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Anti-hyperglycaemic activity of novel synthesized carboxamide derivatives in alloxan-induced diabetic rats

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Abstract

Despite the availability of several chemotherapeutic agents for the management of diabetes mellitus, its global prevalence is on the increase, and the disease therefore poses a significant and complex health challenge that calls for intensified research efforts. The present study evaluated the anti-hyperglycemic activity of a series of novel pyrrolidine carboxamide derivatives synthesized by coupling different amines on a proline-glycine based sulphonamide. The reaction products of glycine with either phenylsulphonylproline or tosylproline were coupled in a condensation reaction with different amines to give the target carboxamide derivatives, which were purified using column chromatography, and their structural characteristics elucidated using mass spectrometry (MS), Fourier-transform infrared (FT-IR), carbon-13 nuclear magnetic resonance (^{13}C-NMR) and proton nuclear magnetic resonance (^{1}H-NMR) spectral data. The condensation reaction gave products (Compounds A – D) with outstanding yield. The synthesized compounds were administered to alloxan-induced hyperglycemic rats and their anti-hyperglycemic effect compared with that of a reference drug. The new compounds were also evaluated for their anti-oxidant activity in vitro, using DPPH and FRAP methods. Single dose administration of the new carboxamides compounds caused significant ($p < 0.05$) reduction in fasting blood glucose of alloxan-induced hyperglycemic rats compared to an untreated hyperglycemic control. Compound B with methyl group para-substituted at the aromatic amine of a tosylpyrrolidine carboxamide gave the best anti-hyperglycemic effect six hours post administration. It was concluded that carboxamide derivatives synthesized by combining glycine proline-based sulphonamides with carboxylic functional group and an amine showed anti-hyperglycemic activity in experimentally induced diabetic rats. These compounds may therefore be considered potential candidates in the development of new class of anti-diabetic drugs.

Keywords: Carboxamides; Anti-diabetic activity; Fasting blood glucose; Hyperglycemia; Alloxan.

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Introduction

Diabetes mellitus (DM) is a disease of metabolic origin characterized by persistent hyperglycemia, dyslipidemia and symptoms such as polyuria, polydipsia and polyphagia (Loria *et al*, 2013). It affects both humans and animals and it is associated with various complications, which include nephropathy, retinopathy, neuropathy and cardiovascular disorders (Bhatt *et al*., 2019; Padhi *et al*., 2020; Rand, 2020). Globally, the prevalence of diabetes mellitus continues to increase, and it is estimated that DM will be affecting 629 million people by 2045 (Glovaci *et al*., 2019; Forouhi and Wareham, 2019). Despite available treatment options for the disease, the incidence of the disease has exponentially grown in the past decades across the globe as reported by the World Health Organization, and it caused the death of 1.5 million people in 2019 (WHO, 2023). There is thus need for more research to develop new chemotherapeutic agents that will help stem the scourge of the disease.

Carboxamides are compounds that contain the carboxamide functional group (-CONH2)**.** They are a constituent of many drugs, and several research works have reported their potency as anti-diabetics (Jo *et al*., 2021), anti-oxidants (Nithyabalaji *et al*., 2019), anti-convulsants (Ho *et al*., 2018), anaesthetics (Al-Otaibi, 2018), antibiotics (Xue *et al*, 2020; Ahmad *et al*., 2023), anti-viral drugs (Pinto *et al*., 2020), among others. Glibenclamide, a sulfonylurea anti-diabetic drug, which acts by inhibiting the ATP-sensitive K+ channels leading to a depolarization of the pancreatic *β* islet cells and insulin secretion (Zhang *et al*., 2017), is synthesized by the conversion of carboxamides to sulfonamide (Wermuth *et al*, 2007). Glibenclamide contains a carboxamide-like moiety, which is a urea functional group (-NH-CO-NH). The present study evaluated the in vivo anti-hyperglycemic and in vitro anti-oxidant activities of a new carboxamide derivatives synthesized by chemically combining glycine with different amines.

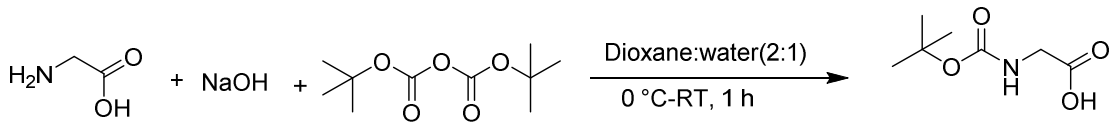
Materials and Methods

Preparation of the test compounds: The compounds and solvent utilized were procured from Sigma-Aldrich, Germany and AVRA Chemical Pvt. Ltd, Hyderabad, India. Proton nuclear magnetic resonance (1H-NMR) and carbon-13 nuclear magnetic resonance (13C-NMR) spectra were captured using Advanced 400 and 500 MHz spectrometers in CDCl3, with TMS as an internal standard. Fourier-transform infrared (FT-IR) spectra were documented with PerkinElmer Spectrum Version 10.03.06. Mass spectra were generated using an Agilent liquid chromatography mass spectroscopy (LCMS) instrument. High-resolution mass spectrometry (HRMS) was performed using Agilent Technologies 6510, Q-TOFLC/MS ESI technique. Melting points were assessed using an open glass capillary tube on a Stuart melting point apparatus and were not corrected. All reactions were tracked using thin-layer chromatography (TLC) on pre-coated silica gel. The spots were observed under ultraviolet (UV) light and in an oven with Ninhydrin. Merck neutral aluminum oxide activated (60-325 mesh) was employed for chromatography purposes.

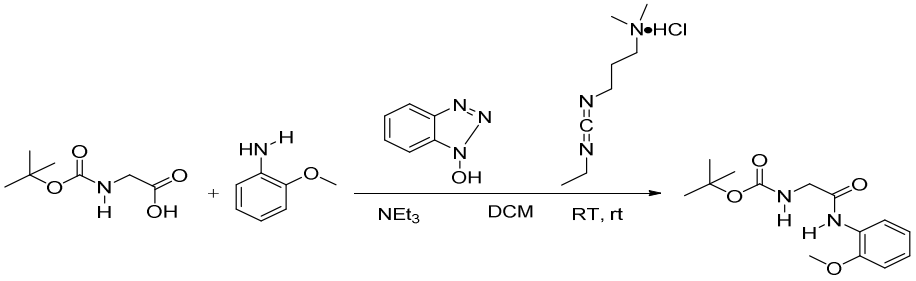
The compounds were synthesized following the methods reported by Attah *et al.* (2022). The steps were as follows:

Protection with Tert-butoxycarbonyl (boc): Two grammes (equivalent to 26.3 millimoles) of glycine obtained from Cambridge Isotope Labs were dissolved in a solution containing 60 ml of a 2:1 mixture of dioxane and water (volume: volume). Then 1.064 grams (equivalent to 26.6 millimoles) of sodium hydroxide was added to the solution and allowed to cool to a temperature of 0 degrees Celsius. Gradually, 6.4 grams (equivalent to 29.2 millimoles) of boc anhydride was

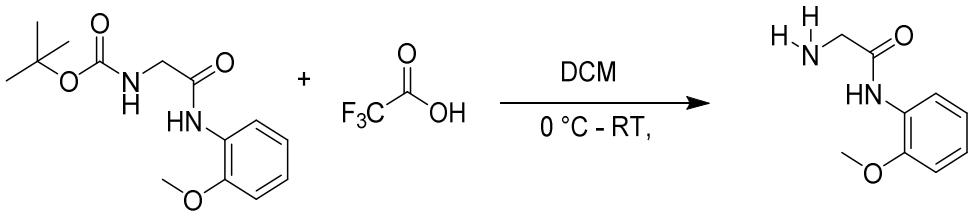
introduced to the solution, allowing it to reach room temperature over the course of one hour while being stirred continuously. Solvent removal was done under vacuum. Water (100 ml) was used to dissolve the residue that remained, which was then washed twice in 50 ml of ethyl acetate. The aqueous solution was then adjusted to a pH of range of 1 – 2 by adding concentrated hydrochloric acid. The aqueous component was subsequently extracted with three 75 ml portions of ethyl acetate and the organic fraction dried using $MgSO_4$, which was concentrated to dryness. The yield obtained was 4.37 g (equivalent to 24.8 millimoles), which corresponds to a 94% yield.



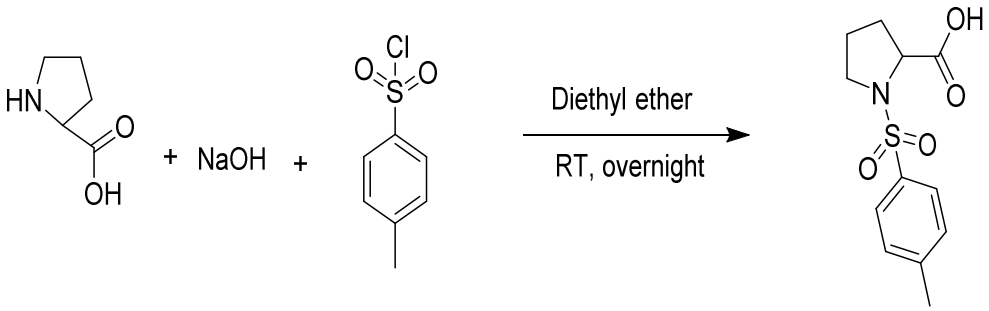
Reaction of boc-protected glycine with amine: A mixture comprising analytical-grade boc-glycine (0.45 g, corresponding to 1.8 millimoles), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.53 g, equivalent to 2.76 millimoles), 1-hydroxybenzotriazole (HOBT) (0.24 g, representing 1.84 millimoles), triethylamine (TEA), and amines (0.51 g, equivalent to 1.84 millimoles) was dissolved in 50 ml of dichloromethane and stirred for 16 hours at 25° C. Throughout this duration, the progress of the reaction was observed with the use of TLC. Upon the successful completion of the reaction, the mixture was then washed with 220 ml of water followed by 110 ml of brine. It was subsequently dried utilizing anhydrous sodium sulfate and the solvent removed by evaporation at low pressure. The crude product obtained, was subsequently purified through column chromatography using silica gel.



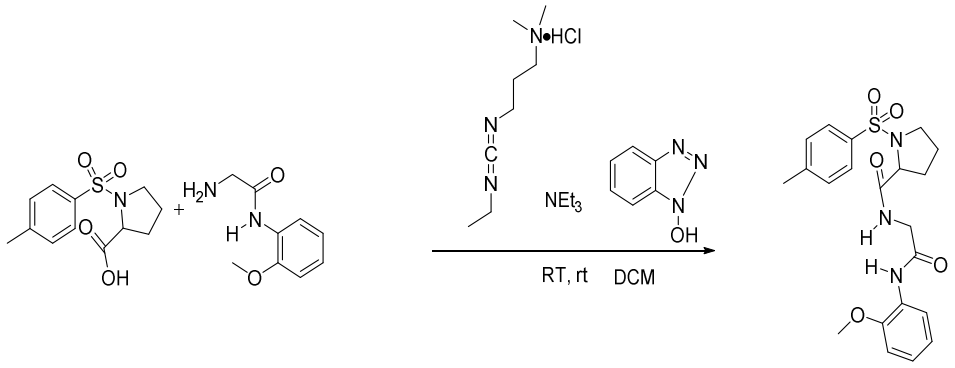
Procedure for the deprotection: Trifluoroacetic acid (TFA) (8 ml) was introduced into a stirred solution of compound 4 (2.80 mmol) in dry dichloromethane (DCM) (10 mL) at 0°C, and the resulting solution was stirred at room temperature for 6 hours. The progress of the reaction was monitored using TLC. Once compound 4 had completely disappeared, the reaction mixture was concentrated under reduced pressure. Subsequently, a 2N NaOH (sodium hydroxide) solution was added to achieve a solution with a pH of 12. This mixture was subjected to extraction using ethyl acetate (three times with 20 ml portions), followed by drying over Na_2SO_4 (sodium sulfate) and filtration. The solvents were then removed under reduced pressure, resulting in the formation of the alkylamino triflates (Ekoh *et al.,* 2022).



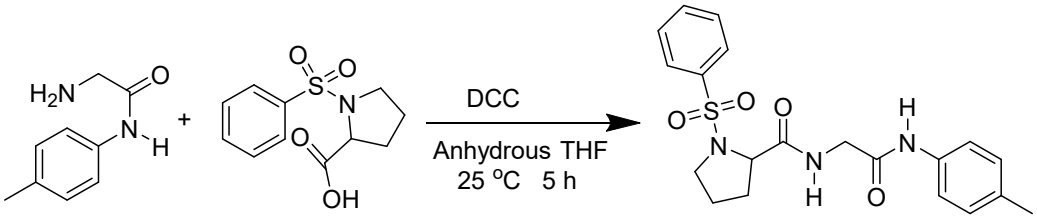
Synthesis of sulphonamide containing carboxylic functional group: A mixture of proline (2.0 g, 17.37 mmol), Et_2O (70 mL), and NaOH (12.0 mL, 1.5 M) was poured in 250 mL dry two-necked pear-shaped flask, which was fitted with a magnetic stirrer, followed by a portion of Tosyl chloride (0.953 g, 6.0 mmol). At 25° Celsius, the mixture was stirred for approximately 20 hours. Afterward, it was extracted with Et_2O. The aqueous layer was adjusted to a pH of approximately 2-3 using 1N HCl (hydrochloric acid) solution and then subjected to extraction with ethyl acetate (AcOEt). The combined organic layers were dried over Na_2SO_4 and subsequently evaporated under reduced pressure. The crude material was finally purified by recrystallization from DCM/hexane, to quantitatively yield pure sulphonamide 2a (Alani *et al.,* 2024).



Coupling of compounds A and B: A mixture comprising analytical-grade boc-glycine (0.45 grams, corresponding to 1.8 millimoles), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride or EDC (0.53 g, equivalent to 2.76 millimoles), 1-hydroxybenzotriazole or HOBT (0.24 g, representing 1.84 millimoles), triethylamine or TEA, and amines (0.51 g, equivalent to 1.84 millimoles) was dissolved in 50 ml of dichloromethane, then stirred at room temperature over a 16-hour duration. Throughout this period, the reaction progress was observed with the use TLC. Upon the successful completion of the reaction, the mixture was then washed with 220 ml of water followed by 110 ml of brine. It was subsequently dried using anhydrous sodium sulfate, following which the solvent was removed by evaporation at low pressure. The crude product obtained, was subsequently purified through column chromatography using silica gel (Attah *et al.,* 2022).



Coupling of compounds C and D: A mixture of 0.618 g, (3.0 millimoles, 1.05 equivalents) of a sulphonamide containing a carboxylic functional group and 0.5 g, (2.85 millimoles, 1.04 equivalents) of 1,3-dicyclohexylcarbodiimide added to a solution containing 0.265 g, (2.85 millimoles, 1.0 equivalent) of deprotected amine in 8 ml of anhydrous THF was prepared at room temperature and stirred at the same temperature for 5 hours. The mixture was then diluted with 25 ml of ethyl acetate and concentrated to yield 5.50 g (100%) of crude product after filtration to eliminate the urea byproduct. The product was subsequently purified using column chromatography (Attah *et al.,* 2022).



Evaluation of Biologic Activity

Animals: Adult male albino rats weighing between 150g to 200g were used for this study. The rats were housed in clean metal cages at the Veterinary Physiology and Pharmacology Animal Unit, and acclimatized for two (2) weeks. During this time, the animals were dewormed with Albendazole suspension and fed *ad libitum* with commercial rat pellets (Grand Cereals Nig. Ltd, Jos, Nigeria), along with clean drinking water available ad libitum.

The animal experiment procedure was approved by the Ethics and Animal Use Committee, Faculty of Veterinary Medicine, University of Nigeria, Nsukka prior to the commencement of various tests (Approval Ref. No.: FVM-UNN-IACUC-2023-11/133).

Induction of experimental diabetes mellitus and evaluation of anti-diabetic activity of the test compound: Thirty five (35) adult overnight-fasted rats weighing 150 -200 gram were used for the study. Their normal fasting blood glucose (FBG) levels were determined with Accu-Chek active glucometer kit. They were assigned into 7 groups (1 – 7), (n = 5). Groups 2 – 7 were given intra-peritoneal injections of 160 mg/kg alloxan monohydrate. Seventy-two (72) hours post alloxan treatment, the FBG was determined and the rats of groups 2 – 7 whose FBG were higher than 120 mg/dl were selected for the next

phase of the study. Group 1 rats served as the normal control, while Group 2 rats were the negative control, and both received only normal saline. Rats in Groups 3, 4, 5, and 6 received the respective test compounds A, B, C and D at 35 mg/kg, respectively. Group 7 served as a positive control and received glibenclamide (2 mg/kg). The FBG levels of all the rats in the various groups were then measured at intervals of 1 hour, 3 hours and 6 hours post-treatment with the drugs.

In vitro anti-oxidant activity of test compounds: i. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) photometric assay – To determine the free radical scavenging activities of the test compounds the DPPH assay method as described by Mensor *et al.*, (2001) was used. Various concentrations of each compound (25, 50, 100, 200 and 400) µg/mL were mixed with 1 ml of 0.5 mM DPPH (in methanol) in a cuvette. Blanks were prepared using 1.0 ml of methanol in 2.0 ml of the novel compounds while the negative control was prepared using 1.0 ml of the 0.5 mM DPPH solution in 2.0 mL of methanol. Ascorbic acid (vitamin C) was used as reference standard (Iwalewa *et al.,* 2008). The absorbance at 517 nm wavelength was read off a spectrophotometer after 30 minutes of incubation in the absence of light at 25 degree Celsius. The experiment was done in triplicate and the antioxidant activities were calculated in percentage as follows: % Antioxidant activity (AA) = 100-[{(ABS sample—ABS blank) ×100}/ABS control].

***In vitro* anti-oxidant activity of test compounds: ii. Ferric reducing antioxidant power:** The ferric reducing antioxidant power (FRAP) assay was conducted following the method outlined by Benzie and Strain (1999). The reagents used included: Reagent 1 = 300 mM Acetate buffer at pH 3.6 (prepared by dissolving 3.1 grams of sodium acetate.$3H_2O$ and adding 16 mL of glacial acetic acid in 1000 mL of buffer solution); Reagent 2 = 10 mM 2,4,6-triphridyl-s-triazine (TPTZ) in 40 mM HCl; and Reagent 3 = 20 mM, FeCl3·6H2O in distilled water. A working solution containing ten parts of Reagent 1, with one part of Reagent 2 and one part of Reagent 3 respectively (ratio of 10:1:1), was freshly prepared a served as the FRAP reagent.

In the FRAP assay procedure, 100 µl sample solution of each test compound prepared at different concentrations of 25, 50, 100, 200, and 400 µg/ml were respectively mixed with 3 ml of the FRAP reagent and allowed to incubate for 4 minutes. The absorbance of each was measured using a spectrophotometer at 593 nm, at a temperature of 37 degrees Celsius. A parallel mixture was prepared with Ascorbic acid in place of the test compound, which was used as the control. The absorbance of each test tube was recorded at both 0 and 4 minutes after the addition of the sample. The FRAP value was calculated as follows: FRAP value = Absorbance at 4 minutes – Absorbance at 0 minutes.

Data Analysis: Data generated were subjected to one-way analysis of variance (ANOVA), followed by Duncan's post hoc test for separating the means of variants. Significance was accepted at $p < 0.05$. The results are expressed as mean ± standard error of mean (SEM).

Results

Products of the synthesis: The products of the synthesis were (Figure 1):
Compound A: N-(3-methoxyphenylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;
Compound B: N-(p-Tolylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;
Compound C: N-(3-ethylphenylamino)-2-oxoethyl-1-(phenylsulfonyl)pyrrolidine-2-carboxamide; and
Compound D: N-(3-ethylphenylamino)-2-oxoethyl-1-(m-tolylsulfonyl)pyrrolidine-2-carboxamide.

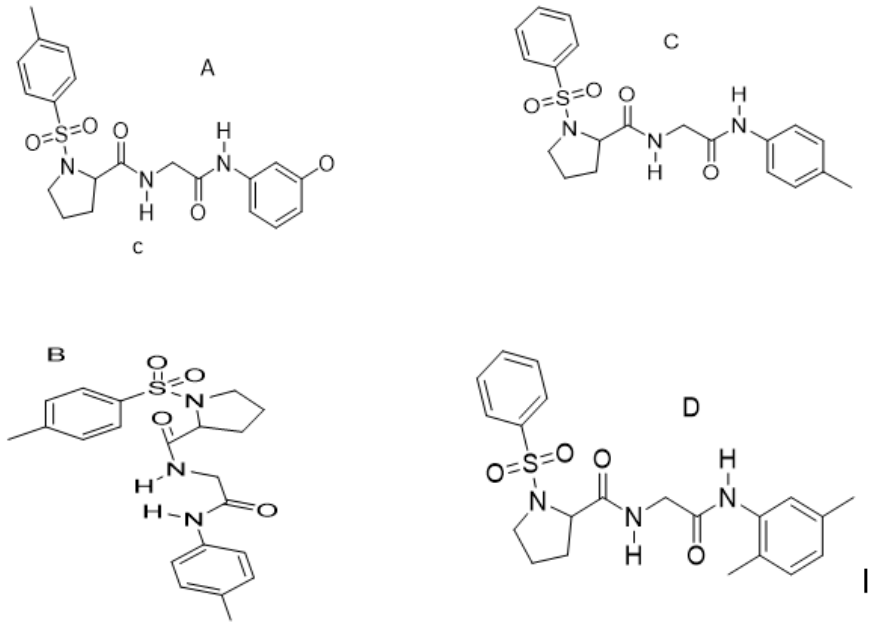


Figure 1: Products of synthesis of Carboxamide derivates: Compound A: N-(3-methoxyphenylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide; Compound B: N-(p-Tolylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide; Compound C: N-(3-ethylphenylamino)-2-oxoethyl-1-(phenylsulfonyl)pyrrolidine-2-carboxamide; and Compound D: N-(3-ethylphenylamino)-2-oxoethyl-1-(m-tolylsulfonyl)pyrrolidine-2-carboxamide.

Spectral Analysis:

Compound A. N-(3-methoxyphenylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide – The amine was 2-methoxyaniline, amino acid was glycine and tosylproline yield (2.8 g, 88 %).

FT-IR (KBr, cm^{-1}): 3314, 3276 (NH), 3193, 3115, 3062(C-H aromatic), 2930, 2853 (C-H aliphatic), 1696, 1643 (2C=O), 1591, 1537, 1490, 1446 (C=C aromatic), 1393, 1348 (2S=O), 1290,1248, 1160, 1117 (S-N), 1096, 1072, 1022, 1007 (C-N).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.52 (s, 1H), 7.90 – 7.83 (m, 2H), 7.55 (dt, *J* = 33.1, 7.4 Hz, 3H), 7.39 (d, *J* = 8.8 Hz, 2H), 7.25 (d, *J* = 4.5 Hz, 2H), 4.69 (dd, *J* = 7.1, 3.5 Hz, 1H), 4.46 – 4.39 (m, 1H), 3.75 (t, *J* = 6.4 Hz, 2H), 3.55 – 3.44 (m, 1H), 3.37 – 3.26 (m, 1H), 2.25 (dd, *J* = 9.4, 5.6 Hz, 1H), 2.07 (dtt, *J* = 24.3, 12.6, 6.4 Hz, 5H), 2.00 – 1.94 (m, 1H), 1.83 (s, 1H).

^{13}C NMR (400 MHz, Chloroform-*d*) δ 171.33 (C=O), 169.61(C=O), 137.53, 137.34, 136.88, 135.34, 132.56, 131.30, 129.03, 127.19, 121.02, 116.01 (ten aromatic carbons), 61.47, 60.23, 48.46, 47.41, 30.19, 27.75, 25.17, (seven aliphatic carbons).

ESI-HRMS (m/z): found 431.1367 (M+H), calcd 431.120.

Compound B. N-(p-Tolylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide: The amine was 4-methylaniline, amino acid was glycine and tosylproline, yield (1.50 g, 99%).

FTIR (KBr, cm^{-1}): 3314, 3276 (NH), 3193, 3115, 3062(C-H aromatic), 2930, 2854 (C-H aliphatic), 1696, 1643 (2C=O), 1591,

1537,1490, 1447 (C=C aromatic), 1393, 1348 (2S=O), 1290,1248, 1160, 1117 (S-N), 1096, 1072, 1022, 1007 (C-N).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.21 (s, 1H), 7.91 – 7.81 (m, 2H), 7.61 – 7.48 (m, 1H), 7.01 (d, *J* = 7.6 Hz, 2H), 6.81 (d, *J* = 7.3 Hz, 2H), 4.80 (d, *J* = 7.7 Hz, 2H), 3.55 (t, *J* = 9.1 Hz, 1H), 3.49 – 3.34 (m, 2H), 2.28 (s, 3H), 2.21 (s, 3H), 2.17 (s, 2H), 2.12 (s, 3H), 1.81 (d, *J* = 7.4 Hz, 4H).

^{13}C NMR (400 MHz, Chloroform-*d*) δ 171.89 (C=O), 169.35 (C=O), 136.68, 136.53, 133.34, 130.55, 129.51, 127.84, 125.86, 125.42 (eight aromatic carbons), 61.33, 60.62, 59.76, 48.79, 31.06, 27.02, 26.03 (seven aliphatic carbons). ESI-HRMS (m/z): found 415.1317 calcd 415.2001.

Compound C. N-(3-ethylphenylamino)-2-oxoethyl-1-(phenylsulfonyl)pyrrolidine-2-carboxamide: The amine was 4-chloroaniline, amino acid was glycine and (phenylsulphonyl)proline, yield (1.91 g, 89.1 %) white solid.

FTIR (KBr, cm^{-1}): 3407 (NH), 3061 (C-H aromatic), 2952, 2878(C-H aliphatic), 1685,1657 (2C=O), 1528,1485, 1436, 1460 (C=C aromatic), 1344, 1389 (2S=O), 1159, 1115 (S-N), 1096, 1072, 1047 (C-N, C-O).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.81 (s, 1H), 8.27 (d, *J* = 9.1 Hz, 1H), 7.89 (d, *J* = 7.4 Hz, 2H), 7.59 – 7.46 (m, 3H), 6.98 (d, *J* = 9.0 Hz, 1H), 6.89 (t, *J* = 7.5 Hz, 1H), 6.82 (d, *J* = 7.5 Hz, 1H), 4.75 – 4.61 (m, 2H), 3.81 (s, 3H), 3.62 (t, *J* = 7.0 Hz, 1H), 3.49 – 3.35 (m, 2H), 2.44 – 2.35 (m, 1H), 2.22 – 1.94 (m, 8H).

^{13}C NMR (400 MHz, Chloroform-*d*) δ 171.80 (C=O), 169.35 (C=O), 148.28, 138.87, 132.90,129.12, 127.72, 127.53, 123.92, 120.96 (eight aromatic carbons), 61.15, 58.99, 55.72, 48.52, 47.38, 30.74 (six aliphatic carbons). ESI-HRMS (m/z): found 401.1881 (M+H), calcd 401.1701

Compound D. N-(3-ethylphenylamino)-2-oxoethyl-1-(m-tolylsulfonyl)pyrrolidine-2-carboxamide: The amine was 2,5-dimethylaniline, amino acid was glycine and (phenylsulphonyl)proline, yield (2.10 g, 98 %).

FTIR (KBr, cm^{-1}): 3314, 3276 (NH), 3193, 3115, 3062(C-H aromatic), 2930, 2854 (C-H aliphatic), 1696,1643 (2C=O), 1591, 1537,1490, 1447 (C=C aromatic), 1393, 1348 (2S=O), 1290,1248, 1160, 1117 (S-N), 1096, 1072, 1022, 1007 (C-N).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.21 (s, 1H), 7.91 – 7.81 (m, 2H), 7.61 – 7.48 (m, 1H), 7.01 (d, *J* = 7.6 Hz, 2H), 6.81 (d, *J* = 7.3 Hz, 2H), 4.80 (d, *J* = 7.7 Hz, 2H), 3.55 (t, *J* = 9.1 Hz, 1H), 3.49 – 3.34 (m, 2H), 2.28 (s, 3H), 2.21 (s, 3H), 2.17 (s, 2H), 2.12 (s, 3H), 1.81 (d, *J* = 7.4 Hz, 4H).

^{13}C NMR (400 MHz, Chloroform-*d*) δ 171.89 (C=O), 169.35 (C=O), 136.68, 136.53, 133.34, 130.55, 129.51, 127.84, 125.86, 125.42, 123.49, 122.59 (ten aromatic carbons), 61.33, 60.62, 59.76, 48.79, 31.06, 27.02, 25.53 (seven aliphatic carbons).

ESI-HRMS (m/z): found 415.2870 (M+H), calcd 415.1901

The effects of novel carboxamides derivatives on the FBG level of alloxan-induced hyperglycemia in rats: Table 1 shows the results of the evaluation of the anti-hyperglycemic effects of the test compounds. At zero hour, the FBG levels of the alloxan-treated groups (2 – 7) were significantly ($p < 0.05$) increased when compared with their pre-induction FBG levels. The test compounds (A – D) and glibenclamide produced time-dependent decrease in the FBG of the treated rat groups while the FBG of the negative control group increased in a time-dependent manner. At zero and 1 hr post-treatment, no significant ($p>0.05$) difference in the FBG of the test compounds and glibenclamide treated groups were observed when compared with the FBG of group 2 (negative control). However, at 3 and 6 hours post-treatment, the FBG of the test compounds and glibenclamide treated groups were significantly ($p < 0.05$) lower in comparison with the FBG of the

negative control group, but there was no significant ($p > 0.05$) difference when compared with the normal control group.

***In vitro* anti-oxidant effects of the novel carboxamide derivatives:** The results of the in vitro anti-oxidant assay are presented in Tables 2 and 3. The test compounds did not produce any antioxidant effects.

Table 1. The fasting blood glucose levels of alloxan-induced hyperglycemic rats given varied novel carboxamides derivatives.

Groups	Fasting blood glucose levels (mg/dl), with SEM in brackets				
	Pre-induction	Zero hour	1 hour	3 hours	6 hours
Group 1 (Normal Control)	64.3 [a] (3.38)	63.7 [a] (4.26)	66.30 [a] (3.84)	62.30 [a] (5.61)	60.00 [a] (4.36)
Group 2 (Negative control)	67.33 [a] (2.33)	290.67 [b] (29.84)	384.33 [b] (87.10)	428.33 [b] (89.12)	393.00 [b] (103.51)
Group 3 (Compound A, 35 mg/kg)	69.0 [a] (2.08)	253.00 [b] (69.29)	202.70 [ab] (95.49)	193.00 [a] (94.32)	165.70 [a] (70.13)
Group 4 (Compound B, 35 mg/kg)	64.7 [a] (2.73)	235.30 [b] (81.67)	199.70 [ab] (80.54)	164.70 [a] (67.31)	150.00 [a] (47.69)
Group 5 (Compound C, 35 mg/kg)	66.7 [a] (1.76)	255.30 [b] (80.63)	280.30 [ab] (73.45)	228.30 [a] (51.41)	201.30 [a] (50.21)
Group 6 (Compound D, 35 mg/kg)	70.0 [a] (2.06)	281.0 [b] (78.69)	212.00 [ab] (76.37)	169.00 [a] (44.52)	212.00 [a] (43.66)
Group 7 (Glibenclamide, 2 mg/kg)	65.7 [a] (1.76)	326.3 [b] (40.55)	297.30 [ab] (53.50)	150.33 [a] (35.80)	95.33 [a] (4.81)

[a, b, c] Different superscripts show significant difference along the column.

Compound A = N-(3-methoxyphenylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;

Compound B = N-(p-Tolylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;

Compound C = N-(3-ethylphenylamino)-2-oxoethyl-1-(phenylsulfonyl)pyrrolidine-2-carboxamide;

Compound D = N-(3-ethylphenylamino)-2-oxoethyl-1-(m-tolylsulfonyl)pyrrolidine-2-carboxamide.

Table 2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the novel carboxamide derivatives, compared to ascorbic acid.

Concentration (µg/mL)	% inhibition of DPPH radical				
	Compound A	Compound B	Compound C	Compound D	Ascorbic acid
25	NA	NA	NA	NA	95.51 ± 0.10
50	NA	NA	NA	NA	96.45 ± 0.08
100	NA	NA	NA	NA	96.49 ± 0.05
200	NA	NA	NA	NA	96.69 ± 0.11
400	NA	NA	NA	NA	96.78 ± 0.53
800	NA	NA	NA	NA	NT

NA = no activity; NT = not tested.

Compound A = N-(3-methoxyphenylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;

Compound B = N-(p-Tolylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;

Compound C = N-(3-ethylphenylamino)-2-oxoethyl-1-(phenylsulfonyl)pyrrolidine-2-carboxamide;

Compound D = N-(3-ethylphenylamino)-2-oxoethyl-1-(m-tolylsulfonyl)pyrrolidine-2-carboxamide

Table 3. Ferric reducing antioxidant power (FRAP) of the novel carboxamides derivatives, compared to ascorbic acid.

Concentration (µg/mL)	FRAP Value (µM)				
	Compound A	Compound B	Compound C	Compound D	Ascorbic acid
25	NA	NA	NA	NA	0.16 ± 0.00
50	NA	NA	NA	NA	0.37 ± 0.00
100	NA	NA	NA	NA	0.78 ± 0.01
200	NA	NA	NA	NA	1.38 ± 0.01
400	NA	NA	NA	NA	1.67 ± 0.00
800	NA	NA	NA	NA	NT

NA = no activity; NT = not tested;

Compound A = N-(3-methoxyphenylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;

Compound B = N-(p-Tolylamino)-2-oxoethyl-1-tosylpyrrolidine-2-carboxamide;

Compound C = N-(3-ethylphenylamino)-2-oxoethyl-1-(phenylsulfonyl)pyrrolidine-2-carboxamide;

Compound D = N-(3-ethylphenylamino)-2-oxoethyl-1-(m-tolylsulfonyl)pyrrolidine-2-carboxamide.

Discussion

Syntheses and Spectral analyses: To synthesize Compounds A, B, C and D, we adopted the use of classical peptide coupling reagent, dicyclohexylcarbodiimide (DCC) in the amidation of compounds with substituted sulphonamides derived from L-glycine. Also, the activation of the carboxylic acid group glycine was enhanced with DCC. The use of DCC was recommended over 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) as a coupling reagent because DCC gives higher yield, faster and form *O*-acylisourea as by-product which can easily be remove by filtration although DCC activation opens the possibility for racemization of the activated amino acid. Racemization can be circumvented with "racemization suppressing" additives such as the triazoles 1-hydroxy-benzotriazole (HOBT) and 1-hydroxy-7-aza-benzotriazole (HcOAt) (Rathod *et al.,* 2023). These reagents attack the *O*-acylisourea intermediate to form an active ester, which subsequently reacts with the peptide to form the desired peptide bond. Ethyl cyanohydroxyiminoacetate (oxyma), an additive for carbodiimide coupling acts as an alternative to HOAt. In the present study, we synthesized and characterized molecules containing sulphonamide, carboxamide and dipeptides moieties. The reaction of tosylchloride with L-proline afforded tosylproline**.** The reaction of L-glycine with boc-anhydride, in the presence of dioxane and water (2:1) as solvent afforded boc-protected glycine. Protection of glycine using tert-butoxycarbonyl (Boc) group was an important step to prevent undesired reactions during subsequent steps (Karmakar *et al.,* 2018). Thereafter, boc-protected glycine reacted with substituted amines using DCC as coupling reagent in tetrahydrofuran to afford the carbamate derivatives glycine**.** Alkylamino triflates were synthesized through deprotection of the boc-protected glycine with trifluoroacetic acid (TFA) in dichloromethane (DCM). The deprotection of the Boc group using trifluoroacetic acid (TFA) was performed at 0°C to prevent side reactions and resulted in deprotected amides which were required for subsequent coupling steps (López and Salazar, 2013). The amidation of tosylproline with unprotected amides using peptide coupling reagent (DCC) in tetrahydrofuran provided the desired products A, B, C and D.

The spectral analyses of the Compounds A – D confirmed their proposed structures. The FT-IR spectra provided insight into the functional groups present in each compound, indicating the presence of NH, C-H, C=O, C=C, S=O, S-N, and C-N functional groups, consistent with the expected structures. The ^{1}H and ^{13}C NMR spectra provided detailed insights into the hydrogen and carbon environments, confirming the presence of aromatic, aliphatic, and heterocyclic components. The ESI-HRMS data corroborated the molecular weights for each compound, providing strong evidence for the successful synthesis of the target compounds.

In the infrared spectra of the dipeptides, the observed peaks between 3314 cm^{-1} and 3276 cm^{-1} indicated the presence of NH stretching vibrations. Such NH stretching vibrations have been reported to be consistent with secondary amides (Silverstein, *et al.,* 1991). The C=O band appeared between 1696 cm^{-1} and 1643 cm^{-1}. These bands indicate successful coupling of the tosylproline with deboc amines.

Furthermore, in the proton NMR spectra of the derivatives (A - D), the diagnostic peaks at 8.60 – 8.24 ppm were assigned to N-H, 7.78 – 7.02 ppm assigned to aromatic protons, 3.82 – 3.02 assigned to aliphatic carbon and 0.92 – 0.79 ppm assigned to CH_3 were all supportive of the formation of the target product. The carbon-13 NMR showed all the peaks expected of successful coupled products. The 2C=O peaks appeared between δ 171.33 and 169.35. All the aromatic and aliphatic peaks were accounted for in the carbon-13 NMR.

The high-resolution mass spectrometer (HRMS) peaks of the derivatives appeared either as molecular ions (M^+), ($M+H^+$), ($M-H^-$) or (M+Na). The results corresponded to three decimals with the calculated values. The high yields and purity of the products, as evidenced by these spectral analyses, demonstrate the efficiency and reliability of the synthetic methodologies employed. The differences in the spectral data primarily arose from the variations in the aromatic substituents attached to the core structure, highlighting the influence of the different substituents on the electronic environment of the molecules.

Biologic activity: The elevated FBG level in the alloxan-treated group is a confirmation of induction of experimental hyperglycemia. Alloxan causes diabetes through its ability to destroy the insulin-producing beta cells of the pancreas. Alloxan has been reported to exert selective toxicity towards pancreatic beta cells, inducing beta cell necrosis through the generation of reactive oxygen species. This process leads to a significant rise in cytosolic calcium levels, triggering the swift destruction of beta cells. (Belhekar *et al*., 2013; Ighodaro *et al*., 2017). The depletion of beta cells leads to reduced insulin secretion and attendant hyperglycemia (Rutter *et al*., 2015).

At 3 and 6 hours post treatment, rats in the carboxamide-derivatives and glibenclamide treated groups showed significantly lower FBG in comparison with the negative control. These significant reductions in the mean FBG of the treated groups indicate that the novel carboxamide derivatives possess anti-hyperglycemic property. The anti-hyperglycemic property of the carboxamide derivatives may be attributed to the tosyl-protected, amide functional groups present in their structure. Earlier reports have shown the anti-diabetic properties of carboxamide derivatives (1-aryl-N-tosyl-1H-tetrazole-5-carboxamide) possessing such structural arrangements in rats (Selvarasu *et al*., 2021).

The anti-hyperglycemic effects of the novel compounds could also be attributed to their structural similarity with glibenclamide, a known hypoglycemic agent. The compounds possess the sulfonyl group $(S = O)_2$, amide groups (-CONH-), carbonic groups and aromatic substituents which are also found in glibenclamide. These similarities in their structure suggest that they might have similar mechanism of action. The glibenclamide, is a sulfonylurea drug whose action inhibit the ATP-sensitive K+ channels, leading to depolarization of the cells and insulin secretion (Luzi and Pozza, 1997).

At six hours post-treatment, the FBG of compound B treated-group had the lowest mean fasting blood glucose level, when compared with other groups treated with the novel carboxamide derivatives. Thus, we could infer that compound B had better anti-hyperglycemic activity than other compounds used, though a milder effect when compared with glibenclamide. This better anti-hyperglycemic effect of compound B could be linked to two reasons: first, the substitution on the aromatic amide at both ends (the tosyl end and the terminal end) of the pyrrolidine carboxamide with a methyl group each. Methyl groups or a similar sp3 alkyl (R) group can act as an σ-donor, considering the fact that an sp3 carbon is less reactive than an sp2 carbon. This means it can share some of its electrons with a carbon ring, causing the ring to have more electrons around it. This extra electron presence gives the ring a positive inductive effect (referred to as a +I effect) (Bazzini and Wermuth, 2008), thereby activating the ring and making it more reactive. Such effect was also reported by Gouda *et al*, (2018), on improved anti-inflammatory properties of a pyrrolizine-5-carboxamide analogue with a methyl group substitution at the phenyl ring of the compound. Secondly, according to the Hammett equation, electron donating substituents in the *para*-position of a benzene

ring increases the activity of the compound, making it more reactive. Pu *et al*., (2010) reported significant improvement in the photochromic features of diarylethenes with *ortho*- and *para*-methoxy substituents than its *meta*- substituted analogue. Bazzini and Wermuth, (2008) reporting the work of Harms and Nauta (1960), stated that substitution with a methyl group on aromatic ring of spasmolytic diphenhydramine caused a 3.7 fold increase in the anti-histaminic activity of the compound compared to its non-substituted analogue. Therefore, having two *para*-substituted methyl groups on different aromatic rings in compound B may have been the reason for its better performance as an anti-diabetic drug.

It is worthy to note that none of the test compounds reversed blood glucose levels to below the upper threshold for normal blood glucose level of 126mg/dl (Chaudhury *et al*., 2017) as observed for glibenclamide treated group. Therefore, Compounds A, B and D had moderate anti-hyperglycemic activities compared to glibenclamide, though no significant difference ($p > 0.05$) existed between them.

Since the cytotoxic effects of alloxan on the beta cells of the pancreas is majorly via the release of free oxygen radical species (Ighodaro *et al*., 2017), it was also necessary to investigate the effects of the test compounds on reactive oxygen species. However, none of the new carboxamides showed any in vitro anti-oxidant properties in the two models of antioxidant tests used in this study. It could therefore be inferred that the anti-hyperglycemic properties shown by the compounds was not due to any antioxidant properties. This finding however contrasts with that of several authors on the anti-oxidative potentials of carboxamides including N-(2'-nitrophenyl)pyrrolidine-2-carboxamides and N-(2'-nitrophenyl)piperidine-2-carboxamides (Odusami *et al*., 2020), as well as that of benzimidazole substituted coumarin-3-carboxamides (Patagar *et al*., 2023) using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation among others. The lack of antioxidant activity by the compounds A – D may be due to the insolubility of the novel carboxamide derivatives in alcohol and other polar solvents which was the solvent used for the DPPH assay.

Conclusion: In this experimental study, four N-substituted pyrrolidine-2-carboxamide analogues that differed in the sulfonyl groups attached to the carbon 1 of their pyrrolidine ring complexes were synthesized. The synthesized carboxamide derivatives exhibited potent anti-hyperglycemic property, but produced no anti-oxidant effect. Among the synthesized carboxamide derivatives, Compound B with methyl groups para-substituted on both the tosyl and pyrrolidine complexes of the compound had the best anti-hyperglycemic effect six hours post administration of single dose. This compound may therefore be considered a potential candidate for the development of a new class of anti-diabetic drugs.

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Conflict of interest:

Authors declare no competing interests

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