaining the glycosylation inhibitory effect of the Methanolic leaf extract of *H. cannabinus*, as a co-adjuvant in the management of diabetics mellitus complication.

MATERIALS AND METHODS

Reagents: All chemicals and reagents used for the study were of analytical grade.

Plant material: The leaves of *Hibiscus cannabinus* were collected from four different communities around Kaduna State, Nigeria between the months of July and August, 2009 and authenticated by the Department of Biological Sciences, Kaduna State University, Kaduna, Nigeria.

Fresh leaves were collected and dried for four days, crushed to powder and stored in an airtight container at 25^{o}C.

Extraction: The powdered plant material (50g) was soaked in methanol and stirred (with the aid of a magnetic stirrer) for one hour, then stored in the dark for 48 hours after which the mixture was filtered first with muslin then filtered again with cotton wool in funnel. The filtrate was then evaporated with the aid of rotary evaporator.

Blood sample collection and preparation of haemoglobin: Blood sample were collected by venopuncture via the cubital vein. Four New Zealand breed of rabbits were placed on the working bench and their fore limb were shave around the cubital area. With the on a pressure around the cubital area the cubital vein was located, the area disinfected with alcohol and with the aid of syringe and needle 30ml of blood was withdrawn and transferred into a blood bottle containing Ethylene diamine tetraacetic acid (EDTA) as anticoagulant (Williams, 1976).

Haemolysate was prepared following Asgary *et al.,* (1999) as reported by Adisa *et al.* (2004) based on the principle of hypotonic lysis. From the procedure, the red blood collected were washed thrice with 0.14M NaCl solution and 1 volume of red blood cells suspension was lysed with 2 volume 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 2300 rpm for 15 min at room temperature. The haemoglobin rich fraction (upper layer) was separated and dispensed into sample bottle for storage in the refrigerator until required for use.

Estimation of haemoglobin concentration: The haemoglobin concentrations were estimated by Drabkin & Austin (1932) method. Into test tube 20 µl of blood and standard solution were transferred into their respective tubes. To each tube 5 ml of Drabkin's reagent was added, mixed properly and was allowed to stand for 10 min. The absorbance for the test and standard solution were read at 540 nm and the haemoglobin concentration determined (Raphael, 1983).

Estimation of haemoglobin glycosylation: Non-enzymic glycosylation of haemoglobin was estimated by Parker *et al.*, (1981) modification as reported by Adisa *et al.,* (2004). Into three test tubes each containing 1 ml solution of different concentration (2, 10, and 20 mg/ml) of glucose in 0.01M phosphate buffer pH 7.4, 1 ml each of haemoglobin fraction was transferred. The contents were incubated at room temperature for 72 hrs. A blank solution in which the addition of glucose solution was omitted was used as control. The amounts of hydroxymethylfurfural in nanomole released were estimated at different incubation periods of 0, 24hrs, 48hrs and 72hrs which corresponds to the degree of glycosylation.

Effect of Extract on Haemoglobin Glycosylation: To 1 ml of haemoglobin solution 5µl of gentamycin and 25µl of Gallic acid (as standard) 25 µl of the leave extract of *H. cannabinus* was added. The reaction was started by the addition of 1 ml of 2% glucose in 0.01M phosphate buffer pH 7.4 and incubated in the dark at room temperature. The concentrations of glycated haemoglobin at the incubation period of 0, 24 and 72 hrs were estimated colorimetrically at 443 nm (Adisa *et al*., 2004). The test was conducted in triplicate.

Effect of extract at physiological glucose concentration: To 1 ml of haemoglobin solution, 1 ml 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 30 µg/ml of Gallic acid or *H. cannabinus* extract respectively. Different concentration of glucose 1mg, 2mg, 4mg, 6mg, 8mg 10mg, 15mg and 20mg in 20 ml each of 0.01M phosphate buffer, pH 7.4 were used. Haemoglobin concentrations were estimated daily throughout the incubation period (72hrs) as an index for measuring the degree of haemoglobin glycosylation; the absorbance reading read colorimetrically at 443 nm. The assay was carried out in triplicates (Adisa *et al*., 2004).

RESULTS

The exposure of haemoglobin over a period of 72 hours to varying concentration of glucose (2mg, 10mg and 20mg) showed decrease in the concentration of haemoglobin indicating an increase in the glycosylation of hemoglobin (Table 1). While Table 2 indicate the inhibition of glycosylation of hemoglobin as compared between the plant extract and a standard (Gallic acid). The Effect of *H. cannabinus* methanolic leaf extract at physiologic glucose.

TABLE 1. ESTIMATION OF HAEMOGLOBIN GLYCOSYLATION OVER THE PERIOD OF 72 HOURS

Glucose concentration	Glycosylated haemoglobin (nanomole of Hydroxymethyl furfural)		
	24hrs	48hrs	72hrs
2 mg/ml	0.846 ± 0.005	1.214 ± 0.006	0.405 ± 0.002
10 mg/ml	0.736 ± 0.002	0.883 ± 0.008	0.07 ± 0.003
20 mg/ml	0.920 ± 0.005	0.736 ± 0.009	0.368 ± 0.007

TABLE 2. EFFECT OF *Hibiscus cannabinus* LEAF EXTRACT ON HAEMOGLOBIN GLYCOSYLATION AS COMPARED WITH STANDARD (GALLIC ACID)

Time	Glycosylated haemoglobin (nanomole of Hydroxymethyl furfural)		
	24hrs	48hrs	72hrs
Extract (25 µl)	4.531 ± 0.00	1.288 ± 0.004	0.994 ± 0.004
Gallic acid (25µl)	-	1.104 ± 0.002	0.019 ± 0.007

TABLE 3. EFFECT OF *Hibiscus cannabinus* METHANOLIC LEAVE EXTRACT AT PHYSIOLOGICAL GLUCOSE CONCENTRATION

	Concentration of glycosylated haemoglobin (nanomole of Hydroxymethyl furfural)					
	Hibiscus cannabinus (µg/ml)			Gallic acid (µg/ml)		
Gluc mg/ml	24hr	48hr	72hr	24hr	48hr	72hr
1	0.730±0.013	0.810±0.00	1.619±0.01	1.614±0.014	2.252±0.014	2.331±0.003
2	1.096±0.017	0.810±0.00	1.546±0.105	-	-	-
4	0.626±0.00	1.178±0.00	1.509±0.130	-	-	-
6	1.251±0.122	1.435±0.122	1.914±0.138	-	-	-
8	0.405±0.00	1.067±0.339	1.472±0.224	-	-	-
10	0.00±0.00	0.258±0.1.17	0.92±0.00	-	-	-
15	0.00±0.00	0.810±0.00	0.920±0.202	-	-	-
20	0.00±0.00	1.178±0.00	1.877±0.40	0.011±0.001	0.005±0.002	0.032±0.013

concentration (Table 3), compared with the data for standard (1mg/ml and 20mg/ml), showed an increase in the concentration of hemoglobin over all the periods of incubation, indicating inhibition of glycosylation. In comparison with standard, at 1mg/ml the increase in hemoglobin concentration was higher than that of the plant extract, but at 20mg/ml the increase in hemoglobin concentration was higher for the plant extract than for the standard.

DISCUSSION

This result suggests that the plant extract inhibits the binding of glucose to hemoglobin, since at higher concentration of glucose the concentration was found to be high.

Literature has shown that AGEs is now known to be the source of free radicals in diabetes, thus aggravating the state of an increased oxidative stress in diabetes mellitus (Adisa *et al*., 2004), hindering its formation implies lower level of free radicals in diabetes, and reduced diabetic complication.

In the management of diabetic in appropriate attention has been given to the reducing properties of monosaccharide. In addition to direct glycosylation reactions, monosaccharides can enolize and thereby reduce molecular oxygen under physiological conditions, yielding α-ketoaldehydes, H_2O_2 and free radical intermediates (Wolff & Dean, 1987). *In vivo* the occurrence of this process contributes to the elevated plasma peroxides found in diabetics complication and may contribute to protein modification reactions perform with glucose *in vitro*. Thus with the high level of glucose in the blood, erythrocytes has become important in the pathophysiologic mechanisms in diabetics as shown from several abnormal features demonstrated for red cells of diabetes mellitus patients (Adisa *et al*., 2004).

It is concluded that the administration of *H. cannabinus* methanolic leaf extract inhibits glycosylation of hemoglobin as such the formation of advance glycated end-product (AGEs) may be inhibited by the plant extract. This observed effect might be attributed by the presence of bioactive compounds in the plant extract like flavonoids, alkaloids, phenols and sterols. This needs further investigation specific bio active compound responsible for such activities.

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