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Glycemic Control Potential of Chemically Characterized Extract from *Withania frutescens* L. Roots in Severe Diabetes-Induced Mice

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Abstract: Diabetes mellitus is a metabolic syndrome that causes impairment, mortality, and many other complications. Insulin and several synthetic medications are currently used in the treatment of diabetes. However, these pharmaceutical drugs are costly, and therefore medicines place priority on alternatives to fight this lethal disease. This modest study aims to investigate the chemical composition, antidiabetic and antihyperglycemic potentials along with subacute toxicity (bodyweight change and biochemical parameters) of hydroethanol extract from *Withania frutescens* L. roots (WFRE). The chemical analysis was carried out using GC–MS after extract silylation. The chemical analysis identified many potentially active compounds that may determine the antidiabetic results of WFRE. The antidiabetic effect of WFRE was evaluated in mice with severe diabetes using oral administration of doses up to 400 mg/kg for 28 days. The results of the antidiabetic and antihyperglycemic tests indicate that WFRE possesses promising glucose-lowering effects and, as a result, it may serve as an antidiabetic alternative for long-term use. The 4-week treatments with different doses of plant extract did not alter the bodyweight appearance of the diabetic mice nor their biochemical parameters (AST and ALT). The findings obtained indicate that the studied plant extract controlled severe diabetes in mice. Therefore, *Withania frutescens* L. can serve society as it provides natural agents to control diabetes.

Keywords: *Withania frutescens* L.; roots; diabetes mellitus; glibenclamide; antidiabetic; antihyperglycemic

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1. Introduction

Sugar diabetes is a metabolic condition with elevated levels of blood sugar arising from an insulin insufficiency in Langerhans pancreatic islet cells or from its inefficacy [1]. Classified as a chronic medical condition, people with diabetes struggle with unstable blood glucose and the complications that come with it during their entire lifetime [2]. Chronic hyperglycemia leads directly to long-term damages and dysfunctions, altering multiple organs, especially the heart, kidney, and eyes [3]. In 2000, there were about 171 million cases of diabetes across the world, and by 2030, this number could exceed 366 million according to the international diabetes federation [4]. In 2012, more than 1.5 million deaths were directly attributed to diabetes [5]. A healthy lifestyle that leaves room for physical activities can help manage diabetes mellitus [6]. Different medicinal plant extracts have also historically been used to treat diabetes worldwide, and they are known to be relatively

cheap, less harmful, and with few or no secondary effects [7]. Diabetes treatment without any side effects remains largely appreciated since many commercially antidiabetic agents have severe side effects including hypoglycemia, malabsorption of lactic acidosis and folate, edema, gastrointestinal symptoms, and weight gain, as reported in earlier work [8]. It is thus fitting that medicines have placed priority on safer and efficient hypoglycemics to control diabetes [9].

For many years, plants have been seen as a promising source of therapeutic agents. Nowadays, several developed drugs, even those used in modern medicines, could be derived from natural products or their chemically modified derivatives [10]. Medicinal plants have been used as an extensive medication source to control disease since more than 80% of populations use medicinal plants to fulfill their primary health care needs [11]. A variety of active ingredients have been purified from plants for direct use as medicines or to function as lead compounds or drug candidates [12].

Withania frutescens L. (*W. frutescens*) is a woody plant belonging to the family Solanaceae with enormous chemical compounds in its composition. The genus Withania possesses several activities, including anti-inflammatory, antituberculosis, and antioxidant ones [13]. To discover further biological activities that may exist in the genus Withania, the current work was undertaken to investigate the chemical composition and the antidiabetic and antihyperglycemic effects of *W. frutescens* roots using alloxan-induced diabetic methods.

2. Materials and Methods

2.1. Chemicals and Instruments

Alloxan monohydrate, glibenclamide, and an ACCU CHEK Performa Glucometer were obtained from Sigma-Aldrich. All chemicals used to perform analysis were of analytical grade.

2.2. Plant Material

W. frutescens. L. was harvested from the province of Fez city, Morocco (34.01300500° N; 4.75206833° W), in March 2019 (the season where the plant undergoes maximum development and flowering). Next, the plant material was authenticated by the botanist Amina Bari and was given the number BPRN69 before being deposited at the herbarium of the faculty. Afterward, the roots of *W. frutescens* were washed with distilled water and then dried in an oven set to 35 °C for 3 days.

2.3. Extract Preparation

After being dried at room temperature in a shady place, the roots of *W. frutescens* were ground into powder using an electric grinder. Afterward, 10 g of the obtained plant powder was extracted by maceration with 100 mL 70% ethanol for 24 H. Next, the mixture was meticulously filtered through the Whatman filter and then the crude extract was concentrated under vacuum before being saved at 4 °C until further use.

2.4. Experimental Animals

Adult Swiss albino mice whose weights ranged from 21 to 27 g were used in this study. Experimental animals were segregated into groups of 5 mice/cage before being acclimatized in the controlled animal's house (23 ± 2 °C with a 12 h/12 h dark/light cycle) for at least one week before starting the experiment. Animals had free access to standard pellets and water during the whole period of dosing except the fasting period. This research work was revised and authorized by the ethical committee of the Faculty of Sciences of Fez and given the ethical clearance N-ANI-BPRN-1382.5 [12]

2.5. Preparation of the Test Solutions

The plant extract was dissolved in physiological water (9 g/L NaCl) and then shook for 5 min using a magnetic stirrer. Next, the obtained solution was stored in a closed

stained glass bottle. The administered dose and volume were determined by the formula following formula:

$$V = (D \times P)/C$$

where V = volume of test solution (ml), D = dose (mg/kg), P = weight of the animal (kg) and C = concentration of the test solution (mg/mL) (Moussaoui et al., 2020b).

2.6. Antidiabetic and Antihyperglycemic Activities

As shown in Figure 1, the antidiabetic and antihyperglycemic effects of WFRE were assessed for 4 weeks using alloxan agent-induced diabetic mice, as reported elsewhere [14]. Animals were placed under monitoring immediately after dosing to control potential changes that occurred in mice, such as the effect of plant extract on the diabetic state, behavior, food and water intake changes. During the whole period of dosing, body weight and blood glucose changes were measured weekly.

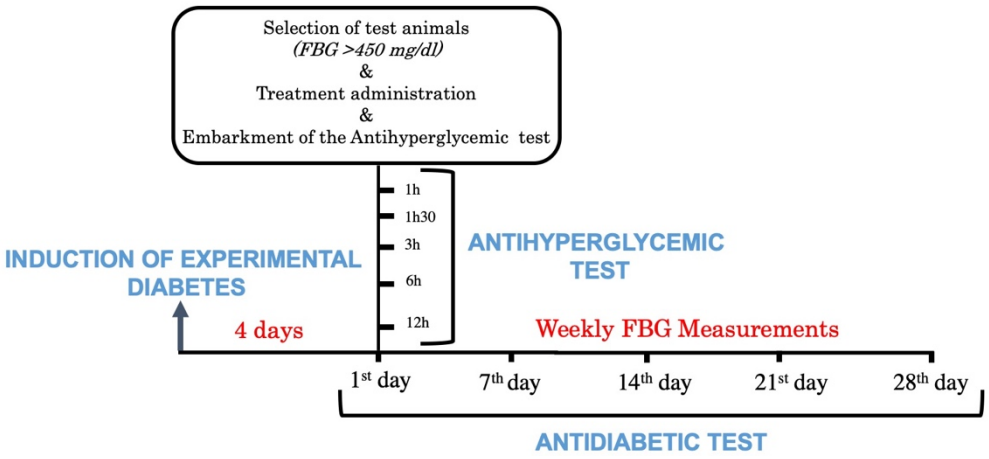


Figure 1. Experimental design.

2.6.1. Experimental Diabetes Induction

After being fasted overnight, animals were given alloxan monohydrate solution in single administration (180 mg/kg b.w.) via intraperitoneal injection. Simultaneously animals were orally given a glucose preparation (0.2 mL/4 g/L) to mitigate any potential hypoglycemic shock due to the action of the alloxan chemical. After 96 h of alloxan administration, the plasma blood glucose was measured. Fasted mice with blood glucose levels (BGL) higher than 450 mg/dL were considered in the findings.

2.6.2. Evaluation of the Antidiabetic Activity

The antidiabetic activity of *W. frutescens* roots was assessed by using five animal groups with five in each. The groups were designed as follows:

Groups 1 and 2 were selected to be normal and diabetic controls, respectively, and were given a vehicle (0.2 mL saline).

Group 3 was daily given glibenclamide that was used as a standard drug (oral administration, 2 mg/kg).

Groups 4 and 5 were given plant extract daily at 200 and 400 mg/kg, respectively.

During the experimental period, blood sugar levels in animals were measured at 1, 7, 14, 21, and 28 days. All animals were placed under control and body weight changes were recorded.

2.6.3. Antihyperglycemic Activity Evaluation

The antihyperglycemic test was carried out for 12 h followed by the 1st blood glucose level measurement on the first day of antidiabetic activity conditions. The antihyper-

glycemic test was conducted by measuring BGL within 1, 1:30, 3, 6, and 12 h of mice after being treated with the plant extract at doses 200 and 400 mg/kg. To perform the comparison, glibenclamide was used as a drug reference standard.

2.7. Identification of Phytochemical Compounds in the Plant Extract

The phytocomponents contained in the plant extract were silylated using N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) before being characterized by GC–MS, as reported by Kabran and al. (Kabran et al., 2014). In summary, 0.003 g of plant sample was mixed with 200 µL of MSTFA before being heated at 37 °C for 30 min. Afterward, 0.1 µL of the sample was injected into the GC–MS apparatus for analysis. To achieve this goal, GC–MS (Model 5973 from Brand Agilent Technologies) was used under the following conditions: Carrier gas: helium with 0.9 mL/s; oven temperature: 60–300 °C at 10 °C/min and 300 °C for 20 min; detector temperature: 250 °C; injector temperature: 260 °C. The silylated chemicals were identified by comparing their retention times with those of the standards obtained from the database Wiley 7n.l [15].

2.8. Statistical Analysis

Qualitative data were statistically analyzed using Graph Pad Prism version 7.0. Results are expressed as means ± SD of triplicate assays. Differences between groups were determined by using one-way ANOVA analysis. Values were considered statistically significant at a p-value ≤ 0.05.

3. Results and Discussion

3.1. Acute and Subacute Toxicity Study of Plant Extract

In vivo, acute and subacute toxicity of the studied plant extract have been well investigated elsewhere [13]. It was reported that the oral administration of single doses at 500, 1000, and 2000 mg/kg for 14 days did not alter the physical appearance of mice treated under acute toxicity conditions. Alongside acute toxicity, subacute toxicity investigated with repeated oral administrations of 500 and 2000 mg/kg/day for 28 days showed no histopathological nor biochemical alterations in mice treated under subacute toxicity conditions. Therefore, the plant, in both acute and subacute toxicity conditions, was deemed healthy for animals up to 2000 mg/kg.

3.2. Evaluation of the Antidiabetic Potential of W. Frutescens Root Extract

The present study aimed to investigate the in vivo antidiabetic effect of hydroethanol extract from *W. frutescens* roots in both alloxan-induced diabetic and normal mice. Alloxan is selectively toxic to pancreatic beta cells by the formation of reactive oxygen species, including nitric oxide [16]. As a result of the alloxan monohydrate induction dose (180 mg/kg), the diabetic mice model suffered from a severe hyperglycemic state (FBG > 450 mg/dL) when compared to the normal mice. Figure 2 represents the different changes that occurred in fasting blood glucose (FBG) during the whole experimental period. Within the first week of treatment, no significant decrease was observed for both doses of WFRE (200 and 400 mg/kg b.w) when compared to the positive control (glibenclamide), $p < 0.001$. This result indicated that the WFRE may not have a direct action on the main actors involved in blood glucose regulation, unlike glibenclamide, as an oral hypoglycemic drug that directly controls the release of insulin by beta cells [17].

From the second week of the experimental period, a significant reduction in BGL was observed in both WFRE extracts and glibenclamide (366 ± 27 mg/dL for the dose of 200 mg/kg; 355 ± 18 mg/dL for the dose of 400 mg/kg and 281 ± 26 for glibenclamide). At the end of the test, there was an important reduction in WFRE since it nearly brought the FBG levels back to those of the positive control (223 ± 19 mg/dL for 200 mg/kg; 199 ± 14 mg/dL for 400 mg/kg; 133 ± 18 mg/dL for glibenclamide). These results indicate a moderate antidiabetic effect of WFRE when compared to the standard antidiabetic drug. At the subacute level, we can suggest that the plant extract does not react directly with the

main receptors involved in the development of hyperglycemia since the reduction was not significant until the second week of the treatment period. In this sense, the control of the blood glucose levels of alloxan-induced diabetic mice may be due to stimulation of the residual pancreatic mechanism and potentially by increasing peripheral consumption of glucose or glycogen production in the liver as well as the reduction in gluconeogenesis [18]. A previous work reported that the plant extract could react when raising the concentration of hepatic glycogen and glycogenesis and glycogenolysis and gluconeogenesis could decrease whilst enhancing glucose utilization by peripheral tissues [18,19]. Moreover, the antihyperglycemic activity of WFRE may have resulted from the potentiation of the insulin effect either by raising the release of insulin from beta cells of the pancreas or by raising the peripheral consumption of glucose [20,21]. Thus, detailed molecular studies are necessary to deepen the knowledge about the exact mechanism of action that is involved in the antihyperglycemic activity. The roots of *W. frutescens* could be beneficial for long-term use as their effects can take a certain amount of time to show up. Concerning the long-term appearing effect, the hyperglycemic test approved this theory, as displayed in Figure 3, showing that during the 12 h of the test, the different doses of the root extract did not manage to lower the FBG. The blood glucose level was subjected to a slight decrease over time until the sixth hour of the test (256 mg/dL for 200 mg/kg and 211 mg/dL for 400 mg/kg); however, when the time-effect finished, the BGL started increasing again at the 12th hour of the test (297 mg/dL for the dose 200 mg/kg and 267 mg/dL for the dose of 400 mg/kg). Thus, the antihyperglycemic and the antidiabetic tests confirmed our suggestion on the long-term effect of the plant root extract.

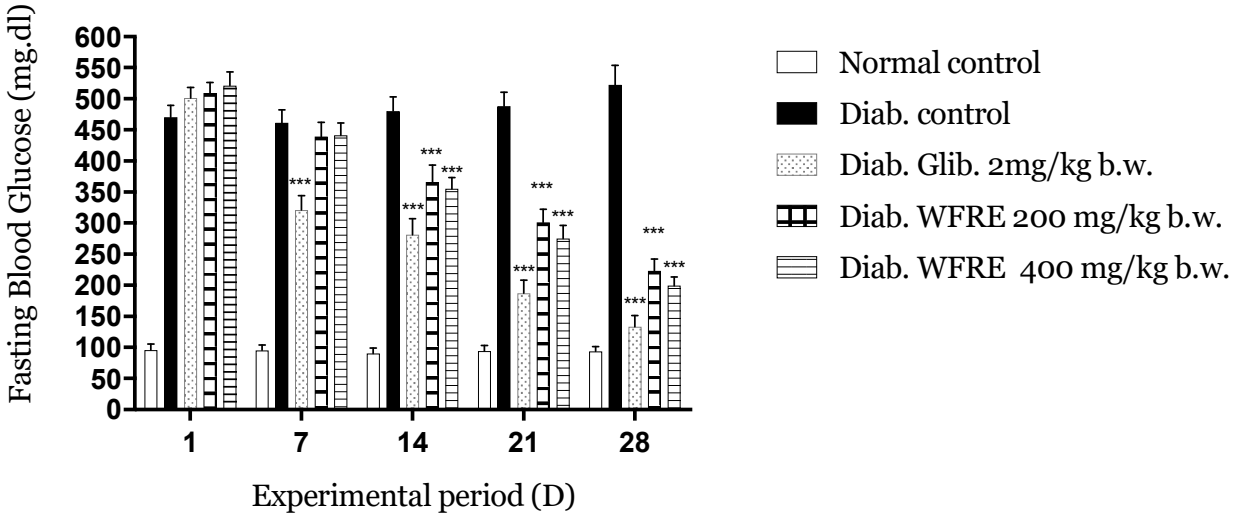


Figure 2. Fasting blood glucose changes occurred in alloxan-induced diabetic mice treated with plant extract for 28 days. (*** $p < 0.001$ means there was a highly significant difference between controls and treatments).

Table 1 represents changes that occurred in body weight and biochemical parameters of alloxan-induced diabetic mice during the whole period of dosing (28 days). Nontreated diabetic mice showed a large decrease in body weight associated with hyperglycemia. The bodyweight alteration of diabetic mice was potentially due to the massive catabolism of lipids and proteins used as an alternative source of carbohydrates. This hypothesis is well confirmed elsewhere [22,23], where it was reported that insulin supply is involved in protein synthesis and proteolysis regulations in the skeletal muscle. During the whole experimental period, diabetic mice treated with the plant extract showed an increase in body weight, which could confirm the protective role of WFRE since bodyweight alteration frequently occurs under toxicity conditions [24].

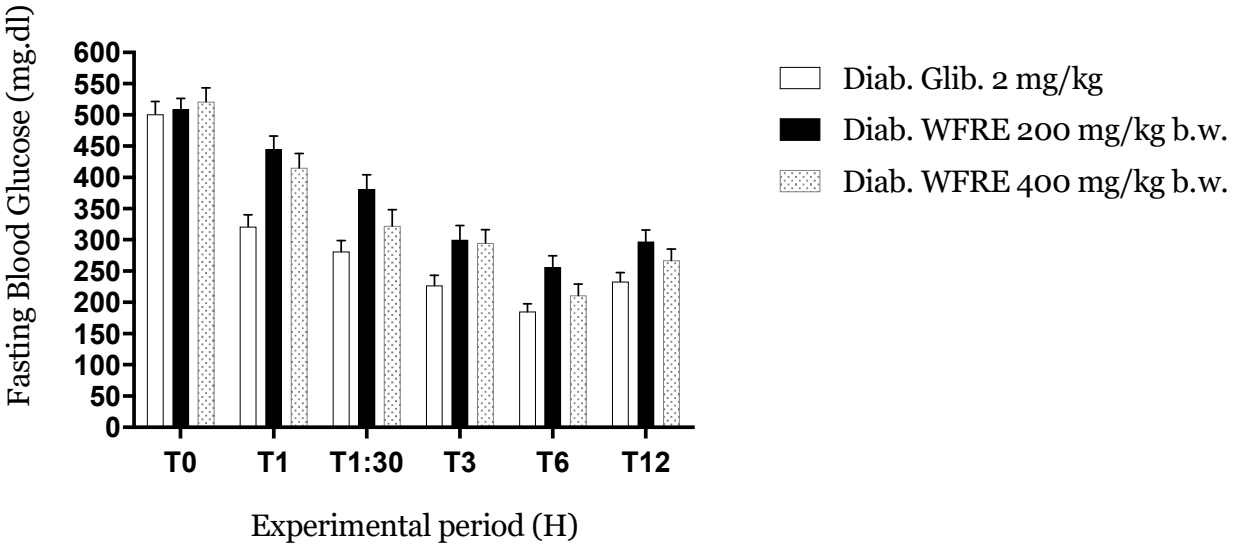


Figure 3. Plant extract effect on alloxan-induced diabetic mice during the 12 h of the antihyperglycemic test.

Table 1. Effect of plant extract on body weight and biochemical parameter development.

Treatment	Bodyweight Development (mg/kg b.w)					Biochemical Parameter			
	Day 1	Day 7	Day 14	Day 21	Day 28	AST (U/L)	ALT (U/L)	Urea (g/L)	Creatinine mg/L
Normal control	23.4 ± 3.3	24.7 ± 3.5 *	25.3 ± 2.4 ***	26.9 ± 3.3 ***	27.2 ± 3.5 ***	253 ± 33.73 ***	45 ± 6.58 ***	0.25 ± 0.04 **	3.3 ± 0.44 *
Diab. Control	23.9 ± 2.1	21.8 ± 3.2	20.2 ± 2.7	19.3 ± 2.4	17.8 ± 2.2	502 ± 48.85	134 ± 21.2	0.64 ± 0.06	5.9 ± 0.83
Diab. Glib. 2 mg	24.4 ± 2.6	23.2 ± 1.6	24.3 ± 2.3 **	25.9 ± 2.5 ***	25.8 ± 1.77 ***	298 ± 25.66 ***	77 ± 9.80 ***	0.29 ± 0.04 ***	4.3 ± 0.44 **
Diab. WFRE 200 mg/kg	24.8 ± 2.2	24.0 ± 1.9	24.4 ± 1.8 **	25.0 ± 2.0 ***	26.2 ± 2.2 ***	243 ± 27.42 ***	57 ± 7.26 ***	0.35 ± 0.05 ***	3.80 ± 0.46 ***
Diab. WFRE 400 mg/kg	23.9 ± 2.4	23.4 ± 2.3	24.1 ± 1.9 **	24.9 ± 2.2 ***	26.4 ± 2.1 ***	276 ± 23.01 ***	52 ± 6.82 ***	0.34 ± 0.04 ***	3.70 ± 0.32 ***

Values are expressed as mean ± SD (n = 5 mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with diabetic control.

The biochemical parameters reported in Table 1 demonstrate elevated levels of transaminases in nontreated diabetic mice, which are biomarkers of leakage of cellular content associated with loss of functional integrity of the hepatic cell membrane [25]. A substantial decrease in the levels of transaminases (ASAT and ALAT) was reported in diabetic animals treated with various doses of plant extracts when compared to diabetic controls. These results can suggest that the extract had a hepatoprotective function. Moreover, high blood levels of urea and creatinine were detected in the untreated diabetic populations, which may inform about potential renal damage attacked mice [15]. We noticed that the plasmatic level of urea and creatinine returned to values near to normal in diabetic mice subjected to treatment with WFRE. Therefore, we can confirm that WFRE had a renal protective effect in addition to its hepatoprotective function.

3.3. Phytochemical Identification of Plant Extract

The phytochemical characterization of plant extract identified fourteen potentially bioactive compounds in *W. frutescens* hydroethanolic extract, which represent 99.98% of the total recovered extract that reacted with MSTFA and could be detected by GC–MS analysis. As shown Figure 4 and Table 2, the extract was found to be majorly constituted of methanamine,N,N-di (2-trimethylsilyloxyethyl), bicyclo [3.1.1] heptane, 6,6-dimethyl-2-methylene-, (1S), and bicyclo [2.2.1] hept-2-ene, 2,7,7-trimethyl, with scores of 34.12, 28.48 and 10.19%, respectively. These chemicals cannot be excluded from being involved in the biological activities investigated in the present work such as antidiabetic and antihyperglycemic activities [25–27]. The present chemicals can react individually or in synergy without excluding potential potentiation effects. The synergistic function of constituents

present in the crude extract can also feature the antidiabetic nature of the plant extract. However, the minor components could interaction with the major chemicals which might affect antidiabetic and antihyperglycemic activities. Thus, testing the entire extract rather than its components is more fitting. Antidiabetic and antihyperglycemic activities can be related to methanaminee,N,N-di (21-trimethylsilyloxyethyl), a major compound of the plant extract. The results obtained agreed with those reported by Ullah et al. (2017) [28] who showed that methyl salicylate as closer compounds isolated from *Pericampylus glaucus* Lam Merr had significant hypoglycemic properties in STZ-induced diabetic rats [28]. The other major compounds detected in the extract such as bicyclo1 [3.1.1] heptane, 6,6-dimethyl-2-methylene-, (1S) and 1bicyclo [2.2.1] hept-2-ene, 2,7,7-trimethyl can also be responsible for the antidiabetic activity even though no previous data have shed light on their activities to the best of our knowledge.

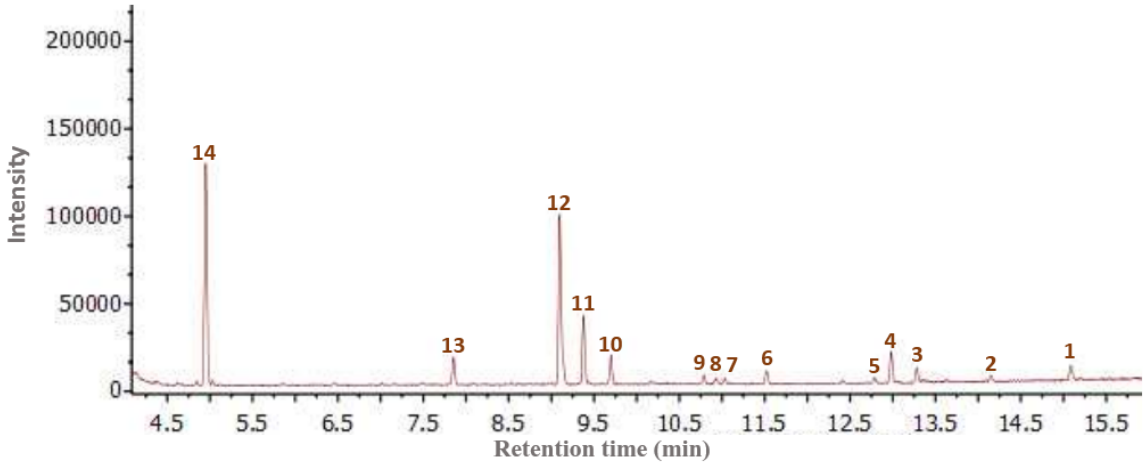


Figure 4. Chromatographic profile of *W. frutescens* characterized extract.

Table 2. Phytochemical compounds identified in the plant extract.

Peak	RT	Compound Name	Formula	Area (%)
1	015.087	3,3,6-dTrimethyl-1,4dheptadien-6-ol	$C_{10}H_{18}O$	2.541
2	014.151	Tricyclo [2.2.1.0(2,6)] d heptane 3-methanol, 2,3-dimethyl-	$C_{10}H_{16}O$	1.141
3	013.280	Bicyclo [3.1.0] hexan s3-one, 4-methyl-1-(1-methylethyl)-, [1S-(1α,4β,5α)]-	$C_{10}H_{16}O$	2.351
4	012.983	Bicyclo [2.2.1] heptan-2-ol, 1,7,7-trimethyl-	$C_{10}H_{18}O$	4.841
5	012.782	Bicyclo [3.1.1] eheptan-3ol,2,6,6-trimethyl-, (1α,2β,3α,5α)-	$C_{10}H_{18}O$	11.82
6	011.525	2,6-Octadien 1-ol, 3,7-dimethyl-, (Z)-	$C_{10}H_{18}O$	12.04
7	011.035	2,6-dOctadien-1 ol, 3,7-dimethyl-, (E)-	$C_{10}H_{18}O$	11.41
8	010.931	Benzenes, 1-ethyl 2,3-dimethyl	$C_{10}H_{14}$	01.26
9	010.790	Benzene, e1-methyl 3-(1-methylethyl)-	$C_{10}H_{14}$	01.33
10	09.698	Bicyclo [2.2.1] ehept-2 ene, 1,7,7-trimethyl-	$C_{10}H_{16}$	04.56
11	09.377	Bicyclo [2.2.1] ehept-2-ene, 2,7,7-trimethyl-	$C_{10}H_{16}$	010.19
12	09.100	Bicyclo [3.1.1] eheptane,6,6-dimethyl-2-methylene-,e (1S)-	$C_{10}H_{16}$	028.48
13	07.855	Tricyclo [2.2.1.e0(2,6)] heptane 1,7,7-trimethyl-	$C_{10}H_{16}$	03.90
14	04.952	MethanamineN,sN-di (2-trimethylsilyloxyethyl)-	$C_{11}H_{29}NO_2Si_2$	034.12
		Total		099.98

4. Conclusions

The present work sheds light on the chemical composition and the antidiabetic potential of *W. frutescens* L. The findings obtained showed that the studied plant extract well-controlled the blood glucose levels in alloxan-induced diabetic mice. Thus, the outcome of the present work supports the utilization of the plant by the local population as an antidiabetic alternative for long-term use. Since the studied extract exhibited interesting antidiabetic and antihyperlipidemic activities, further research should take place to deepen the knowledge of the action mechanism as well the determination of the responsible compounds of the plant extract.

Author Contributions: A.E.M., H.M., and M.B.: writing—original draft; I.E.-s.: formal analysis; A.M.S., M.S.A., and H.K.A.: reviewing and editing; D.B., A.B.: methodology and supervision. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data reported here is available from the authors upon request.

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Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

Abbreviations

WFRE	Hydroethanol extract of Withania frutescens L. roots
FBG	Fasting blood glucose
BGL	Blood glucose level

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