Afr. J Med med Sci (1998) 28, 219-223

Hypoglycaemic activity of α, α trehalose-6-phosphate

J.M. Oke and R.A. Watt
London School of Pharmacy, University of London, 29/30 Brunswick Square, London.

Summary
α-α-trehalose-6-phosphate, synthesized by Oke was screened for hypoglycaemic activity. Alloxan-induced diabetic albino rats and fasted-rabbits were used in the study. The inhibitory activity of trehalose-6-phosphate on trehalase was also assayed. The study shows that α-α-trehalose-6-phosphate is a glucose analogue with potent anti- hyperglcaemic activity as shown by its hypoglycaemic response in fasted rabbits. The ability of α-α-trehalose-6-phosphate to attenuate the diabetic toxicity in alloxan-induced diabetic rats confirmed its potent anti-diabetic activity. The mechanism of action of this synthesized compound may be linked with its ability to inhibit trehalase, and increase the activity of the superoxidase dimutase present in the β-cells of the alloxan-diabetic rats and also being a glucose analogue according to Puls principle, α-α-trehalose-6-phosphate is able to influence the intermediate metabolism of carbohydrate.

Keywords: *α-α-trehalose-6-phosphate, fasted-rabbits trehalase, anti-diabetic, alloxan-induced-diabetic-rats.*

Résumé
L` α-α trehalose-6 phosphate, synthetisé par oke a été examiné pour leur activité hypoglycemique. Les rats d'origine albino chez lesquels de diabete a été induite par l'alloxan et les lapins à jeun ont été utilisé pour l'étude. L'activite inhibitive du trehalose-6 phosphate sur la trehalase a aussi été examiné. L'étude a montré que l' α-α trehalose-6 phosphate est un anologue du glucose avec un potentiel áctivite hypoglycemique, telque le montre la reponse hypoglycemique chez les rats à jeun. L'abilite de l'α-α trehalose-6 phosphate à atenuer la toxicite du diabete induite par l'alloxan chez les rats confirme son potentiel autosynthetisé serait lié à son abilité à inhiber la trehalose superoxidase presente dans les cellules des rats don't le diabete a été induit à l'alloxan, et son role d'analogue du glucose selon le principe de puls, fait de l'α-α trehalose-6 phosphate un element inter-mediaire du metabolisme des glucides.

Introduction

An ideal oral hypoglycaemic agent at low dosage-regimen should at least be equipotent with insulin and with minimal side effects However, the undesirable side effects of insulin, coupled with its prolonged mode of administration, have led to the search for and eventual discovery of some oral hypoglycaemic agents. Since there is yet no ideal oral hypoglycaemic agent that could fully replace insulin, the search must continue. This led to the synthesis of 5-alkyl derivatives of 1,3,4 thiadiazole-2-oxamic acid and demonstrated that these compounds have potential oral hypoglycaemic activity [1]. Further work [2] reported that the dioxamide derivatives of 1,3,4-thiadiazole were more potent and acted for a longer period when compared with the oxamide derivatives of 1,3,4-thiadiazole which were previously reported [1]. In continuation with this study, some researchers reported the prerequisite chemical functional groups necessary for oral hypoglycaemic activity [3]. Recently, further study of the hypoglycaemic activity of the hydrazino-oxamido derivatives of 1, 3, 4-thiadiazolyl-2-oxamic acid reported that the hydrazino-oxamido derivatives of 1, 3,4 thiadiazolyl-2-oxamic acid were the most active of the 1,3,4 - thiadiazolyl series [4, 18].

In this study, α,α-trehalose-6-phosphate was synthesized and screened for hypoglycaemic activity and its mechanism of action investigated.

Correspondence Dr. J.M. Oke, Faculty of Pharmacy, College of Medicine, University of Ibadan, Ibadan, Nigeria

Materials and methods

(a) Chemicals and reagents [1] α-α-trehalose-6-phosphate was obtained as follows: (Figure 1).

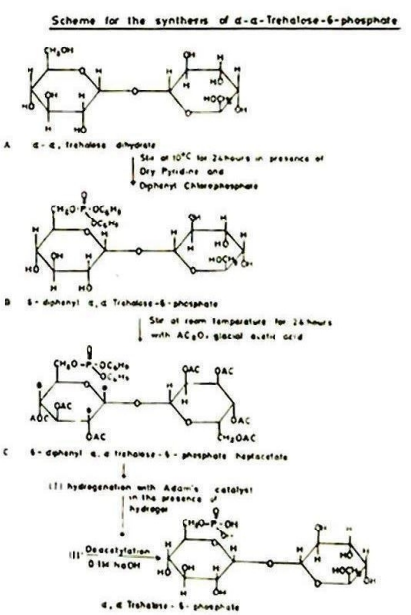

Fig. 1 Scheme

Equimolecular quantities of α, α trehalose dihydrate (A) and diphenyl chlorophosphate were heated together in a distillation flask in the presence of dry pyridine to produce 6-diphenyl-trehalose-6-phosphate (B) which on further acetylation with glacial acetic acid gave 6-diphenyl α-α-trehalose-6-phosphate hepta acetate (C). On dehydrogenation of (C) with Adam's catalyst and further deacetylation in 1M NaOH solution, α-α-trehalose-6 phosphate (Figure 1) was obtained. Spectrophotometric analysis (NMR, IR) confirmed the molecular structure of this new compound.

Equipment

(i) Varian XL-300 spectrometer (IR)

(ii) Bruker WP805Y spectrometer operated at 20.14 MHz (For NMR)

(iii) IR spectra (KBr disc) γ_{max}^{cm-} 2920 - 2830 = CH; γ_{max}^{cm-1} 1670-1630 C = 0; γ_{max}^{cm-} 1030-1040 P = 0 - alkyl.

(iv) H-NMR (C_DCl_3)
$\delta(7.99) = (CH_2)$; $\delta(4.9)$ = OH alcholic; $\delta(2.2)$ = OH; $\delta(4.4) = CH_2$; $\delta(1.4)$ = C-P
pka of this new compound was determined by potentiometric method (8) and found to be pka_A = 10.32; pka_B = 8.11.
MP = 198, $[\alpha]_D$ = +197°

Other reagents included phosphate buffers 0.1M. Substrate solutions, reagents for the determination of glucose by glucose oxidase method [5], and finally glucose standard.

(b) Animals: All animals used in this study were obtained from the University of Ibadan animal house. These included 100 white albino rabbits each weighing 3 kg to 3.500 kg and 250 Wister strain rats each weighing 250-300 gm.

(c) Drug - Gilbenclamide was the control drug in this study.

Pharmacological Investigations - The procedures were as follows:

Experiment I

Determination of hypoglycaemic activity of tested compound

Healthy albino rabbits of the same sex were used for this investigation. Each rabbit weighed between 3 kg and 3.50. Five sets of six rabbits in a set were employed. Each set of rabbits was labelled set A,B,C,D and set E.

All sets of rabbits for the study except set A and E were fasted for 72 hours before the start of the experiment. A preliminary test-dose study for the synthesized compound was conducted as follows:

(a) *Determination of preliminary test-dose value of α-α-trehalose-6-phosphate*

Set A rabbits received no treatment and were not fasted through out the study period. These rabbits served as the control. Set E rabbits received 10 mg, 25 mg and 40 mg/kg body weight, of A-α-α-α-trehalose-6-phosphate. This set of rabbits continued to be fed on the normal diet; they were not fasted.

Sets B, C, and D rabbits were fasted for 72 hours before the start of the study and given only a measured quantity of water ad libitum. Set C rabbits were in addition fed orally with 10 mg, 25 mg and 40 mg/kg per body weight, respectively, of the synthesized compound while set D of rabbits received orally 5 mg, 25 mg, 40 mg/kg body weight, respectively, of gilbenclamide - a drug used for treatment of diabetics in the clinics. Thus sets C and D of rabbits represented, respectively, treated diabetics; while set B of rats were untreated hyperglycaemic rabbits. The basal (fasting) blood glucose levels of the rabbits were determined [5] before and after the treatment and recorded.

The preliminary test study revealed that 25 mg/kg of α-α-trehalose-6-phosphate was the optimum dose for this investigation.

(b)(i) *Determination of the hypoglycaemic activity of α-α-trehalose-6-phosphate*

The experiment was repeated with fresh sets of rabbits as described above, but feeding orally 25 mg/kg body weight of the synthesized compound and 25 mg/kg of gilbenclamide to set C and D of rabbits, respectively, while sets A, B, D and E rabbits were treated as in (a) above. Blood (2ml) were collected from all sets of rabbits at interval of 2, 4, 6, 8 and 10 hours, respectively, and the blood glucose levels were estimated using the glucose oxidase method [5].

(ii) *Assay of trehalase activity (17)*

The serum obtained from the blood samples collected above was used for the estimation of serum trehalase activity. The trehalase activity was determined according to the method of Van Handel in 1970 [6] and expressed as the microgramme of glucose produced from trehalose per hour per ml of serum at 37 °C (Table 3).

Experiment II

Investigation of the Anti-diabetic activity of α-α-trehalose-6-phosphate

The procedure was as follows:

(a) *Preliminary test-dose value*

In this experiment, 250 Wister strain rats each weighing between 200-250 g, were used. The procedure was the same as that described in Experiment I, but with the following differences. (a) 150 mg/kg body weight of alloxan-monohydrate dissolved in 5 mls of distilled water was injected to sets B, C, and set D of Wister rats to render them diabetic. Initial (basal) blood glucose levels of all sets of rats were determined and recorded (b). 72 hours thereafter, sets C and D rats were each fed orally with the test-doses of 4 mg, 5 mg, 8 mg and 10 mg/kg body weight, respectively, of the synthesized compound and gilbenclamide, respectively; the blood glucose levels were then determined [5] and recorded. Set A rats were not treated at all, while set E rats received 5 mg/kg of the synthesized compound orally. These served as the control for both normoglycaemics treated and untreated rats, respectively.

The preliminary test-dose study revealed that 5 mg/kg of synthesized compound was the optimum dose for the investigation of anti-diabetic activity of this compound.

This investigation was then repeated as in experiment 1b (i) above, but with a test-dose of 5 mg/kg of the gilbenclimide and α-α-trehalose-6-phosphate, respectively. The blood glucose levels were determined as in Experiment 1b(i) above and recorded (Table 2).

Each experiment was repeated five times and the average recording displayed in Tables 1 and 2, after they have been statistically analyzed by the student's *"t"* test [7] and analysis of variance [8].

Results

The results showed that α-α-trehalose-6-phosphate was a potent hypoglycaemic agent causing a gradual, lowering of blood glucose in both fasted rabbits and alloxan-induced diabetic rats. Also, for every □g/ml reduction in the blood glucose level caused by α-α-trehalose-6-phosphate, 100 units/ml reduction in the activity of serum trehalase (Table 3) was noticed. The study also evaluated the acid-base property of α-α-trehalose-6-phosphate and found that the synthesised compound was basic in character.

Discussion

Within two hours post treatment with 25 mg/kg of α-α-trehalose-6-phosphate, the blood glucose level in fasted-rabbits was reduced by 49.5% (i.e., from 1495.4±5.0 to 740.0±5.0□g/ml) and at the end of 10th hour of treatment, the reduction in the blood glucose levels of these experimental animals was further depressed to 900.0±5.0 μg/ml - a reduction of 60%. Gilbenclamide at the same time forced a reduction of 58.0% (Table 1). Similarly, in diabetic-induced rats, at a dose-regimen of 5 mg/kg, α-α-trehalose-6-phosphate produced a depression of blood glucose level of 44.6%, six hours after treatment and at 24 hours post treatment the glucose level had further dropped by 50.5% (820□g/ml±5.0□m/ml). This activity of α-α-trehalose-6-phosphate is very significant ($P < 0.050$).

Structurally, trehalose-6-6phosphate is a derivative of trehalose, to which the phosphate group is attached. A study of the chemistry of trehalose shows that trehalose 1-(α-D-glucopyranosyl) α-D-glucopyranoside) is a non reducing sugar and is much more stable in dilute acid than sucrose, reducing disaccharides or non-reducing trisaccharides. On hydrolysis, D-glucose is the only identifiable product. On total oxidation with four moles of periodic acid, trehalose gave oxalic and glyceric acids as the end products. The presence of D-glucopyranosyl in α-α-trehalose-6-phosphate - a unit characteristic of typical pseudo oligosaccharide α-glucosidase inhibitors as found in acarbose [9] allowed the author to classify α-α-trehalose-6-phosphate as a pseudo- oligosaccharide α-glucosidase inhibitor. Furthermore, according to the observation of some workers, the greater the number of glucose residues after the hydrolysis of these pseudo-oligosaccharide α-glucosidase inhibitors, the more pronounced is the inhibitory effect on oligosaccharidases, such as α-amylase, [10] the smaller the number of the glucose units, the more pronounced is the inhibitory effect on oligosaccharidases such as sucrase and maltase [11]. This fact may be submitted for explaining of the ability of α-α-trehalose-6-phosphate to inhibit the activity of the α-glucosidase enzyme - trehalase as shown in Table 3 and hence the reduction or inhibition of the blood glucose in the experimental animals. Hence it may be postulated that α-α-trehalose-6-phosphate is a potent, fully competitive trehalase inhibtor, competing for the substrate trehalose

Table 1: Blood glucose levels in μg/ml produced by α-α trehalose-6-phosphate and glibenclamide respectively in fasted rabbits

Group of rabbits	Dose in mg/kg	Initial BDGL in μg/ml (± S.D)	Pre-Treatment BDGL in Fasted Rabbits in μg/ml (± S.D.)	Post-treatment Average BDGL in μg/ml (± S.D) obtained by α-α-trehalose-6-phosphate and GILB fasted rabbits					
				2Hr	4Hr	6Hr	8Hr	10Hr	Category of rabbits
A	25	985.2 ± 4.0	-	985.20 ± 4.0	985.0 ± 4.0	985.10 ± 4.0	985.00 ± 4.0	986.10 ± 4.0	Normal rabbit untreated
B	25	985.2 ± 4.0	1495.2 ± 4.0	1492.3 ± 5.0	1493.6 ± 5.0	1492.2 ± 5.0	1493.6 ± 5.0	1492.4 ± 5.0	Diabetic (fasted) untreated rabbits
C	25	984.2 ±4.0	1495.4 ± 5.0	(50%) 740.0 ± 5.0	(47.2%) 789.0 ± 5.0	(44%) 830.0 ± 5.0	(41%) 880 ± 5.0	(39%) 900 ± 5.0	Diabetic fasted rabbits treated with T6P
D	25	987.5 ± 5.0	1500.1 ± 4.0	(51.3%) 730.0 ± 5.0	(49.6%) 755 ± 5.0	(46.8%) 798.0 ± 5.0	(44%) 840 ± 5.0	(41%) 880 ± 5.0	Diabetic fasted rabbits treated with GILB
E	25	985.3 ± 5.0	-	985.20 ± 4.0	985.0 ± 4.0	985.20 ± 4.0	985.10 ± 4.00	985.3 ± 4.00	Normal rabbits treated with T6P

Student'S t-test Final values are compared with initial values. Blood sugar significantly decreased in the treated (C and D) groups ($P <$ 0.001).

Values of variation in blood glucose levels are means of ± S.D. of six rats in each group.

Group C blood glucose level is significant as compared to Group B. ($P \leq 0,01$)

Table 2: Blood glucose levels in µg/ml produced by α-α-trehalose-6-phosphate and glibenclamide respectively in alloxan induced diabetic rats

Group of rats	Dose in mg/kg	Initial blood glucose level µg/ml (± S.D)	Pre-treatment blood glucose level in alloxan-induced diabetic rats (± S.D.)	Post-treatment Average BDGL in µg/ml (± S.D) obtained by α-α-trehalose-6-phosphate and rats GILB in alloxan-induced-Diabetic				
				6Hr	12Hr	18Hr	24Hr	Category of rabbits
A	5	864.6 ± 5.0	-	865.5 ± 5.0	865.4 ± 5.0	866.01 ± 5.0	865.0 ± 5.0	Normal rat untreated
B	5	866.5 ± 5.0	1654.5 ± 5.0	1655.0 ± 5.0	1654.8 ± 5.0	1654 ± 5.0	1654.2 ± 5.0	Diabetic untreated rats
C	5	865.6 ± 4.0	1654.6 ± 5.0	(56.4%) 720.2 ± 5.0	(54.0%) 760.0 ± 5.0	(51.5%) 801.5 ± 5.0	(51.5%) 820 ± 5.0	Diabetic rats treated with T6P
D	5	869 ± 4.0	1654.7 ± 5.0	(57.6%) 701.6 ± 4.0	(56.4%) 720.2 ± 4.0	(52.4%) 786.3 ± 4.0	(50.7%) 815.4 ± 4.0	Diabetic rats treated with GILB
E	5	864.6 ± 5.0	-	865.0 ± 5.0	865.6 ± 5.0	864.0 ± 4.0	865.0 ± 5.0	Normal rats treated with T6P.

Student's t-test Final values are compared with initial values. Blood sugar significantly decreased in the treated (C and D) (P = 0.001) Values of variation in blood glucose levels are means ± S.D. of six rats in each group.
Percentage reductions in blood glucose level are shown in parenthesis when compared with initial value.
Group C blood glucose level is significant as compared to Group B. (P=0.01)

Table 3: Comparative tolerance of α-α-trehalose-6-phosphate on blood glucose levels on serum trehalase activity in fasted rabbits

Blood glucose level in µg/ml	Initial BDGL/serum trehalase level normal (± S.D)	Pre-Treatment Serum trehalase levels after 72 hours in fasted rabbits (± S.D)	Post-treatment Trehalase in µg/ml after feding 24 hours fasted rabbit with 25 mg/kg of α-α-trehalose-6-phosphate (± S.D.)				
			2HR	4HR	6HR	8HR	10HR
Blood glucose level in µg/ml	Normo-glycaemic 993.1 ± 5.0	Diabetic rabbits 1493.1 ± 5.0	1199 ± 5.0	1201.2 ± 5.0	1396.8 ± 5.0	1371.6 ± 5.0	1341.8 ± 5.0
Serum trehalse Units/ml	Normal Rabbits 531.87 ± 5.0	Diabetic rabbits (Untreated) 886.07 ± 5.0	628.7 ± 5.0	648.8 ± 5.0	632.8 ± 5.0	632.8 ± 5.0	630.6 ± 5.0

Comment: For every µg/ml reduction in blood glucose caused by α-α-trehalose-6-phosphate, there is always 100 units/ml reudction in the activity of serum trehalase as shown Table 3 above

In their separate studies, Aulgad [12] and Eze [13] in their separate studies, reported that the enzyme-trehalase, found in man and some invertebrates, has been indicative of trehalose's hydrolysis into small glucose molecules and later for reabsorption of these glucose molecules in the distal tubules for the formation of trehalose. Trehalose is the only disaccharide sugar found in the blood stream of man [14]. Evaluation of the inhibiting capacity of α-α-trehalose-6-phosphate on trehalase, however, revealed that for every □g/ml depression in the blood glucose level caused by α-α-trehalose-6-phosphate, there is always a-100unit/ml reduction in the activity of the *serum trehalase* (Table 3). The results of this evaluation complimented our previous statement and also collaborated Eleanders earlier discovery [15] that α-α-trehalose-6-phosphate is a competitive inhibitor of the enzyme trehalase found in yeast cells for the substrate binding site [13]. Consequently, the hypoglycaemic response of α-α-trehalose-6-phosphate may be correctly linked with this inhibitory action on trehalase

Pual and Keup [10,16] observed that a pharmacological interference with intestinal carbohydrate digestion by suitable α-glycosidase inhibitor should be a feasible way to regulate and retard carbohydrate digestion and in this way influence the intermediate metabolism of carbohydrates. This was the principle that gave birth to the use of *acarbose* for the treatment of diabetics . Acarbose-a pseudo-oligosaccharidic α-glucosidase inhibitors is a potent, fully competitive sucrase inhibitor which is been used successfully for the treatment of diabetes [9]. Similarly, as described above, α-α-trehalose-6-phosphate on acid hydrolysis gave D-glucose which has been shown to be a potent α-glucosidase inhibitor [15]. Thus the results of this study complemented the earlier reports of Allen that glucose analogues were able to inhibit the uptake of glucose in some organisms [1,13] and also agree with Plus' principle. [16]

In this respect, the response of α-α-trehalose-6-phosphate as an antihyperglycaemic agent could not be unconnected with the fact that, α-α-trehalose-6-phosphate is a glucose analogue, like acarbose, inhibiting the uptake of glucose molecules in the fasted rabbits.

Although the precise mechanism of alloxan-induced hyperglycaemia remains unclear, there is increasing evidence that it involves the degeneration of the Islet's β cells by the accumulation of cytotoxic free radicals However, it has been demonstrated that increase in the Islet β cells' superoxide demutase (SOD) activity could prevent or even decrease alloxan toxicity (12). Hence the above facts might be proposed to explain scientifically the anti-diabetic activity of α-α-trehalose-6-phosphate. α-α-trehalose-6-phosphate attenuated alloxan-induced hyperglycaemia in category C rats (Table 2) without inflicting any adverse effects on normoglycaemic (category E) rats also fed with α-α-trehalose-6-phosphate. Considering the fore-mentioned facts, the author was tempted to postulate that α-α-trehalose-6-phosphate could be a very suitable oral hypoglycaemic agent.

In conclusion, this study has shown α-α-trehalose-6-phosphate to be a very good agent capable of reducing the glucose blood levels in fasted-rabbits and alloxan-induced diabetic rats. Hence it could be labelled as a potent anti-diabetic agent. The ability of α-α-trehalose-6-phosphate to attenuate the alloxan toxicity in alloxan-induced diabetic rats coupled with its inhibitory action on enzyme - trehalase, leading to its hypoglycaemic response in fasted rabbits are factors that led to the classification of α-α-trehalose-6-phosphate as a potent hypoglycaemic agent. Furthermore α-α-trehalose-6-phosphate is a glucose analogue, hence it is able to influence the intermediate metabolism of carbohydrates. All these facts could be used to elucidate the mechanism of action of α-α-trehalose-6-phosphate as a potent blood glucose level reducing agent. Furthermore, the fact that α-α-trehalose-6-phosphate was able to lower the blood sugar level in the selected experimental models to a level comparable to that of gilbenclamide - the control drug in this study - revealed that α-α-trehalose-6-phosphate is a potent hypoglycaemic agent.

Work is in progress in our laboratories to confirm the postulates of this study and thus bring α-α-trehalose-6-phosphate to the clinics for treating diabetics.

Acknowledgements

This study was partly supported by the Link-Programme London School Pharmacy and Faculty of Pharmacy University of Ibadan.

The authors is grateful to Mr. J.L. Fajimi of the Department of Pathology University College Hospital Ibadan, for his technical assistance.

References

1. Oke J.M. and Fajimi JL. (In press). O-Hydroxyl-Benzamide Derivatives of Oxamic acid as potential oral hypoglycaemic agents - AJMPS
2. Oke J.M and Olowokorun M.O. The oral hypoglycaemic activity of 2,5-dioxamide derivative of 1,3,4-Thiadiozole. Japan J Pharmacology 1989; 49: 135-138
3. Oke J.F. An investigation into the acid-base properties of some derivatives of 1,3,4 thiadiazolyl-2-oxamic acid. *N.J.P.* 1986; 17(3): 34-39.
4. Oke J.M and Cherynk V.P. Sugar reducing activities of the substituted amides of 5-alkyl, 1,3,4-thiadiazole -2-oxamic acid J. Pharm (U.S.S.R.) 1981; 4: 34-38.
5. Trinder P. Method for determination of glucose in Blood. *Cin. Pathol.* 1969; 22: 246-248.
6. Van Handel E. Serum trehalase. Assay and normal values. Clin. Chim. Acta 1970; 29: 3349-3353.
7. Armitage P. "Statistical methods in medical research" Blackwell Scientific Publicatfons. Oxford, London 1970; 104-106.
8. Snedecor GW and Cochram WG. Statistical method (1967), 6th Edition New Delhi, India.
9. Schmidt DO. Glucosidase inhibition studies Nature 1977; 64: 535.
10. Puls, Kemp U., Krause H.P. Front. Horm Res. 1980 7: 255
11. Schmidt DO, Junge B, Frommer W, Muller L, and Truscheit E. In U. Brodback (Ed) Enzyme Inhibitors, Proc. Meet. Basel March 20-21 1980: Verlag Chemie Winhem 1980: 106.
12. Auigad O; Zui P. Activity of trehalase in yeast. Biochem J 1965; 97: 115-118.
13. Eze L.C and Prince D.A. Plasma trehalase activity in diabetes mellitus. Clin. Chem. Acta 1970; 29: 349-353.
14. Allen O. Elbin Metabolism of trehalose. Adv. Carboh Chem and Biochem 1971; 3: 277-286.
15. Eleander M. Ark. Mechanism of action of α-α-trehalose-6-phosphate Hemi 1968; 31: 17.
16. Puls, and Keup α-glucosidase inhibitor as regulators of carbohydrate meetabolism Diabetologia 1973; 9: 99
17. Halliwels B. and Gutteridge J.M.C. In "Free Radicals in Biology and Medicine". 2nd Ed. Clarendon Press, Oxford 1989;.308.
18. Oke J..M and Fajimi J.L. Hydrazino-oxamido derivatives of 1,3,4-thiadizolyl-2-oxamic acid as potential oral hypoglycaemic agent. AJBR. 1998; 1: 53-62.