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Original Research Article

Synergistic Antidiabetic Effects of *Vernonia amygdalina* Leaves and Glibenclamide on Alloxan-Induced Wistar Rats

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Abstract

Purpose: One chronic illness that affects millions of people worldwide is diabetes mellitus. If left untreated, it can hasten the body's degenerative processes. *Vernonia amygdalina* (*V. amygdalina*) is used as a vegetable and herb, mostly found in tropical Africa. *V. amygdalina* is one of the many plants with anti-diabetic properties. The study's objective was to understand the anti-diabetic activity of *V. amygdalina* leave's methanol extract and its synergistic effect with glibenclamide on alloxan-induced rats.
Methods: Injection of alloxan monohydrate (160 mg/kg) intraperitoneally (i.p.) led to the induction of diabetes in the Wistar rats. The rats were grouped into five: normal (group 1) and diabetic (group 2) controls, glibenclamide at 2 mg/kg (group 3), 200 and 2 mg/kg of *V. amygdalina* and glibenclamide (group 4), respectively and methanol extract of *V. amygdalina's* 200 mg/kg (group 5). Each treatment was orally. The consequence of *V. amygdalina* on blood glucose, lipid profile, oxidative stress markers, haematological, and renal function indices was determined by standard methods.
Results: At $p < 0.05$, *V. amygdalina* extract significantly reduced the fasting blood glucose concentration in alloxan-induced rats relative to untreated/diabetic rats. Group 4 showed relatively lower concentrations of TCL, LDL, VLDL, and TAG compared to group 5. Higher levels of reduced glutathione and catalase was observed in group 4 relative to group 1 and group 5. Equally, a comparatively higher level of malondialdehyde were observed in group 5 than in group 4. In the haematological profile, the extract-treated groups exhibited relative elevation in RBC count, PCV, and Hb concentration compared to group 2.
Conclusion: The most promising treatment group is group 4 rats treated with extract and glibenclamide. The results demonstrated that sulphonylurea medications like glibenclamide can increase the potential anti-diabetic impact of *V. amygdalina* leaves.

Keywords: *Vernonia amygdalina*, anti-diabetic, anti-oxidant, oxidative stress, glibenclamide

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INTRODUCTION

Hyperglycemia, or high blood glucose, and insufficient insulin production or action by the pancreas within the body are hallmarks of diabetes mellitus, a set of metabolic illnesses.[1] Increased blood glucose levels over time are linked to both macro and microvascular problems that can result in heart disease, stroke, blindness, and renal failure.[2] Other factors that contribute significantly to the pathophysiology of diabetes, in addition to hyperglycemia, include oxidative stress and hyperlipidaemia, which increase the risk of complications.[3] The tropical regions of Africa, including Nigeria are home to *Vernonia amygdalina*. Despite being most commonly used for food, its therapeutic qualities have long been recognized.[4] The aqueous extract is taken to cure fever and soothe piles and mild stomach ailments. The fresh leaves are squeezed and applied to skin rashes and ringworms.[5] It is not recommended to consume the leaf juice while pregnant because local women in Guinea Bissau use it to contract the uterus after giving birth.[6] The entire plant has pharmaceutical value. In phytomedicine, the roots and leaves are used to cure a variety of illnesses, including fever, hiccups, kidney illness, and stomach pain.[7] There have also been reports of antitumourigenic[8] and anthelmintic and antimalarial[9] qualities. Hence, this study seeks to investigate and establish a scientific report on the anti-diabetic effect of *Vernonia amygdalina* and its combination with a known diabetic drug (glibenclamide).

MATERIAL AND METHOD

Plant Material

The leaves of *Vernonia amygdalina* were collected from the parent plant in its native environment in the Amogbo Community in Nsukka, Nsukka L.G.A. of Enugu State, Nigeria, and verified at the University of Nigeria Nsukka's Herbarium.

Drug

A glibenclamide dose of 2.5 mg/kg b.w was used as a standard (reference) drug (Nigerian German Chemical Plc.).

Animal Management

In this investigation, 200–250 g adult Wistar rats were employed. These rats were acquired from the University of Nigeria, Nsukka's Department of Zoology. Before the trial started, the animals were housed for 14 days, with free access to water and food.

Preparation of Plant Extracts

Fresh leaves of *V. amygdalina* were dried at room temperature, crushed, and macerated in methanol for 72 hours. The mixture was filtered and evaporation of the crude extract was achieved at room temperature via a rotary evaporator.

Experimental Design

A total of thirty (30) animals were grouped into five (5) made up of six (6) rats per group. Treatment was carried out orally using an intubation tube as follows

Group 1: Normal/negative control rats administered distilled water.
Group 2: Diabetic untreated rats.
Group 3: Diabetic rats given 2 mg/kg b.w dose of glibenclamide.
Group 4: Diabetic rats given 2 and 200 mg/kg b.w of glibenclamide and methanol extract of *V. amygdalina* leaves, respectively.
Group 5: Diabetic rats given 200 mg/kg b.w. of methanol extract of *V. amygdalina* leaves

The animals were bled via ocular puncture 21 days after treatment, and blood samples were collected and placed in sterile test tubes for the measurement of biochemical markers.

Induction of Diabetes

Prior to induction, for 24 hours, the animals were fasted. Intraperitonally injection of Alloxan monohydrate (Sigma, USA) (160 mg/kg b.w) after dissolution in cold normal saline. After seventy-two (72) hours, glucometer (Accu-chek) was used to assess the blood glucose concentrations. Antidiabetic and antioxidant properties of *V. amygdalina*'s methanol leaves extract was analyzed in rats whose blood glucose levels ≥ 200 mg/dl.

Determination of Fasting Blood Glucose Concentration

Glucometer (Accu-check Active by Roche Diagnostics Turkey, A.S) was used to assess the Fasting blood sugar.[10]

Determination of Lipid Profile

Cholesterol level was determined spectrophotometrically with QCA assay kits.[11] Estimation of HDL and TAG concentrations were in the QCA commercial kit used. [12] LDL (in mg/dl) and VLDL were estimated through calculation.[13]

Determination of Oxidative Stress Markers

MDA concentration was based on the method.[14] The concentration of reduced glutathione was estimated according to the method.[15] Catalase was assayed by the method.[16]

Determination of Haematological Indices

The determinations of RBC count, PCV, and Hb concentration were carried out according to the methods.[17]

Determination of Renal Function Indices

Urea in serum estimation was based on the Urease Berthelot method.[18] Creatinine determination was in accordance to Jaffe's alkaline picrate reaction via Direct Endpoint.[19]

Statistical Analysis

Data were shown as mean ± SEM. The data were analyzed using two-way ANOVA. Posthoc analysis employed the Duncan multiple test range. $P < 0.05$, were deemed significantly different.

RESULT AND DISCUSSION

Effect of LMEVA on Fasting Blood Glucose Concentration in Alloxan-Induced Rats

During the pre-induction phase, for both the control groups and the test groups, no significant difference ($p > 0.05$) were seen in the fasting blood glucose concentrations. At 1 Hour, the glucose concentrations in all the groups showed significant ($p < 0.05$) elevation in comparison to the group 1; however, at $p > 0.05$ no significant variations were observed in the glucose levels of rats in groups 2 - 5. At 3 hours, at $p < 0.05$, the glucose concentration of group 5 rats was found to be non-significant in comparison to group 2. Similar trend was seen in groups 3 and 4. Similar observation was made for the 6th hour. At 24 Hours, they were a significant ($p < 0.05$) reduction in the glucose concentrations of group 4 relative to the glucose levels of groups 2 and 5 rats. At $p < 0.05$, group 5 rats exhibited significant higher glucose concentration relative to the group 1 but significantly lower relative to group 2. On Days 7, 14, and 21, the glucose concentration of groups 3 and 4 were observed to be significantly lesser at $p < 0.05$ relative to group 5 as shown in Table 1.

Effect of LMEVA on Lipid Profile Concentrations of Alloxan-Induced Rats

Alloxan-induced rat's lipid profile result on days 7, 14, and 21 are shown in Tables 2a and 2b. Group 4 showed relatively lower concentrations of TCL, LDL, VLDL, and TAG relative to group 5. Time-dependent non-significant ($p > 0.05$) increases were observed in TCL, LDL, VLDL, and TAG concentrations of groups 4 and 5 rats compared to group 1.

Effect of LMEVA and Glibenclamide on Pro- and Anti-Oxidant Status in Alloxan-Induced Diabetic Wistar Rats

Results obtained in the pro- and anti-oxidant analyses showed clearly that there were relatively higher concentrations of reduced GSH and catalase in group 4 diabetic rats that were administered with both the extract and the glibenclamide relative to the levels observed in group 1 and group 5. Conversely, a relatively higher level of MDA was observed in group 5 rats than the level observed in group 4 rats. However, the level of the MDA across the treatment groups were not significantly ($p > 0.05$) higher relative to the normal control group (Table 3).

Effect of LMEVA on Haematological Indices of Alloxan-Induced Diabetic Wistar Rats

In the haematological studies, the treatment groups (groups 4 and 5) exhibited relative elevation in RBC count, PCV, and Hb concentration compared to group 2 which showed relative reduction as shown in Table 4. It is very pertinent to state that the most promising treatment group is group 4 rats that were treated with both the extract and glibenclamide after the induction of diabetes. Group 4 rats had similar values to that of group 1.

Effect of LMEVA on Renal Function Indices of Alloxan-Induced Diabetic Wistar Rats

Alloxan-induced diabetic rat's renal function indices for rats treated with methanol extract of *V. amygdalina* and glibenclamide are shown in Table 5. At $p < 0.05$, urea and creatinine concentrations were observed to be significantly lower in the treatment groups (groups 4 and 5) relative to group 2.

Synergistic and duration-dependent variations were observed in the glucose concentrations of both the treated and control groups. Relative time-dependent reduction was observed in the glucose concentrations of the rats in all the groups with special observation in group 4. The lowest glucose concentrations were witnessed in group 4 rats administered glibenclamide and the extract across all the durations except rats in group 1. With careful observation of the glucose results shown, group 4 exhibited the most promising outcome based on the

primary aim of the research which is anchored on the proper management of diabetes mellitus. The hypoglycaemic act of the methanol extract on blood glucose in diabetic rats is comparable to that of the standard drug, glibenclamide which is an effective hypoglycaemic agent; hence suggesting that the methanol extract of *V. amygdalina* leaves contain active components or principles with possible potent hypoglycaemic property. The extract may have demonstrated this potential hypoglycemic effect by inhibiting endogenous glucose synthesis, increasing insulin secretion, and peripheral glucose utilization, or by inhibiting intestinal glucose absorption. The extract may have demonstrated this potential hypoglycemic effect by inhibiting endogenous glucose synthesis, increasing insulin secretion, and peripheral glucose utilization, or by inhibiting intestinal glucose absorption as reported.[20,21]

Relative elevations in lipid profile indices such as the concentrations of TCL, LDL VLDL, and TAG may be due to the deployment of lipid from adipose tissues for energy production based on the fact that these groups had lost appetite which is among the potential symptoms of diabetes mellitus complications. Conversely, a reverse trend of result was witnessed in the outcome of HDL as shown in Table 2a. The findings indicate that the extract's single dose decreased the hepatic synthesis of triacylglycerol and promoted cholesterol redistribution among the lipoprotein molecules. This discovery is found to be consistent with the research findings[22] where the extract of *V. amygdalina* significantly reduced diabetic rat's hepatic triacylglycerol and LDL concentrations. HDL, being known for its reverse transport mechanism, and has anti-atherogenic effect in part by opposing LDL oxidation. The reverse cholesterol transport route is necessitated by HDL through the induction of efflux of accumulated cellular cholesterol via the prevention of the generation of LDL that is oxidatively potentiated.[23] On the strength of these findings, the extract with the synergistic effect of glibenclamide might have played an anti-atherogenic function via lipids oxidation inhibition due to its anti-lipoperoxidation effect including HDL's cholesterol elevation.

The German physician Helmut Sies originally used the term "oxidative stress" in 1985 to denote a disproportionate in the levels of oxidants and antioxidant defenses that is deleterious to biological systems.[24] Numerous illnesses, including diabetes and its associated pathologies have been linked to oxidative stress. The biochemical relationship between oxidative stress indices and lipid profile indices (TCL, LDL, VLDL, and TAG) was ascertained in this research, where reduced GSH which is a potential marker of the antioxidant defense system, showed a negative correlation with the concentrations of diabetic rat's TCL, LDL, VLDL, and TAG, but a positive correlation with the extract-treated rats. This finding suggests that *V. amygdalina* may trigger or elicit some health benefits through the regulation of biochemical processes, such as the atherogenic lipid profile.[22] It is documented that the pancreatic beta cell's destruction by alloxan occurs selectively manner and this is mediated through the generation of free radicals as an outcome of oxidative stress.[25] Because of the aforementioned discovery, studies have focused on protecting pancreatic beta cells from chemically induced damage by using antioxidants. Glutathione reduces alloxan to dialuric acid, which causes toxicity. The redox recycling mechanism produces ROS that harm beta cells.[25]

MDA, is a degradative product of the cell membrane's PUPA peroxidation which is an index of lipid peroxidation. One of the underlying causes of diabetes mellitus has been linked to oxidative stress and lipid peroxidation, which are indicated by a relatively high serum quantity of MDA.[26] Table 3 shows increased levels of reduced GSH and catalase in group 4 rats compared to the levels seen in group 1 and group 5 . On the other hand, a relatively higher level of MDA was observed in group 5 rats than the level observed in group 4 rats. This could be ascribed to the synergistic attributes of glibenclamide and the extract with active components and bioactive potentials anchored on some antioxidant phytochemicals. The methanol leaves extract of *V. amygdalina* contains numerous medicinal active phytochemicals particularly phenols as demonstrated.[27] Several mechanisms are linked to the antioxidant activities of phenolic compounds.[28,29] Our study is consistent with the discoveries of [30] who showed relative elevations and reduction in the antioxidants (GSH and catalase) levels and MDA, respectively in the treatment groups relative to the normal control group.

Group 4 exhibited relative elevation in RBC count, PCV, and Hb concentration compared to that of the positive control. The promising result (Table 4) obtained in group 4 could be credited to the synergistic effect of both the extract and the glibenclamide.

Table 1: Effect of LMEVA on Fasting Blood Glucose Concentration in Alloxan-Induced Rats

Grp	Full Blood Glucose Concentration (mg/dl) at Different Durations							
	Pre-Induction	1 Hour	3 Hours	6 Hours	24 Hours	Day 7	Day 14	Day 21
1	63.33±2.52[a]	71.00±1.00[a]	64.00±2.65[a]	63.00±1.00[ab]	72.67±10.97[d]	61.33±1.53[a]	60.33±3.21[a]	58.33±3.51[a]
2	63.00±0.00[a]	243.67±30.99[b]	226.33±24.70[ab]	220.67±17.04[ac]	218.33±17.56[ab]	213.33±32.15[b]	208.33±18.93[b]	200.67±16.77[b]
3	63.67±11.85[a]	205.00±5.00[b]	141.67±41.93[ac]	104.00±8.72[ad]	77.00±23.39[ac]	69.00±9.54[c]	63.67±6.35[a]	62.00±1.00[a]
4	61.33±6.03[a]	192.00±8.19[b]	102.33±2.52[ac]	83.00±13.00[ab]	74.67±8.39[ab]	67.00±8.72[c]	61.67±1.53[a]	48.00±6.08[a]
5	64.00±1.00[a]	229.00±13.11[b]	220.67±19.01[ab]	197.006.08[ac]	102.33±3.06[ac]	90.33±0.58[d]	100.00±1.00c	86.67±6.11c

Values are expressed in Means ± SD; n = 6, Values are presented as Mean ± SD; n = 4. Values with different letters down the group are significantly ($p < 0.05$) different.

Table 2a: Effect of LMEVA on the Concentrations of TCL, HDL and LDL in Alloxan-induced Diabetic Rats

Grp	Total Cholesterol Conc. (mg/dl)			HDL Conc. (mg/dl)			LDL Conc. (mg/dl)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
1	130.33±4.16	136.33±5.69	133.33±2.56	54.67±4.93	57.67±1.15	58.00±1.00	45.40±9.34	47.27±6.67	44.73±3.16
2	147.33±7.09	174.33±6.03	197.33±6.43	48.67±1.52	31.33±3.21	26.67±1.53	59.00±7.80	101.27±2.00	127.47±6.83
3	150.33±2.52	146.67±3.06	133.00±1.00	52..33±2.08	50.00±1.00	58.67±2.08	64.60±0.92	63.07±3.24	43.40±2.23
4	143.33±2.52	138.67±8.08	130.33±1.53	54.00±4.36	59.00±1.00	59.67±1.15	55.20±4.90	45.00±9.32	40.13±2.50
5	143.67±3.21	148.33±4.04	148.33±7.64	47.67±2.08	48.67±1.53	52.00±3.00	60.60±2.23	61.07±4.67	63.53±10.71

Results are expressed in Means ± SD; n = 6

Table 2b: Effect of LMEVA on the Concentrations of VLDL and TAG in Alloxan-induced Diabetic Rats

Grp	VLDL Conc (mg/dl)			TAG Conc. (mg/dl)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
1	30.27±0..31	31.40±0..20	30.60±0.53	151.33±1.53	157.00±1.00	153.00±2..65
2	39.67±1.60	41.73±1.55	43.20±1.06	198.33±8.02	208.67±7.77	216.00±5..29
3	33.07±0.61	33.60±0.40	30.93±1.30	165.33±3.06	168.00±2.00	154.67±6.51
4	34.20±0.40	34.67±0.64	30.53±0.81	171.00±2.00	173.33±3.21	152.67±4.04
5	34.52±3.27	38.87±0.90	32.80±1.64	177.00±2.00	194.33±4.51	164.00±6.62

Results are expressed in Means ± SD; n = 6

Table 3: Effect of LMEVA on the Concentrations of Pro- and Anti-oxidant Status in Alloxan-induced Diabetic Rats

Grp	MDA Conc (mg/dl)			GSH Conc (mg/dl)			Catalase Activity (IU/L)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
1	4.43±0.15	4.63±0.58	4.70±0.10	164.67±4.16	165.00±5.00	167.33±3.79	5.27±0.56	5.18±0.25	5.80±0.11
2	5.63±0.31	7.53±0.47	9.00±0.40	121.67±3.06	115.67±4.04	106.67±5.77	2.66±0.28	2.10±0.11	1.20±0.28
3	5.63±0.60	5.20±0.70	4.83±0.06	170.00±10.54	164.33±2.08	171..67±5.51	5.47±0.51	6.23±0.28	5.78±0.30
4	5.30±0.60	5.33±0.21	4.20±0.30	165.33±5.69	168.67±1.53	176.67±4.51	5.91±0.09	6.12±0.28	6.26±0.37
5	7.43±0.32	6.37±0.15	5.33±0.67	157.67±7.09	153.00±15.72	156.67±21.55	5.10±1.11	5.04±0.10	5.05±0.15

Results are expressed in Means ± SD; n = 6

Table 4: Effect of LMEVA on Haematological Indices of Alloxan-Induced Diabetic Rats

Grp	RBC Count (X^{12}/L)			PCV (%)			Hb Conc (g/dl)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
1	7.20±0.44	7.23±0.49	7.47±0.42	39.00±1.00	39.67±1.15	40.00±1.00	12.77±1.07	13.90±0.10	13.83±0.29
2	7.37±0.45	6.77±0.21	5.63±0.21	33.00±2.00	29.33±1.53	28.33±1.53	11.03±1.05	10.40±0.53	9.90±0.44
3	6.63±0.55	6.87±0.58	6.97±0.58	40.00±1.00	38.67±0.58	39.33±1.15	11.83±0.29	11.33±0.76	12.67±0.76
4	7.73±0.12	6.97±0.21	7.40±0.44	39.33±1.53	40.67±1.53	41.00±1.00	13.60±0.61	13.80±0.26	13.27±1.10
5	6.87±0.15	6.37±0.57	6.60±0.95	38.33±0.58	37.67±1.15	38.001±1.00	13.00±1.00	12.83±1.44	12.47±1.27

Values are expressed in Means ± SD; n = 6, Hb: haemoglobin, PCV: packed cell volume, RBC: red blood cell

This research outcome is in agreement with the research of [31] who attributed the low level of the haematological parameters to the condition of anaemia which is a common pathophysiology related to diabetes mellitus. In support of the aforementioned assertion, [32] further demonstrated that hypochromic anemia is a consequence of diabetes mellitus that arises from a decrease in the body's iron level, ultimately leading to a condition linked to oxidative stress.[32] The marked elevated levels of RBC, PCV, and Hb propose that the extract might possess some anti-anaemic properties which could be anchored on the high iron content [33] who stated the ability of some extracts' potential to advance bone marrow functions which is a key site for erythropoiesis. Relative reduction in PCV is considered to be an early sign of anaemia which is a cardinal symptom associated with infections or diabetic state. The outcomes of this study also point to the fact that the extract used in this study improved the counts of red blood cells as reported by [34] and [35] who used aqueous and ethanolic extracts of *V. amygdalina* respectively in improving the formation of RBC counts in experimental animals. Synergistic and duration-dependent variations were seen in the treated groups, particularly the normal and diabetic control groups. Interestingly, in group 4, marked reduction was observed in the concentrations of urea and creatinine relative to the levels observed in group 5 (Table 5).

Table 5: Effect of LMEVA on Renal Function Indices of Alloxan-Induced Diabetic Rats

Group	Urea Conc (mg/dl)			Creatinine Conc (mg/dl)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Group 1	11.71±0.44^{a}	11.88±0.29^{a}	11.97±0.25^{a}	0.65±0.06^{a}	0.59±0.06^{a}	0.62±0.04^{a}
Group 2	14.98±1.08^{b}	16.28±0.81^{b}	16.96±0.35^{b}	0.91±0.07^{b}	0.94±0.04^{b}	0.94±0.05^{b}
Group 3	15.00±1.33^{c}	13.74±1.41^{c}	13.11±2.75^{a}	0.69±0.08^{a}	0.90±0.07^{b}	0.73±0.03^{a}
Group 4	13.74±0.97^{b}	11.67±0.58^{a}	12.17±0.76^{a}	0.64±0.10^{a}	0.55±0.04^{a}	0.58±0.03^{ab}
Group 5	15.17±1.06^{c}	13.97±0.58^{c}	12.82±1.66^{a}	0.67±0.06^{a}	0.69±0.06^{ab}	0.70±0.07^{a}

Values are presented as Mean ± SD; n = 6. Values with different letters down the group are significantly ($p < 0.05$) different.

This result could be attributed to the renal protective potentials of the extract with a more appreciative effect when used synergistically with a standard antidiabetic drug such as glibenclamide which was used in this study. Urea – an end product of protein metabolism could be attributed to the multifactorial difficulties of diabetes mellitus such as renal disease, urinary obstructions, shock, congestive heart failure, or burns.[36] Marked elevation of the levels of urea and creatinine has a medical or clinical interpretation of renal dysfunction.[37] This finding is steady with the view of [38] who observed a positive effect of the ethanol extract of *V. amygdalina* leaves on the renal functions of experimental animals.

CONCLUSION

The point of attraction in this research is anchored anti-diabetic potential exhibited by the synergistic potential initiated by the co-administration of the extract and the standard drug as observed in group 4 rats. The findings showed, glaringly, that the possible anti-diabetic effect of methanol leaves extract of *V. amygdalina* can be boosted by using sulphonylurea drugs such as glibenclamide as used in this study. From the result, it can be deduced that the combined administrations of glibenclamide and *V. amygdalina* possess a hypoglycemic effect possibly by increasing the release of insulin from β-cells of islets of Langerhans and via glucosidase inhibition. The combined therapy was also found to have a lipid-lowering effect through the reduction of total serum cholesterol, TAG, LDL, and VLDL concentration owing to inhibition of fatty acid synthesis. High-density lipoprotein increased; thus increasing reverse cholesterol transport. It was also observed that the combined therapy reduced MDA concentration. Catalase activity and GSH concentrations were also restored by the combined therapy. The therapy also reduced the concentrations of creatinine and urea and thus prevented kidney damage. The effectiveness of the combined therapy was found to be better than single therapy. The combined therapy therefore has a synergistic effect which may be because they fact that they act on different receptors.

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Conflict of interest

The authors declare no conflict of interest.

Authors Declaration

The authors hereby declare that the works presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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