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Influence of *Rhamnus alaternus L* leaf extract on normal and alloxane-induced Diabetic rats

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The present study aimed to test the influence of methanol extract from *Rhamnus alaternus L.* leaves on the biochemical parameters of normal and diabetic rats. Diabetes mellitus was induced by an intra-peritoneal injection of 150 mg / kg.bw of alloxane monohydrate after 72 hr of the injection. Rats with a blood glucose level greater than or equal to 280 Mg/dl were considered diabetic. Blood glucose concentration of the tested animals were measured at the beginning as well as on the 3rd, 7th and 14th day after the start of the experiment. On day 14, retro-orbital blood samples taken under anesthesia with petroleum ether. Chromatographic analysis of the crude methanol extract of *Rhamnus alaternus L* leaves revealed the presence of multiple phenolic components in the major ones: rutin, cinnamic acid, ellagic acid, benzoic acid, and vanillic acid. The methanol extract from *leaves of Rhamnus alaternus L.* showed a non-significant reduction in blood sugar (P> 0.05). This extract also reduced body weight in a very highly significant way (p <0.0001) as well as improved other biochemical parameters such as serum urea, serum protein, serum creatinine, serum cholesterol and serum triglycerides.

Keywords: *Rhamnus alaternus L*, Methanol extract, Diabetic effect, Liquid chromatography, Biochemical parameters

INTRODUCTION

Studying the chemistry of plants is always burning news despite its seniority. This is attributed to the fact that the plant kingdom represents an important source of a wide variety of bioactive molecules (Bahorun et al., 1996)

According to the French Diabetes Federation, 425 million people worldwide have diabetes. This qualifies the phenomenon as a real pandemic because the progression is considerable. For example, WHO estimates 622 million diabetics by 2040 (Atlas IDF 201) Diabetes Atlas (2017)

Diabetes mellitus is a metabolic disorder of a multiple etiology characterized by chronic hyperglycemia, which affects carbohydrate, protein as well as lipid metabolism and which results from a deficiency of insulin secretion (Alberti and Zimmet, 1998)

Rhamnus Alaternus L. belongs to *Rhamnaceae* family and present in North Africa, the Middle East and the south of Europe. In Algeria, it grows in scrubland and limestone hills very sunny.

In traditional medicine *R. alaternus L.* has been used as a hypotensive, digestive, laxative, diuretic and for the treatment of hepatic and dermatological complications (Bhouri et al., 2012)

These berries have a purgative action, a bitter taste, used in veterinary medicine Gubb AS. (1913).The leaves are astringent gargles (Jacques et al., 2005)Also, all the aerial parts are used by the local population of Laghouate city (Algeria) as an antidiabetic plant (Khachba and Benamar 2008).

MATERIALS AND METHODS

Plant material

Our study is carried out on the leaves of *Rhamnus alaternus L* harvested at maturity during 2015 in the region of Seraidi wilaya of Annaba, Northest of Algeria.

Preparation of *Rhamnus alaternus L.* extract

The shade-dried plant leaves were crushed, pulverized and extracted with methanol. After 3 h of maceration with continuous stirring at 200 rpm, the mixture was filtered. The filtrate was evaporated to dryness under vacuum using a rotary evaporator. This operation is repeated four times with renewal of the solvent to increase the yield. The extract was then stored in the refrigerator until used.

Test animals

The present study was carried out on female Wistar rats, aged 2 to 3 months with a body weight of 130 -175g, and adult albino mice with a body weight of 24-27g. The pharmacological study was carried out in the laboratory of Pharmacology of the Research and Development Center (CRD), SAIDAL Algiers. The rats were housed in transparent polypropylene cages with a stainless steel lid. They were kept under favorable breeding conditions (20-24 ° C and 50% humidity) and arranged with tap water and standard food, litter composed of sawdust renewed every other day. The experimental protocol has been proven by the CRD.

Determination of phenols compounds in *Rhamnus alaternus L.* extract

This was done by HPLC liquid chromatography, using the method of Agilent Application Note, (Publication number 5991-3801EN, 2014).

Instrument condition for phenolic compounds

Agilent1260 infinity HPLC Series (Agilent, USA), equipped with Quaternary pump, a Zorbax Eclipse plusC18 column 100 mm x 4.6 mm i.d., (Agilent technologies, USA), operated at 25 ºC. The separation is achieved using a ternary linear elution gradient with (A) HPLC grade water 0.2 % H_3PO_4 (v/v), (B) methanol and (C) acetonitrile. The injected volume was 20 µL . Detection: VWD detector set at 284 nm .Condition of test item: -21°C.

Acute toxicity determination

The albinos mice female, 2-3 month old with an average weight of 24 à 27 g, were used for the study. To determine the toxicity, a single oral administration of methanol extract of *Rhamnus alaternus* L. leaves in different dose (150, 300,450, and 600 mg/kg) were administrated to different groups of animals. Mortality and general behavior of animals were observed periodically for the next 48 hr during the 14 days.

Effect of *Rhamnus alaternus L.* leaf extracts on alloxane-induced diabetic rats

Fasting Wistar rats (16h) were treated with a single intra-peritoneal dose of alloxan monohydrate (≥98.0%) at 150mg / kg. PC prepared just before injection into a normal saline solution NaCl (0.9%)(Alarcon-Aguilar et al.,2000) Because alloxane is capable of producing lethal hyperglycemia following massive release of pancreatic insulin, the rats were then treated with 30% glucose solution orally at a different time interval after 6 hours of treatment. Induction of alloxane and a 5% glucose solution was stored in baby bottles in their cages for the next 24 hours.
After 72 hours of alloxane injection, rats with blood glucose level greater than or equal to 280 mg / dl were considered diabetic and are used for this experiment. 30 normal and diabetic rats were divided into 6 groups of 5 rats for each of these groups (n = 5). The rats were treated with a single daily dose orally for 14 days.

Group I: Normal rats: positive control served as 300 mg/kg.bw of glibenclamide
Group II: Diabetic rats: positive control served as 300 mg/kg.bw of glibenclamide
Group III: Normal rats: negative control served as 1ml NaCl 0.9%
Group IV: Diabetic rats: negative control served as 1ml NaCl 0.9%
Group V: Normal rats: served as 300 mg/kg.bw of *Rhamnus alaternus L.* extract of leaves
Group VI: Diabetic rats: served as 300 mg/kg.bw of *Rhamnus. alaternus L.* extract of leaves

Retro-orbital blood samples are taken for each lot at 0 day (Basal Glycemia), 3rd day 7th day and 14th day. Whole blood was used for the blood glucose measurement using a glucometer, while for the assay of the other blood parameters, at the 14th day of the study, blood was recovered in tubes containing heparin, and then centrifuged at 1000 rpm for 15min. The recovered plasma was assayed for serum urea, serum creatinine, serum protein, serum triglycerides and serum cholesterol.

Statistical analysis

Data were represented as the mean ± SEM. Statistical comparison of data was made by means of one way ANOVA using Student's test to compare two lots. The following notation is used for any significant difference: Not significant (*): p > 0.05, Significant (**): p <0.01, Very significant (***): p <0.001 highly significant (****): p <0.000

RESULTS

The acute oral toxicity study of the methanolic extract of leaves from *Rhamnus alaternus L*. showed median lethal LD50 of 680.79 mg / kg p.c administered orally in albino mice. alloxane is widely used to induce experimental diabetes in animals. The mechanism of their action in pancreatic B cells has been extensively studied and is now fairly well understood. The cytotoxic action of this diabetogenic agent is mediated by reactive oxygen species. Alloxan and the product of its reduction, dialuric acid, establish a redox ring with formation of superoxide radicals. These radicals undergo a disproportionation with hydrogen peroxide. Then, highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of B cells Szkudelski (2001).

The variation in body weight of rats is a very important parameter. The regular monitoring of the animals led us to obtain the values relating to Table 1 and Figure 1. From the results obtained, we recorded a regular gain of body weight of normal rats treated with *Rhamnus alaternus L.* extract as well as positive and negative control groups. This increase in weight is very highly significant throughout the experience p <0.0001.

It was observed that injection of alloxane at a dose of 150g / kg.bw intraperitoneally induced weight loss in diabetic rats. This decrease in body weight is very highly significant p <0.0001. After one week of treatment with *Rhamnus alaternus L.* extract, we observed a very highly significant increase in body weight in diabetic rats treated with *R. alaternus L.* extract, following the same route as the positive and negative control.

The effect of the *Rhamnus alaternus L.* extract leaves administered orally on the fasting blood glucose in normal rats and diabetic rats by the intra-peritoneal injection of 150 g / kg.bw of alloxane was shown in Table 2. The results obtained show that there is no significant difference p> 0.05 between normal rats treated with *Rhamnus alaternus L*. extract and normal positive and negative control rats. Fasting blood glucose was stable during the 14 days of treatment and followed the same route as rats in positive and negative control groups.

During a two-week follow-up of diabetic rats, hyperglycemia was noted following the pretreatment of the rats with alloxane (150 mg / Kg.bw). A 4-fold increase in fasting blood glucose has been noted since the injection of alloxane. The daily treatment of the rats by the methanol extract succeeded in reducing this hyperglycemia. No significant difference p >0.05 was observed in this experiment between the diabetic rats treated with the *Rhamnus alaternus L.* extract and the positive and negative control diabetic rats.

Alloxane resulted in the establishment of a diabetic syndrome characterized by polyphagia, polyuria, polydipsia and weight loss and the rats exhibited a table characterized by hyperglycemia, hypercholesterolemia, hypertriglyceredemia in rats Kebieche, M (2009)

The variation of serum urea was shown in Table 3. We noted a significant p <0.01 increase of urea in diabetic rats compared to normal rats treated with *Rhamnus alaternus* L. extract, very highly significant increase p <0.0001 urinary serum between normal rats and diabetic rats underwent 1ml of NaCl to 0.9%, and finally a non-significant p> 0.05 increase in urea serum between normal rats and diabetic rats treated with 300mg / kg.bw of *Rhamnus alaternus L.* extract.

The variation in serum protein is shown in Table 3. The results show that total protein levels were very similar in all six experimental groups. The statistical study of these results revealed no significant difference between normal and diabetic rats treated with glibenclamide and NaCl 0.9% p> 0.05, and a very highly significant difference (p <0.0001) in protein between normal and diabetic rats treated with *Rhamnus alaternus L.* extract.

Serum creatinine variation was shown in Table 3. We noted a non-significant p> 0.05 increase in creatinine between diabetic rats compared to normal rats treated with *Rhamnus alaternus* L. extract. The groups treated with glibenclamide showed a significant increase p <0.01 between the diabetic and normal rats of these lots. Finally, there was a very highly significant increase p <0.0001 between normal rats and diabetic rats that have undergone 1 ml of 0.9% NaCl orally.

From the results obtained in Table 4 we highlighted a very highly significant (p <0.0001) increase of serum cholesterol in diabetic rats treated with *Rhamnus Rhamnus alaternus L.*

extract compared to normal rats treated with this same extract.

Table1. The effect of *Rhamnus alaternus L.* extract on body weight in normal rats and diabetic rats

Group	Body weight (mg)			
	0 day	3rd day	7th day	14th day
I	133.00± 3.26****	136.30 ± 3.11****	141.62± 3.24****	148.65 ± 1.31****
II	142.90± 2.15****	137.06 ± 1.43****	135.54± 1.75****	153.80 ±2.36****
III	139.76± 1.50****	146.64 ± 1.30****	157.06± 1.06****	163.70± 2.27****
IV	172.86± 1.65****	166.76 ± 1.05****	162.60 ± 1.54****	157.32 ± 1.56****
V	131.08± 1.67****	131.36± 1.32****	139.24± 1.38****	147.62±1.45*****
VI	138.20± 0.80****	131.34± 1.44****	131.24± 0.65****	141.20 ± 0.52****

Values are the mean ± SEM; n=5, Highly significant (****): p <0.0001. Group I: Normal positive control, group II: diabetic positive control, group III: normal negative control, group IV: diabetic negative control, group V: normal *Rhamnus* extract, group VI: diabetic *Rhamnus* extract

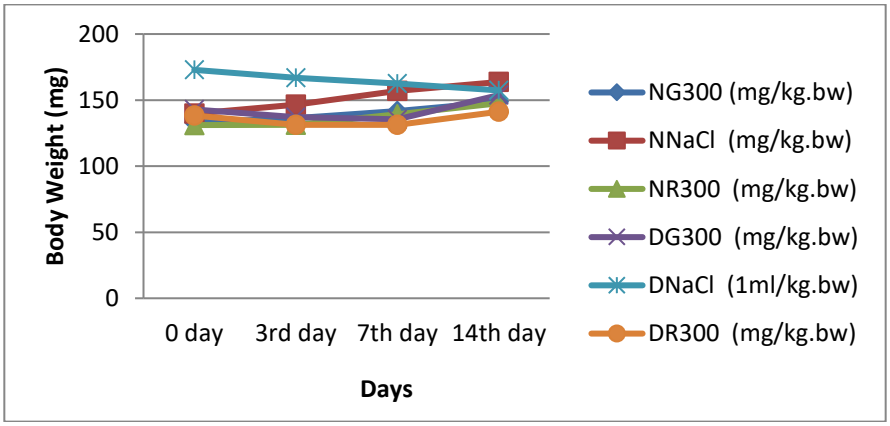

Figure 3.Variation of body weight in normal (N) and diabetic (D) rats treated with methanol extract of *Rhamnus alaternus L* for 14 days. p <0.0001

Table 2.The effect of *Rhamnus alaternus L.* Extract on blood glucose level in normal rats and diabetic rats

Group	Blood glucose level (mg/dl)			
	0 day	3rd day	7th day	14th day
I	77.4 ± 6.27*	89.00 ± 7.78*	82.40 ± 7.92*	77.60 ± 8.08*
II	81.80 ± 9.36*	372.20 ± 3.24*	298.80 ± 43.80*	232.00 ±52.40*
III	78.40 ± 10.90*	88.40 ± 7.89*	83.60 ± 6.35*	81.40 ± 8.26*
IV	84.80 ± 5.45*	307.60 ± 2.52*	282.20 ± 18.50*	256.80 ± 32,0*
V	81.80 ± 5.76*	84.60 ± 3.97*	88.20 ± 12.90*	88.00 ± 4,69*
VI	80.20 ± 9.50*	352.80 ± 36.80*	292.60 ± 9.10*	264.40 ± 28.4*

Values are the mean ± SEM; n=5, Not significant (*): p > 0.05, Group I: Normal positive control, group II: diabetic positive control, group III: normal negative control, group IV: diabetic negative control, group V: normal *Rhamnus* extract, group VI: diabetic *Rhamnus* extract.

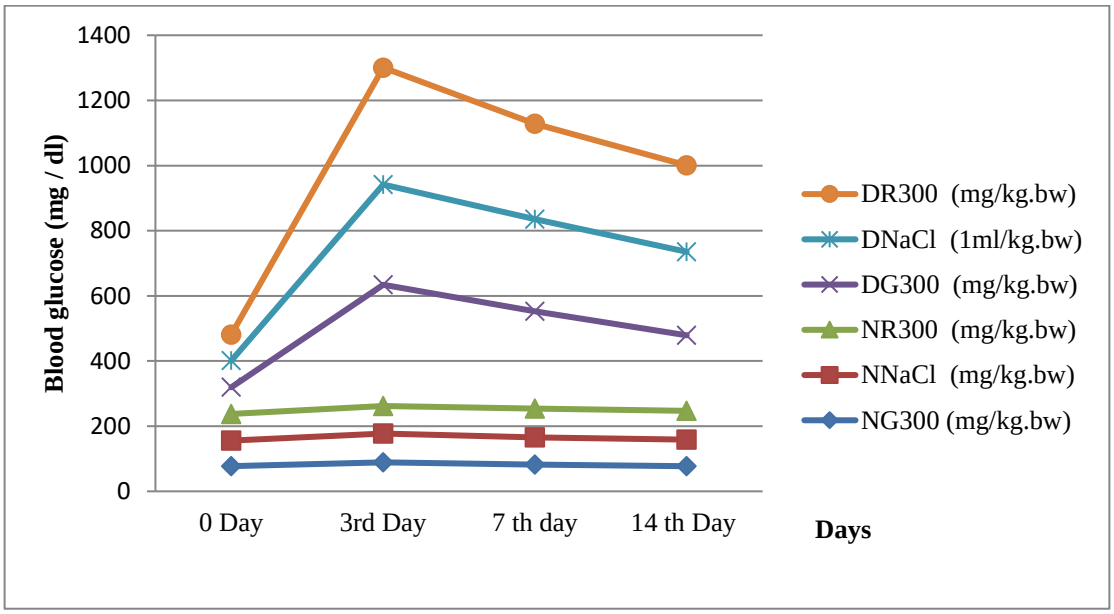

Figure 2. Variation of blood glucose levels in normal (N) and diabetic (D) rats treated with methanol extract of *Rhamnus alaternus L* for 14 days. p > 0.05.

Table 3. Effect of *Rhamnus alaternus L.* extract on serum urea, serum protein and serum creatinine in normal rats and diabetic rats

Group	Blood parameter		
	Serum urea (mg/dl)	Serum protein (g/dl)	Serum creatinine (mg/dl)
I	40.40± 7.40 *	8.20± 0.10*	0.67± 0.04**
II	45.60± 4.88*	8.01± 0.19*	0.72± 0.01**
III	46.40± 3.65****	8.47± 0.13*	0.69± 0.03****
IV	76.80± 2.59****	8.59± 0.10*	1.24± 0.03****
V	40.60± 2.41***	8.03± 0.13***	0.73± 0.01*
VI	49.60± 1.67***	8.45± 0.09***	0.74± 0.01*

Values are the mean ± SEM, n=5, Student's test. Not significant (*): p > 0.05, Significant (**): p <0.01, Very significant (***): p <0.001 Highly significant (****): p <0.0001. Group I: Normal positive control, group II: diabetic positive control, group III: normal negative control, group IV: diabetic negative control, group V: normal *Rhamnus* extract, group VI: diabetic *Rhamnus* extract.

Table 4. Effect of *Rhamnus alaternus L.* extract on serum triglycerides and serum cholesterol, in normal rats and diabetic rats

Group	Lipid blood parameter	
	Serum triglycerides (mg/dl)	Serum cholesterol (mg/dl)
I	62.60 ± 5.68***	54.00 ± 3.16*
II	148.80 ± 39.20***	83.80 ± 9.55*
III	70.40 ± 6.58****	56.00 ± 1.58****
IV	220.00 ± 16.97****	215.00 ± 15.89****
V	59.00 ±5.61**	55.60 ± 2.88****
VI	77.80 ± 8.70**	173.40 ± 14.47****

Values are the mean ± SEM, n=5, Student's test. Not significant (*): p > 0.05, Significant (**): p <0.01, Very significant (***): p <0.001 Highly significant (****): p <0.0001. Group I: Normal positive control, group II: diabetic positive control, group III: normal negative control, group IV: diabetic negative control, group V: normal *Rhamnus* extract, group VI: diabetic *Rhamnus* extract.

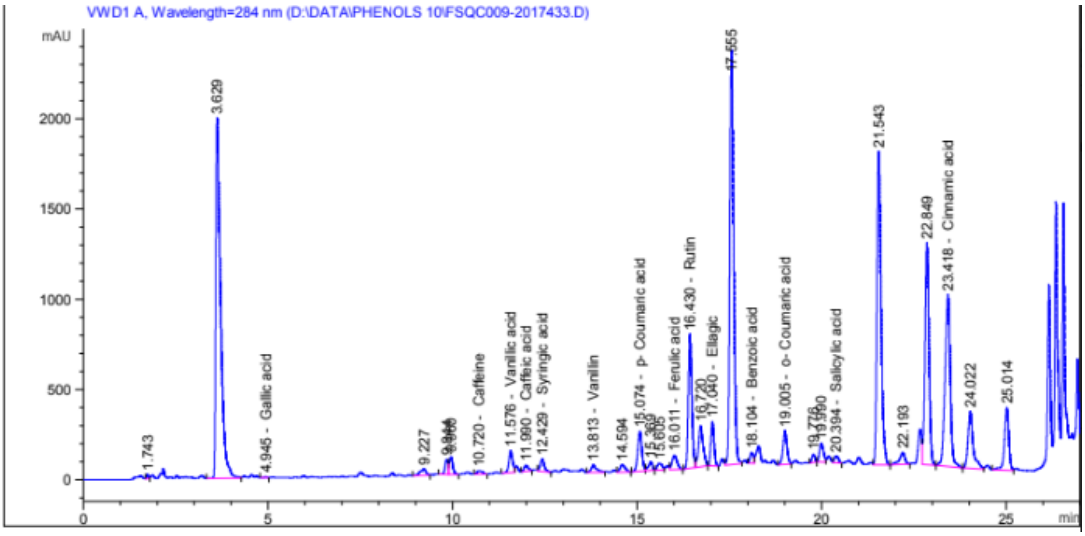

Figure 3.The HPLC of the methanol *Rhamnus alaternus L.* leaves extract

Table 5. Phenol compounds of *Rhamnus alaternus L* leaves methanol extract.

Ret Time [min]	Area[Mau*s]	Amount ug/ml]	Name	Molecular formula	Conc. Mg/100g
4.945	23.69825	1.62334e-1	Gallic acid	$C_7H_6O_5$	0.27
9.004 1	-	-	Catechol	$C_6H_6O_2$	ND
10.395	-	-	p- Hydroxy benzoic acid	$C_7H_6O_3$	ND
10.720	186.0243	1.50699	Caffeine	$C_8H_{10}N_4O_2$	2.15
11.576	885.83978	7.35340	Vanillic acid	$C_8H_8O_4$	12.26
11.990	216.25833	1.03343	Caffeic acid	$C_9H_8O_4$	1.72
12.429	531.07904	3.08806	Syringic acid	$C_9H_{10}O_5$	5.15
13.813	429.64291	2.16553	Vanillin	$C_8H_8O_3$	3.61
15.074	1745.08740	4.97898	p-Coumaric acid	$C_9H_8O_3$	8.30
16.011	755.71997	4.00358	Ferulic acid	$C_{10}H_{10}O_4$	6.67
16.430	4929.67773	110.97772	Rutin	$C_{27}H_{30}O_{16}$	671.36
17.040	1513.43726	22.16.35	Ellagic	$C_{14}H_6O_8$	36.93
18.104	370.06271	29.04409	Benzoic acid	$C_7H_6O_2$	48.41
19.005	1393.65222	4.55254	O-Coumaric acid	C9H8O3	7.59
20.394	305.46872	4.99870	Salicylic acid	$C_7H_6O_3$	8.33
23.418	9422.40430	32.66224	Cinnamic acid	$C_9H_8O_2$	54.44

This increase was 173 ± 14.17 mg/dl compared to normal rats which are 55.6 ± 2.88 mg/dl. This increase is greater than that of rats treated with glibenclamide, which are 83.80 ± 9.55 mg/dl in diabetic rats and 54.00 ± 3.16 mg/dl in normal rats.

All these obtained observations were very highly significant p <0.0001, except that in rats that undergone oral 1ml of 0.9% NaCl, the increase in serum cholesterol was not significant p> 0.05.

The variation of serum triglyceride was shown in Table 4. The results showed that the serum triglycerides level in diabetic rats treated with *Rhamnus* extract increased significantly p <0.01 compared to normal rats treated with this same extract. It is was 77.80 ± 8.70 mg / dl in diabetic rats compared to normal rats 59.00 ± 5.16 mg / dl. This increase was lower compared to that of

diabetic rats treated with glibenclamide which was very significant p <0.001 it is 148.80 ± 39.20 mg / dl compared to normal rats 62.60 ± 5.68 mg / dl. In addition, there was a very highly significant increase in serum triglyceride between normal rats and diabetic negative control groups.

The methanol extract of *Rhamnus. alaternus* leaves was subjected to liquid chromatography analysis to identify the different phenolic components (Fig.3). Sixteen compounds were identified whose data were presented by retention time, area, amount, name, molecular formula and concentration in mg/100g extract. The major detected components of *Rhamnus. alaternus* extract were rutin, cinnamic acid, benzoic acid and ellagic. The complete list of compounds identified in the extract of *Rhamnus alaternus L*. were listed in Table 5.

Chromatographic analysis showed that the predominant component was rutin which is a flavonoid widespread in nature. It is one of the most attractive phytochemicals because of its properties. In fact it is considered one of the most important flavonoids used in the pharmaceutical industry. More than 130 rutin-based medicines have been registered around the world. Rutin is characterized by its anti-oxidant, anti-inflammatory and neuroprotective activities. It would also be beneficial in certain chronic diseases such as cardiovascular diseases or diabetes. The anti-diabetic character of rutin was highlighted in 2007 by the Stanley Mainzen Prince group (Matough, et al.,2012) for improvement of glucose homeostasis in diabetic rats. In this study, oral administration of rutin (100 mg / kg) to diabetic rats over a period of 45 days resulted in decreased plasma glucose and increased insulin levels, with increased glycogen content the liver and muscles. There was also a decrease in the glycogen content in the kidneys. Fasting blood glucose was reduced by increasing the activity of hexokinase. In addition, the histopathological study of the pancreas revealed the protective role of rutin (Stanley et al., 2006)

Among the major components of *R. alaternus L*. extract is cinnamic acid, which belongs to the class of auxins, is also recognized as a plant hormone regulating cell growth and differentiation Sharma P. (2011). Cinnamic acid is a natural organic compound, it is replicated in many spices cinnamon, clove, cranberries and prunes. It provides natural protection against pathogenic organisms. Cinnamic acid and its derivatives were studied for its various biological activities as antioxidant, hepatoprotective, antioxidant, insect repellent, antidiabetic and anti cholesterolemic Kebieche, M (2009)

CONCLUSION

The present work was an investigation to confirm the traditional use of *Rhamnus alaternus* L. leaf to decrease blood sugar levels in people with diabetes in the Algerian population. In light of these results we can conclude that methanol extract of *Rhamnus. alaternus* L leaves was rich in phenolic compounds that represent a source of bioactive molecules. The methanol extract of *Rhamnus alaternus* improved the biochemical parameters such as serum glucose, urea, serum protein, serum creatinine, serum cholesterol and serum triglycerides as well as the body weight of diabetic rats.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Seridi Ratiba and Hamed M El-Shora and Ouchtati Sara designed and performed the experiments and wrote the manuscript. Also, all authors read and approved the final version.

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