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Modulation of Antidiabetic and Antioxidative Status in Experimental Diabetic Rats following Intake of a Novel Succinamic Acid Derivative (α-HSA)

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ABSTRACT

Context: *E. jambolana* (EJ) has been traditionally used for the management of diabetes. Even after the isolation of the herbal anti-diabetic compound (FIIc) from the fruit pulp of EJ has been done, yet it is not commercialized due to unavailability of the fruit throughout the year and due to the low yield of the isolation process of FIIc. The structure elucidation of FIIc has been done and it was found to be α-hydroxy succinamic acid (α-HSA). The goal of the present study is to chemically synthesize α-HSA and further to investigate its anti-diabetic and antioxidant potential in streptozotocin (STZ) and nicotinamide (NAD) induced type 2 diabetic rats.

Materials & methods: The synthesis of α-HSA has been done in a step wise manner. Male albino wistar rats were made diabetic by injecting NAD and STZ (230 and 45 mg/kg body weight i.p with a time interval of 15 min). α-HSA was orally administered to STZ+ NAD induced diabetic rats (20mg/kg body weight).

Results: The structure confirmation of α-HSA was done by spectral studies like ^{1}H NMR, ^{13}C NMR, & HRMS. α-HSA treated rats at week 6 of the study showed marked improvement in FBG levels, HbA1c levels, serum insulin levels, malondialdehyde (MDA) and total antioxidant capacity(TAC) levels in comparison to diabetic control rats.

Conclusions: Above findings suggest that α-HSA possesses anti-hyperglycemic and antioxidant activity which makes it a suitable candidate for the management of T2DM. However, to establish its role as potent anti-diabetic agent in STZ+NAD induced type 2 diabetic rats, its mechanism of action needs to laid down.

Keywords: *E. jambolana*, Succinamic acid derivative, T2DM, STZ+ NAD, Antidiabetic, Antioxidant

INTRODUCTION

Type-II diabetes mellitus (T2DM) is a carbohydrate metabolic disorder which is recognized by decreased insulin sensitivity or there may be partial destruction of pancreatic β cells. Presently, it is one of the leading causes of mortality and morbidity [1,2]. The global burden of diabetes was about 465 million in 2019 and its projected to rise to 700 million by 2045 [3]. There are many synthetic drugs (metformin & sulphonylureas) available in the market for the management of T2DM. However, they have limited efficacy, tolerability and mechanism-based side effects like weight gain, itching, hypoglycemia, edema, hepatotoxicity etc., which are not normally associated with herbal drugs. Many medicinal plants which are known to have anti-diabetic activity like *E. jambolana*, *Cassia auriculata*, Glycine max and have been studied in our lab [4-6]. *E. jambolana* (Botanical name-*Syzgium cumini*) is one such plant and its role in the management of diabetes is well mentioned in the scientific literature [7-10].

In the previous studies done by Sharma et. al, the isolation of anti-hyperglycemic compound (FIIc) from the fruit pulp of EJ has been done and granted Product Patent (No. 2,30,753)

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for isolation and identification of herbal compound [10,11]. The chemical structure of FIIc was elucidated (α-hydroxysuccinamic acid, α-HSA) by spectral analysis. Therefore, it was contemplated that chemically synthesized α-HSA will also possess anti-diabetic and antioxidant properties due to its identical structure with FIIc. Hence, the objective of the present study was to synthesize and assess the anti-diabetic & antioxidant potential of α-HSA in STZ+NAD induced diabetic rats.

The structure of α-HSA is displayed in **Figure 1**.

MATERIALS & METHODS

Design and developing a synthetic strategic for the synthesis of α-HSA

Three different routes have been used to synthesize the main compound 4-amino-2-hydroxy-4-oxobutanoic acid (α-HSA) to standardize the experimental conditions. However, route no. 1 has been found to be successful and best yields were obtained **(Figures 2 and 3)**.

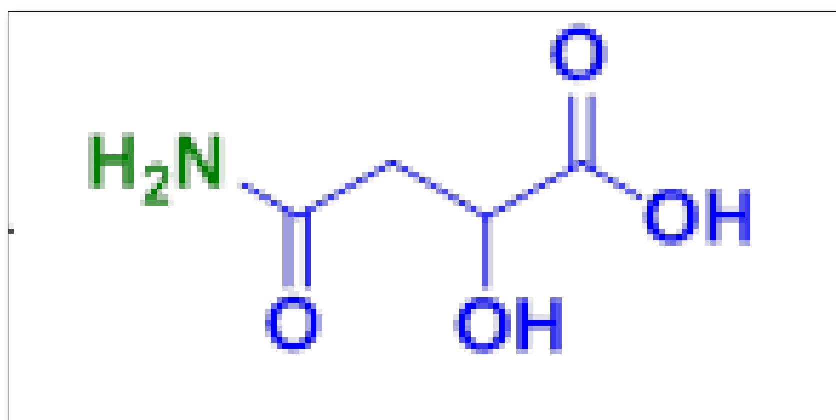

Figure 1. Structure of α-HSA.

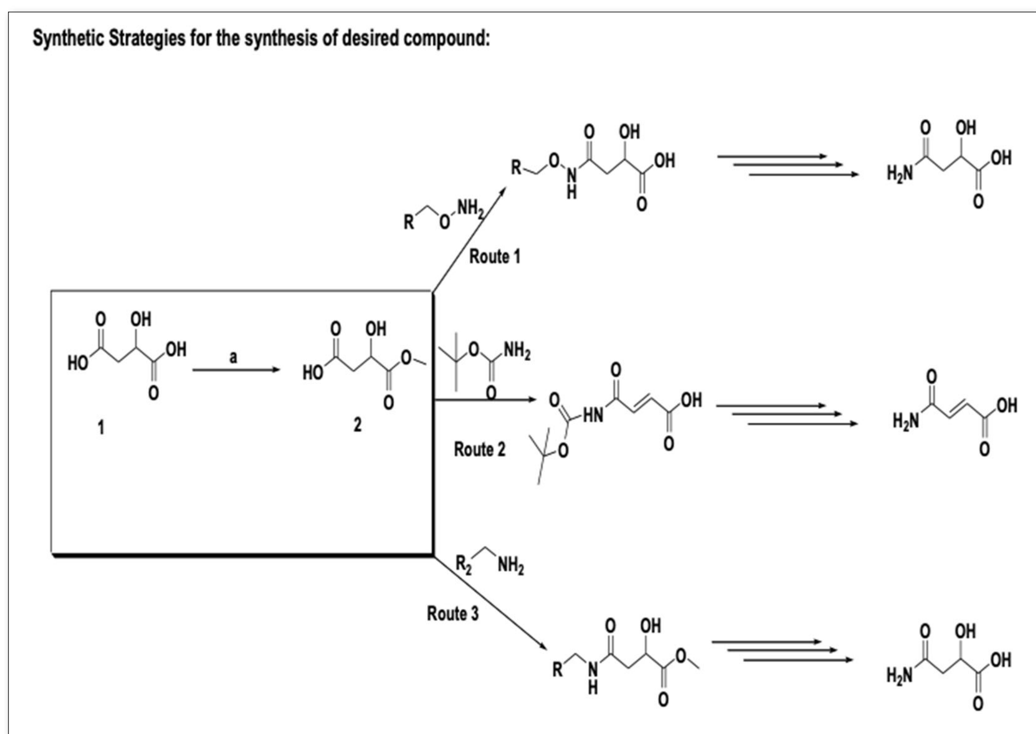

Figure 2. Various strategies adopted for the chemical synthesis of α-HSA.

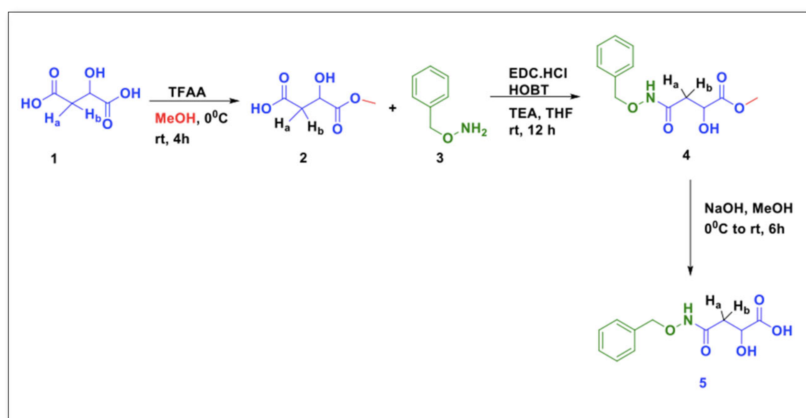

Figure 3. Synthetic strategy for the intermediate derivative [4-((benzyloxy) amino)-2-hydroxy-4-oxobutanoic acid] of α-HSA.

We have already synthesized the intermediate compound (5) 4-((benzyloxy) amino)-2-hydroxy-4-oxobutanoic acid in previous study [12]. In the present study, the herbal anti-diabetic compound4-((benzyloxy) amino)-2-hydroxy-4-oxobutanoic acid (α-HSA) (6) has been chemically synthesized from the intermediate compound 5 **(Figure 4)**.

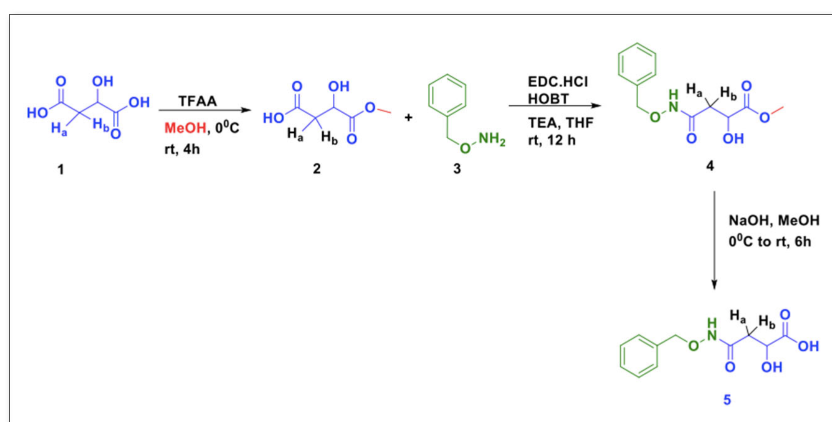

Figure 4. Synthesis of α-HSA [4-amino-2-hydroxy-4-oxobutanoic acid] (6) from its derivative 4-((benzyloxy) amino)-2-hydroxy-4-oxobutanoic acid (5).

Synthesis of 4-amino-2-hydroxy-4-oxobutanoic acid (6) (α-hydroxysuccinamic acid)

Pd/C (0.1 eq) was added to intermediate **5** (0.5 g, 1 eq) in MeOH and was continuously stirred at room temp, under H2 atm for 6 hrs. After the completion of reaction, the progress was evaluated by TLC, the reaction mixture was filtered through sintered funnel, washed with MeOH and concentrated under reduced pressure. The crude compound was purified by prep HPLC. Under high vacuum, the concentrated compound was dried to give the target compound **6** as white solid. Melting-point: 110-114^{0}C; Yield: 30 % ;^{1}**H NMR** (400 MHz, DMSO-d$_6$): 11.81 (br s, 1H, -COOH), 8.314 (br s, 1H, NH), 4.12 (t, 1H), 2.46 (dd, J=14.20 Hz. 1H, H$_a$), 2.36 (dd, J=14.21 Hz. 1H, H$_b$); ^{13}**C NMR** (100 MHz, DMSO) δ: 173.63, 171.64, 78.019, 36.79.; **HRMS** (ESI) $(M+H)^+$Calcd for $C_{11}H_{13}NO_5$: 133.0375, found 133.0454.

ANIMALS EXPERIMENTAL SETUP AND ETHICAL CLEARANCE

The animal study was conducted at Central Animal Service Unit, University College of Medical Sciences (UCMS) & GTB Hospital, Delhi, India. All the animals were acclimatized under standard lab conditions (25°C to 30°C). They are fed with standard pellet diet and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC), University College of Medical Sciences & GTB Hospital, Delhi, India (UCMS/IAEC/26/13). All experimental procedures conducted in the study complied with the relevant guidelines on the care and use of laboratory animals.

Induction of T2DM in Experimental Animals

The induction of diabetes was done by injecting a single dose of freshly made STZ in citrate buffer (0.1 M; pH 4.5) and NAD (45 and 230 mg/kg b.wt.) [13]. Diabetes was

confirmed after 72 h by measuring the fasting blood glucose levels. Wistar rats showing FBG levels 200 mg/dl or more were used for the experimental setup.

ANIMAL EXPERIMENT DESIGN

For the experimental setup, six group of male albinowistar rats having six rats in each group (n=6) were used.

Group I: Control (healthy non diabetic) given normal saline

Group II: Untreated diabetic given normal saline

Group III: Diabetic- administered withFIIc (20 mg/kg b.wt)

Group IV: Diabetic- administered with α-HSA (20 mg/kg b.wt.)

Group V: Diabetic- administered withglibenclamide (600 µg/kg b.wt.)

- The dosage of FIIc (20 mg/kg b.wt) was standardized from the previous studies done in our lab [10].
- The therapeutic dosage of α-HSA (20 mg/kg b.wt.) was calculated after performing the LD_{50}studies.
- The dosage of glibenclamide (600 µg/kg b.wt.) was decided from the previous studies done by Stanley et al. [14].

ACUTE TOXICITY STUDIES AND DETERMINATION OF LD_{50}

Acute toxicity studies on α-HSA were done by following the guidelines given by Organization for the Economic Co-operation and Development (OECD) (Section 4.Test No. 425). Determination of LD_{50} was carried out by the method given by Affifi et al. [15].

For the experimental setup, six group of male albinowistar rats having five rats in each group (n=5) were used. Increasing gradual dose (200, 400, 600, 800, 1000 and 1200 mg/ kg b. wt.) of α-HSA were orally administered to each group respectively. The no of dead animals were recorded 48 hours after the administration of α-HSA. LD_{50} was calculated by applying the following formula.

LD_{50}=The average lethal dose in which all animals are killed – Σ (z.d)/n

z=the mean of dead animals in two progressive doses

d=the steady factor between two progressive doses

n=number of animals in each group

COLLECTION OF SAMPLES

Micro-capillary technique was used to draw blood from retro orbital plexus [16]. For plasma separation, blood was collected in vials containing anti-coagulant (sodium fluoride potassium oxalate) for estimation of fasting blood glucose levels and for serum separation, blood was collected in plain vials for the estimation of insulin levels, MDA & TAC levels. Whole blood was collected in EDTA vials for glycosylated Hb.

BIOCHEMICAL & ANTIOXIDANT ACTIVITIES ASSESSMENTS

Fasting blood glucose levels (Centronic, GmbH, Gemany), serum insulin levels (MercodiaRat ELISA kit, Sweden) and Hb1Ac levels (Biosystems S.A., Costa Brava, Spain) were measured according to the instruction provided by the manufacturer.

TAC & MDA levels using plasma was estimated according to manufacturer's instruction. The kits were provided by BioAssay, USA and ELAB Biosciences, China respectively.

STATISTICAL ANALYSIS

Two-way analysis of variance (ANOVA) followed by Turkey's test were used to perform the comparison of parameters between different groups. $P<0.05$ was considered significant and the results were expressed as mean ± S.D.

RESULTS

1. Acute oral toxicity test

LD_{50} of α-HSA was determined by giving single increasing gradual doses to different groups of normal male albino wistar rats. LD_{50} of α-HSA was found to be 867 mg /kg b.wt. **(Table 1)**. The therapeutic dose chosen for the subsequent study was 20 g/kg b.wt. which is around 1/50 of LD_{50} and considered safe.

LD_{50} = Median lethal dose which kill all animals - Σ(z.d)/n = 1200-2000/6 =867mg /kg b. w.

1/50 of LD_{50} is about 20 mg/kg b. w. which was considered as sub lethal dose that was used as therapeutic dose in the subsequent studies.

2. Effect of α-HSA on Glycemic index

i) Effect of α-HSA on FBG levels

Blood glucose levels were estimated in the overnight fasted experimental rats. After the administration of α-HSA and FIIc to diabetic treated groups (C & D), a significant fall in FBG levels were observed as compared to diabetic control (group B). However, an insignificant difference in FBG levels was observed in group C & D **(Table 2)**.

ii) Effect of α-HSA on HbA1c levels

After the induction of diabetes, the levels of HbA1c increased significantly in diabetic control rats (group B) when compared with normal control rats (group A) ($p<0.005$). After administration of α-HSA, there was significant improvement in HbA1c level α-HSA and FIIc treated groups (group C and D). However, a statistically

insignificant difference in group C & D in HbA1c levels was observed **(Table 2)**.

iii) Effect of α-HSA on insulin levels

In diabetic control rats (group B), the serum insulin levels were found to be significantly decreased ($p<0.001$) as compared to healthy control rats (group A). However, after administration of α-HSA and FIIc for 6 weeks to group C and D animals respectively, a significant increase in serum insulin levels was found when compared to diabetic control rats (group B). The serum insulin levels were slightly higher in group D in comparison with group C. However, the difference was statistically insignificant **(Table 2)**.

3. Effect of α-HSA on Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC) values

A significant increase in MDA levels and a decrease in TAC levels ($p<0.001$) was observed in diabetic control rats (group B) when compared to normal control rats (group A). After administration of α-HSA and FIIc to group C and D respectively for 6 weeks showed a significant decrease in the MDA levels and increase in TAC levels when compared to diabetic control rats (group B) ($p<0.005$). The difference in MDA & TAC values of group C & D was insignificant **(Table 3)**.

Table 1. Determination of LD_{50} of α-HSA.

Dose	Total No. of Animals	No. of Dead Animals	z	d	∑(z.d)
200	6	0	-	200	-
400	6	0	0	200	0
600	6	1	0.5	200	100
800	6	2	1.5	200	300
1000	6	4	3	200	600
1200	6	6	5	200	1000

z: Mean number of dead animals in two successive doses
d: Constant factor between two successive doses

Table 2. Effect of α-HSA on Glycemic index.

Parameters			FBG levels (mg/dl)		HbA1c levels (%)		Serum insulin levels (µU/mL)	
S. No.	Groups	Dose	Week 0	Week 6	Week 0	Week 6	Week 0	Week 6
1	Healthy control (A)	Normal Saline	91.67±5.22	89.67±5.12	5.5±2.12	5.4± 2.58	16.28±3.64	15.68±4.12
2	Diabetic control (B)	Normal Saline	190.6±3.22	220.4±4.56[a]	7.12±3.24	8.1±3.68 [a]	9.2±4.42	7.78±4.56[a]
3	Diabetic (FIIc) treated (C)	20mg/kg b.w.	187.7±4.67	135.56±4.22[b,d,h]	7.18±2.27	6.27±2.48 [b,d,h]	9.98±4.67	11.42±3.22[b,d,h]
4	Diabetic (α-HSA) treated (D)	20mg/kg b.w.	193.45±3.22	129.21±3.28[c,e]	7.24±3.45	6.14±3.24 [c,e]	9.1±5.56	12.26±5.28[c,e]
5	Diabetic (Glibenclamide) treated (E)	600µg/kg b.w.	198.24±2.42	111.38±4.22[f,g]	7.96±3.22	5.42±4.28[f,g]	8.96.3±5.56	11.47±5.28[f,g]

Values are mean ± S.D. (n=6) (p<0.001)
a= Group A vs Group B, b= Group A vs Group C, c= Group A vs Group D, d= Group B vs Group C , e= Group B vs Group, f= Group A vs Group E, g= Group B vs Group E, h= Group C vs Group D

Table 3. Effect of α-HSA on Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC) values.

MDA levels (nmol/L)					TAC levels (µmol/L)	
S. No.	**Groups**	**Dose**	**Week 0**	**Week 6**	**Week 0**	**Week6**
1	Healthy control (A)	Normal Saline	8.5±0.76	8.82±0.58	4.89±0.12	4.58±0.11
2	Diabetic control (B)	Normal Saline	18.35±1.27	20.21±1.67[a]	2.79±0.18	2.16±0.13[a]
3	Diabetic (FIIc) treated (C)	20mg/kg b.w.	16.25±1.45	9.26±1.08[b,d,h]	3.01±0.08	3.48±0.26[b,d,h]
4	Diabetic (α-HSA) treated (D)	20mg/kg b.w.	17.01±1.26	9.1±1.27[c,e]	2.86±0.19	3.56±0.15[c,e]
5	Diabetic (Glibenclamide) treated (E)	600µg/kg b.w.	16.58±1.14	9.98±1.47[f,g]	2.72±0.16	3.82±0.25[f,g]

Values are mean ± S.D. (n=6) ($p<0.001$)
a= Group A vs Group B, b= Group A vs Group C, c= Group A vs Group D, d= Group B vs Group C , e= Group B vs Group, f= Group A vs Group E, g= Group B vs Group E, h= Group C vs Group D

DISCUSSION

Type 2 Diabetes mellitus is considered to be one of the most common chronic diseases and is associated with hyperglycemia and hyperlipidemia. The metabolism of carbohydrates, proteins including lipids and lipoprotein are altered during diabetes.

In the present study, α-HSA was evaluated *in vivo* for its anti-hyperglycemic and antioxidant properties in STZ+NAD induced diabetic rats. Blood glucose, HbA1c, serum insulin and antioxidant/oxidative parameters (TAC & MDA) were analyzed.

In the pancreas, there is partial destruction of β-cells in STZ+NAD induced diabetic rats due to which the levels of serum insulin in the diabetic rats were reduced as compared to normal healthy control rats. After administration of α-HSA to diabetic rats, the serum insulin levels were increased significantly in comparison to diabetic control rats.

The fasting blood glucose levels of diabetic rats after the administration with α-HSA were significantly reduced as compared to diabetic control rats. The anti-diabetic activity of α-HSA is either due to improved insulin secretion or due to enhanced transport of blood glucose to the peripheral tissue [17].

The possible mechanism of action which can be correlated with the way sulphonylureas work i.e. promoting insulin secretion by membrane depolarization, closure of K+ ATP channels and opening of Ca^2+ channels [18,19]. Glycosylated hemoglobin (HbA1c) is as an excellent marker for overall glycemic control and diabetic complications. α-HSA has significantly reduced the glycosylated level in α-HSA treated diabetic rats indicating its efficiency in glycemic control.

The role of lipid peroxidation in the development of diabetes complications is well mentioned in the literature. According to previous studies, the pace of development of MDA, a significant final result of lipid peroxidation, is altogether higher in diabetic rats contrasted with non-diabetic controls [20].

In T2DM there is a direct correlation between levels of MDA and the severity of the disease [21]. The role of free radicals in the onset of lipid peroxidation during T2DM is well mentioned in several scientific reports [21].

In our study, it has been found that α-HSA possess potent antioxidant properties as it decreases the serum MDA levels in α-HSA treated diabetic rats.

Total antioxidant capacity (TAC) is a widely used method that uses antioxidants as reductants in a redox-linked colorimetric reaction, wherein Cu^{2+} is reduced to Cu^{+}. This ability of plasma to scavenge free radicals can be used to exploit its potential to counter the excess RONS observed in diabetes [22,23]. In our study, we have found that after administration of α-HSA, the plasma antioxidant levels of diabetic rats were significantly enhanced as compared to diabetic control rats.

Hence, chemically synthesized α-HSA has shown beneficial effects on hyperglycemia &oxidative stress. α-HSA improves insulin secretion through β-cells restoration in the pancreas and promotes insulin utilization capability in various peripheral tissues and its free radical scavenging property has potential to prevent diabetes associated complications.

CONCLUSION

In the present study, we have concluded that the α-HSA plays a vital role in the management of Type 2 diabetes. Molecular studies need to be done for elucidating the therapeutic action of α-HSA and before administrating it as possible replacement of insulin therapy or as an adjuvant.

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AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no conflict of interests to disclose.

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