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BENEFICIAL EFFECTS OF *ROSMARINUS OFFICINALIS* AND *THYMUS NUMIDICUS* ON KEY ENZYMES OF CARBOHYDRATE METABOLISM IN ALLOXAN-INDUCED DIABETIC RATS

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ABSTRACT

The aim of this study is to evaluate the protective effects of the ethyl acetate fraction of *Rosmarinus officinalis* (RO-EA) and *Thymus numidicus* (TN-EA) plant extracts on carbohydrate metabolism enzymes during diabetes mellitus. To achieve this objective, two experiments were carried out. The first of which was evaluating the hypoglycemic and the anti-hyperglycemic effects on normal rats overloaded with glucose (4 mg/kg b.w), with the use of two doses (150 and 300 mg/kg b.w), for each plant in the presence of negative control (vehicle 0.5 ml/100 g b.w), and positive control (5 mg of glibenclamide /kg b.w). The second experiment aims at studying the dose-dependent effect of the two plants' extracts on carbohydrate metabolism in Wistar rats rendered diabetic by intraperitoneal injection of Alloxan at (120 mg/kg b.w); 54 male rats of the Wistar strain were divided into 09 groups with 06 rats per group. 03 of the groups have normal rats receiving vehicle (0.5 ml/100 g bw), TN-EA (300 mg/kg b.w), RO-EA (300 mg/kg b.w), respectively. The 06 remaining groups were composed of diabetic rats receiving vehicle (0.5 ml/b.w), TN-EA (150 and 300 mg/kg b.w), RO-EA (150 and 300 mg/kg b.w) Glibenclamide (5 mg/kg b.w), respectively. The process lasted for 21 days. Then we proceeded to the assays.

The analysis of the results showed a dose-dependent reduction in glycemia for the anti-hyperglycemic and hypoglycaemic tests of the RO-EA with the dose of 150 and 300 mg/kg b.w, the latter demonstrated a significant increase in insulin and hemoglobin hexokinase enzyme activity, glucose-6-phosphate dehydrogenase and a concomitant effect modulating plasma glucose levels. In addition to decreased plasma HbA1c levels, glucose-6-phosphatase and fructose-1,6-bisphosphatase enzymatic activity in diabetic rats. The administration of this fraction prevented the loss of body weight and led to an improvement in the activity of glycogen synthase and glycogen phosphorylase. In conclusion, the RO-EA fraction at a dose of 300 mg/kg b w has an anti-hyperglycemic power in diabetic rats, these results were confirmed by histological examination of the liver.

Keywords: Alloxan, Carbohydrate metabolic enzymes, Diabetes mellitus, Glycogen, *Rosmarinus officinalis*, *Thymus numidicus*

INTRODUCTION

Diabetes mellitus is a major public health problem in the world. The prevalence of which has increased considerably over the past two decades and is expected to affect 592 million adults by 2035 **(Paneni et Cosentino, 2015; El Gayar *et al.*, 2019)**. Diabetes mellitus is a metabolic syndrome characterized by the presence of hyperglycemia linked to a dysfunction in insulin secretion or the action of insulin on their receptor or on the post-receptor signal **(Vieira *et al.*, 2019; Yong *et al.*, 2021)**, associated with carbohydrate (at the level of the liver on key enzymes), protein and lipids metabolism disorders which will generate a state of chronic hyperglycemia, combined with secondary complications **(Kalra *et al.*, 2021)** which are attached with the diabetic patients for a long period. These complications can be: microvascular (affecting the eyes, kidneys...) or cardiovascular (atherosclerosis) or neurodegenerative (Alzheimer's) **(Schalkwijk et Stehouwer, 2020)**.

Although the prevalence of diabetes is truly growing, an effective treatment is still lacking. Regular administration hypoglycemic agents on the market including insulin and oral drugs (biguanides, sulfonylurea, α-glucosidase inhibitors) **(Iid *et al.*, 2020)** generate undesirable effects or decreased response after a long period of treatment **(Kumar *et al.*, 2017)**, this led World Health Organization to make it a major public health concern in 2011 and led to the use of traditional medicine for the treatment of diabetes because of their therapy soft. Furthermore, it seems that the conventional treatment is very expensive in developing countries **(Bishu *et al.*, 2019)**, so the use of these plants is affordable and also constitutes a natural reservoir of bioactive compounds **(Widayanti *et al.*, 2020)**.

Nowadays, researchers use all the technological advances to highlight the characteristics and properties of these compounds such as flavonoids, which seem effective in reducing the complications linked to diabetes mellitus **(Sundaram *et al.*, 2019; Hussain *et al.*, 2020; Gandhi *et al.*, 2020)**. Flavonoids were found in several plants of different species, with different quantities and qualities **(Testa *et al.*, 2016; Zhou *et al.*, 2020; Jubaidi *et al.*, 2021)**. Based on these observations, we made ethnobotanical investigations in Algeria which allowed us to choose two very used medicinal plants *Rosmarinus officinalis* and *Thymus numidicus*. The latter seems to be promising in the therapy of metabolic syndrome **(Xie *et al.*, 2017; Rahbardar et Hosseinzadeh, 2020; Carresi *et al.*, 2020)**. Much literature has described *Rosmarinus officinalis* and cited their antioxidant, anti-inflammatory, hypolipidemic and hypoglycemic power under different aspects (essential oil, aqueous extract, methanolic...) **(Karadağ *et al.*, 2019; Othman *et al.*, 2021)**. On the other hand, the plant *Thymus numidicus* has been commonly described in essential oil form, while few studies have been carried out on the antidiabetic effect of these plants in vivo, precisely on the key enzymes of carbohydrate metabolism **(M Shatla *et al.*, 2019; Bacanli *et al.*, 2019; Landazuri *et al.*, 2021)**.

The present work studies the effects of the fraction rich in flavonoid compounds of the plant *Rosmarinus officinalis* and *Thymus numidicus* on the key enzymes of carbohydrate metabolism in rats rendered diabetic by Alloxan.

MATERIAL AND METHODS

Rosmarinus officinalis and *Thymus numidicus* were collected in April 2022, during the flowering period from two localities in the east of Algeria, the plant identification was done by a botanist, cleaned, and dried out off the sun. The voucher specimen (05-2022) was deposited in the herbarium of the department of applied biology of Larbi-Tebessi, university, Tebessa-Algeria.

The extraction of the flavonoid fraction was carried out according to the protocol of **(Markham, 1982)**, modified by **(Bruneton, 1993)**. This method includes 2 main phases: a solid-liquid phase and a liquid-liquid phase, the last phase consists of confronting several solvents with the extract to recover different compounds according to their polarity with the solvent, only the ethyl acetate fraction of

Rosmarinus officinalis and *Thymus numidicus* (RO-EA and TN-EA) was the subject of our study.

Experimental animals

Healthy male Wistar rats of the *Rattus novergicus* aged 2 months weighing between 200 and 220g with no prior drug treatment, were used just for the present study, were obtained from the Pasteur Institute, and the animals were acclimatized to laboratory hygienic conditions before 10 days to starting the experiment under these conditions (temperature: 23c° ± 2 c° and natural photoperiod: 12h light and 12h dark), they were fed with pellet diet (ONAB-Elharouche, Skikda-Algeria), and water available adlibitum.

Acute toxicity studies

Before starting our study on diabetes an acute toxicity test was done to evaluate our flavonoid fraction, this test was determined according to the guidelines of the OECD n° 420 (organization for economic cooperation and development). Male rats (200-220g) were used and divided into different groups of 6 rats. Each group received different doses of test sample up to 2000 mg/kg b.w, after that the animals were monitored for 14 days to confirm if there are mortalities or detected behavioral response. In the end, no deaths were found up to the dose 2000 mg/kg b.w, Therefore, 300 mg/kg b.w was chosen as the most advanced experimental dose.

Anti-hyperglycemic activity of R.O-EA and T.N-EA in glucose-loaded normal-animals

The antidiabetic activity of the ethyl acetate fraction was evaluated according to the method of **(Jarald *et al*., 2009)** with a slight modification. The animals were randomly divided into 6 groups, 6 rats for each, the negative control received only the vehicle solution 1% Tween 80 dose 0,5 ml/100g b.w, and the positive control received a dose of glibenclamide 5 mg/kg b.w. The other groups were treated with the extract of RO-EA and TN-EA with different doses (150 mg/kg and 300 mg/kg b.w). The animals received their doses orally with a gavage feeding tube number 7. The blood glucose level of the animals was taken after a period of overnight fasting at a time 0. Just after the first measurement, the animals have received all the treatment and remained to receive the dose of glucose 4 mg/kg b.w, and the other blood glucose measurement was taken at ½, 1, 2, 3h after a glucose dose. Blood glucose was estimated with a glucometer (DIAGNO-CHECK smart) from the tail tips of rats.

Hypoglycemic activity of R.O-EA and T.N-EA in normal fasting animals

The same steps done in the previous test were repeated. However, the rats fasted overnight for 10h, the first group which remained as negative control has received a dose of 0,5 ml/100g b.w of vehicle. The second group received glibenclamide as a reference dose of 5 mg/kg dissolved in water saline (0.9 w/v NaCl). Group 3 up to 6 received the extract dose of RO-EA and TN-EA (150 mg/kg and 300 mg/kg b.w), as mentioned in the table: 2, the blood sample was taken from the tail tip at a time 0 (before oral administration) and ½, 1, 2 and 3h after vehicle, extract and glibenclamide administration. The blood glucose level was done like the previous test.

Dose-dependent effect of extract RO-EA and TN-EA on plasma glucose and insulin level in diabetic rats

Diabetes was induced in the rats (after fasting overnight for 8 hours), with an Alloxan single dose of 120 mg/kg b.w in 0,9 w/v NaCl intraperitoneally. The rats were placed in the cages with bottles filled with 10% glucose for the next 24 h to prevent hypoglycemia. After 72 hours of injection, fasting blood glucose was measured, the rats that showed a blood glucose level more than 300 mg/dl have been accepted for the test. The selected 54 rats were randomly divided into 9 groups, 6 rats for each, as the following table:

Group 1: Normal control rats(vehicle 0,5 ml/100g b.w)
Group 2: Normal + TN-EA (300 mg/kg b.w)
Group 3: Normal + RO-EA (300 mg/kg b.w)
Group 4: Diabetic control rats(vehicle 0,5 ml/100g b.w)
Group 5: Diabetic + TN-EA (150 mg/kg b.w)
Group 6: Diabetic +TN-EA (300 mg/kg b.w)
Group 7: Diabetic + RO-EA(150 mg/kg b.w)
Group 8: Diabetic + RO-EA(300 mg/kg b.w)
Group 9: Diabetic +Glibenclamide(5 mg/kg b.w)

The extract was dissolved in vehicle (1% Tween 80), glibenclamide was dissolved in water saline 0.9 w/v NaCl, water and food consumption were monitored throughout the 21-day period at a fixed time for each group and renewed each day, and the weight of the rats was taken before the start of treatment and before the sacrifice. After 21 days of treatment, rats were fasted overnight then anesthetized and sacrificed by cervical decapitation, blood was collected to measure the serum concentration of glucose and insulin, and the treatment group which appeared to show a good improvement in plasma glucose and insulin level was selected to determine the Hb and HbAc1 level and also for histological part and assay of the key carbohydrate metabolism enzyme, the liver was recovered in 10% of formalin solution and immediately sent for histological study, the liver was rinsed with ice-cold saline, homogenized with a 10% w/v of 0,1 M Tris-HCl buffer (pH 7,4), then centrifuged (10000× g for 15 min at 4° C), the supernatant was used for the determination of hepatic enzyme and glycogen.

Biochemical assays

The serum concentration of glucose was estimated using the COBAS INTEGRA® 400analyzer and commercial kit Spinreact reference (Ref: 1001190) **(Kaplan, 1984)**, Hb and HbAc1 level was measured using the commercial kit Spinreact reference (Ref: 1001230) **(Franco, 1984)** and (Ref: 43099) **(Trivelli, 1971)**, the level of insulin was measured by electro-chemiluminescence method **(Sapin, 2003)**. Carbohydrate metabolism enzymes were estimated according to the following methods: glycogen synthase according to the protocol of **(Leloir et Goldemberg, 1962)**, fructose-1, 6-bisphasphatase according to **(Gancedo et Gancedo,1971)**, glycogen content according to **(Morales *et al*., 1973)**, glucose 6 phosphate dehydrogenase according to **(Ells et Kirkman, 1961)**, glycogen phosphorylase according to **(Cornblath *et al*., 1963)**, glucose-6-phosphatase according to **(Hikaru etToshitsugu., 1959)**, hexokinase according to **(Brandstrup *et al*., 1957)**.

Statistical analysis

The results are expressed as means ± standard deviation, (n =6), Difference between groups was assessed by analysis of variance (One Way ANOVA,) followed by a Tukey post-hoc test using Graph Pad prism 8.0.1. software, the level of significance was set at P <0.05.

RESULTS

Anti-hyperglycemic activity of R.O-EA and T.N-EA in glucose-loaded normal-animals

The rats received its extract at different doses. After 30 min of glucose administration, an increase in serum glucose levels in different groups were observed. The control group reached the level of fasting glucose in 2 hours. On the other hand, the groups treated with glibenclamide and the extract of RO-EA and TN-EA at a dose of 300 mg/kg b.w reached it significantly at the end of the 1st hour compared to the dose of 150 mg/kg b.w (after 2 hours). Comparing the anti-hyperglycemic power of these extracts with glibenclamide, the extract of RO-EA at the dose of 300 mg/kg b.w proved to be significantly P<0.05 very close to glibenclamide (Table 1).

Table 1 Anti-hyperglycemic activity of RO-EA and TN-EA in glucose-loaded normal-animals

Treatment groups (mg/kg b.w)	Blood glucose concentration (mg/dl)				
	0 min	30 min	60 min	120 min	180 min
N.Control	84,35±1,367	130,3±1,528^{b}	109,5±1,515^{b}	84,30±1,399^{b}	82,15±1,412^{b}
N+(Gliben 5)	82,26±1,425	86,32±1,482^{a}	73,54±1,721^{a}	61,37±1,469^{a}	57,28±1,401^{a}
N+TN-EA 150	84,23±1,408	116,4±0,988^{ab}	104,7±0,710^{ab}	92,37±1,446^{ab}	89,53±0,786^{ab}
N+TN-EA 300	86,26±0,573	95,36±1,476^{ab}	86,23±1,380^{ab}	73,39±0,784^{ab}	71,63±0,502^{ab}
N+RO-EA 150	85,50±0,625	102,7±1,459^{ab}	95,31±0,732^{ab}	82,52±1,570^{b}	70,53±0,809^{ab}
N+RO-EA 300	82,67±1,585	91,28±0,882^{ab}	79,37±1,635^{ab}	66,37±0,793^{ab}	59,35±0,456^{ab}

Values were expressed as means ± SEM(n =6), minimal significant level;P< 0,05, significantly difference; ^{a} in respect to N.Control,^{b} in respect to N+Gliben, (ANOVA followed with Tukey test), N; normal, Gliben; glibenclamide, TN; *Thymus numidicus*, RO; *Rosmarinus officinalis*, EA; ethyl acetate fraction.

Hypoglycemic activityof RO-EA and TN-EA in normal fasting animals

The evaluation of the hypoglycemic activity has clearly shown that extract of the RO-EA fraction exhibited a very significant hypoglycemic activity compared to the control group, and a significantly strong $p<0.05$ at the dose 300 mg/kg b.w compared to the dose 150 mg/kg b.w, this activity is dose-dependent, it was observed after the 1st 30 min. On the other hand, with the extract of the TN-EA fraction no hypoglycemic activity was recorded with the two-doses, no significant difference $p\geq0.05$ compared to the control group (Table 2).

Table 2 Hypoglycemic activity of RO-EA and TN-EA in normal fasting animals

Treatment groups (mg/kgb.w)	Blood glucose concentration (mg/dl)				
	0 min	30 min	60 min	120 min	180 min
N.Control	84,41±1,991	82,97±1,162[b]	83,45±0,849[b]	81,19±1,551[b]	83,47±1,913[b]
N+(Gliben 5)	81,45±0,822	47,22±0,708[a]	34,27±1,512[a]	31,35±0,792[a]	32,41±1,435[a]
N+TN-EA 150	82,34±1,441	81,31±1,454[b]	83,31±0,567[b]	80,22±0,711[b]	81,38±3,401[b]
N+TN-EA 300	81,37±1,476	83,53±0,748[b]	83,38±0,726[b]	80,38±1,467[b]	80,36±0,601[b]
N+RO-EA 150	82,43±0,697	74,33±1,419[ab]	67,35±0,765[ab]	63,03±1,123[ab]	59,35±1,501[ab]
N+R.O-EA 300	83,40±1,170	67,33±1,347[ab]	59,50±1,448[ab]	51,51±1,256[ab]	45,27±2,236[ab]

Values were expressed as means ± SEM (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control, [b] in respect to N+Gliben, (ANOVA followed with Tukey test), N; normal, Gliben; glibenclamide, TN; Thymus numidicus, RO; Rosmarinus officinalis, EA; ethyl acetate fraction.

Dose-dependent effect of extract RO-EA and TN-EA on plasma glucose and insulin level in diabetic rats

Analysis of the results showed a significant increase in the level of serum glucose and a significant decrease in the level of plasma insulin in the diabetic control rats. After 21 days of treatment with RO-EA and TN-EA fraction extract, the rats exhibited good improvement, it prevented the increase in plasma glucose and reversed the serum insulin level when compared to the diabetic rat control, moreover, it was observed that the dose of 300 mg/kg b.w of the RO-EA fraction was more promising compared to the other dose and the other fraction (TN-EA), it significantly prevented the increase in glucose and the decrease in insulin, it was observed that the effect of this dose the most similar to glibenclamide, this dose was fixed as an effective dose for the rest of our study. Regarding the administration of RO-EA and TN-EA fraction extract to normal rats, no significant change was observed in insulin and serum glucose levels when compared to normal control rats (Fig. 1).

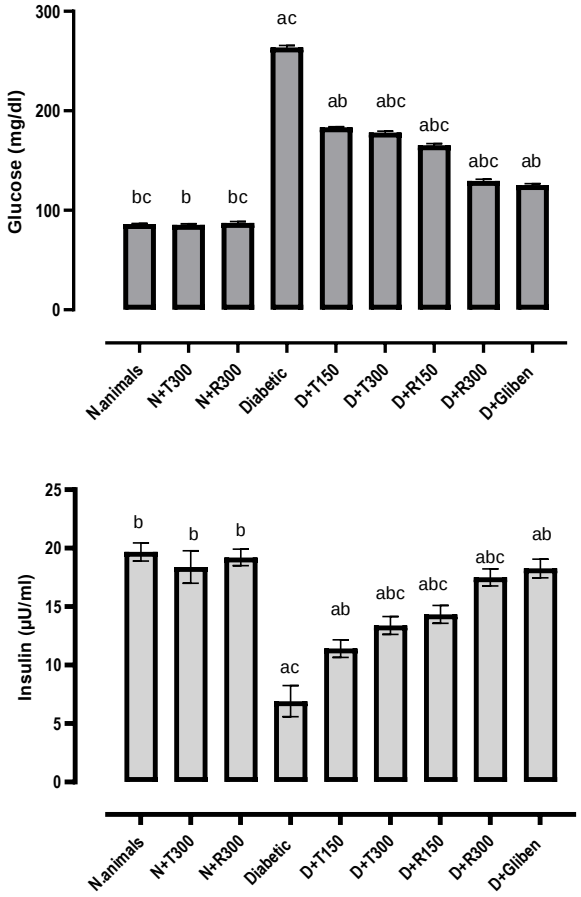


Figure 1 Dose-dependent effect of extract RO-EA and TN-EA on plasma glucose and insulin level in diabetic rats. Values were expressed as means ± SEM(n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control, [b] in respect to Diabetic-induced,[c]in respect to D+gliben, (ANOVA followed with Tukey test), N; normal, Gliben; glibenclamide, TN; *Thymus numidicus*, R.O; *Rosmarinus officinalis*, EA; ethyl acetate fraction.

Effects of the extract R.O-EA fraction in a change in body weight, food, and water intake

The evaluation of the results was shown a significant decrease in body weight in diabetic control rats, and a significant increase in water and food consumption, compared to normal rats, all these changes were significantly restored during treatment with the extract of the RO-EA fraction and glibenclamide. Concerning normal control rats and normal rats treated with the RO-EA fraction no significant change was observed between them (Fig. 2. 3. 4).

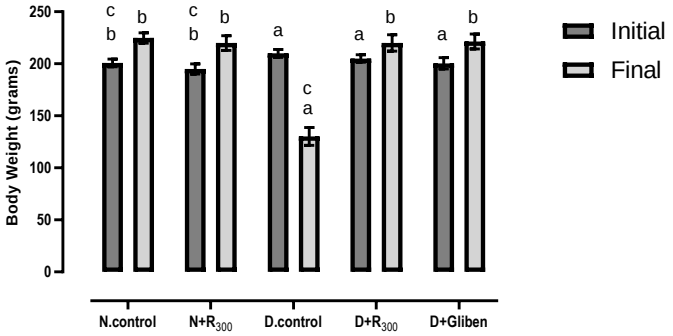


Figure 2 Effect of RO-EA on body weight in control and experimental rats. Values were expressed as means ± SEM (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control, [b] in respect to Diabetic control, [c] in respect to D+gliben, (ANOVA followed with Tukey test), N; normal, D; diabetic, Gliben; glibenclamide, R.O; *Rosmarinus officinalis*, EA; ethyl acetate fraction.

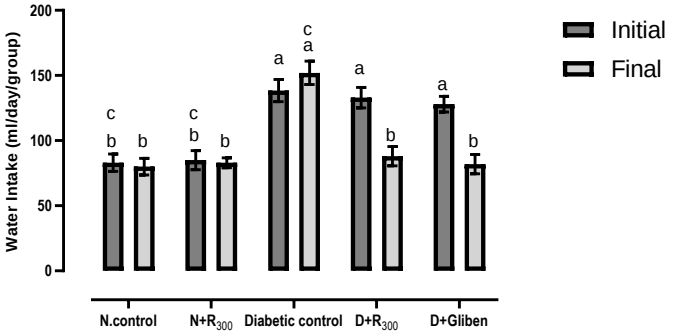


Figure 3 Effect of RO-EA on water intake in control and experimental rats. Values were expressed as means ± SEM (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control, [b] in respect to Diabetic control, [c] in respect to D+gliben, (ANOVA followed with Tukey test), N; normal, D; diabetic, Gliben; glibenclamide, RO; *Rosmarinus officinalis*, EA; ethyl acetate fraction.

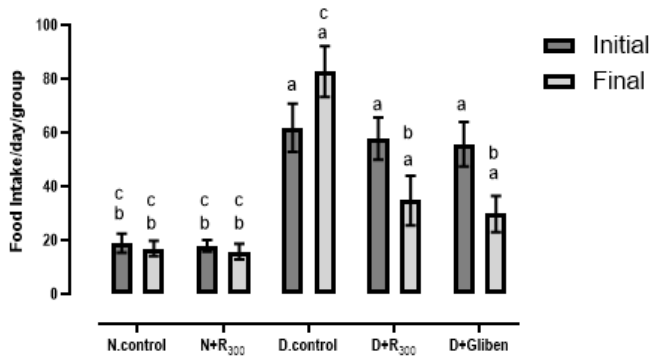


Figure 4 Effect of RO-EA on food intake in control and experimental rats. Values were expressed as means ± SEM (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control, [b] in respect to Diabetic control, [c] in respect to D+gliben, (ANOVA followed with Tukey test), N; normal, D; diabetic, Gliben; glibenclamide, R.O; *Rosmarinus officinalis*, EA; ethyl acetate fraction.

Effect of RO-EA fraction on the level of Hb and HbA1c

Table 3 summarizes the blood Hb and HbA1c levels in the control and experimental rats. This table shows that the diabetic rats suffered a significant decrease in the level of Hb and a significant increase in the level of HbA1c when compared with normal control rats. After treatment with RO-EA extract fraction and glibenclamide, a good improvement was observed with diabetic rats, the blood level of Hb and HbA1c was restored. No significant difference was observed in normal rats treated with the RO-EA fraction at the dose of 300 mg/kg b.wt when compared with normal control rats.

Table 3 Effect of RO-EA fraction on the level of Hb and HbA1c

Groups	Hb (g/dl)	HbA1c (% Hb)
Normal Control	14,43±1,61 [b]	5,554 ±1,43 [b]
N+RO-EA 300 mg/kg b. w	15,59±0,69 [b]	5,480 ±1,35 [b]
Diabetic control	6,418±1,51 [a c]	12,36 ±1,46 [a c]
D+RO-EA 300 mg/kg b. w	12,50±0,79 [b]	7,544 ±2,12 [b]
D+Gliben 5 mg/kg b. w	12,88±0,66[b]	6,246 ±1,57 [b]

Values were expressed as means ± SEM (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control, [b] in respect to Diabetic control, [c] in respect to D+gliben, (ANOVA followed with Tukey test), N; normal, D; diabetic, Gliben; glibenclamide, RO; Rosmarinus Officinalis, EA; ethyl acetate fraction.

Effect of RO-EA fraction extract on activities of carbohydrate metabolic enzymes

The analysis of the results from (Table 4) shows the effect of the RO-EA fraction extract on the activity of carbohydrate metabolism enzymes in the liver of control and experimental rats, we noticed a significant decrease in hexokinase and glucose-6-phosphate dehydrogenase activity and a significant increase in glucose-6-phosphatase and fructose-1,6-bisphosphatase activity in diabetic rats compared to normal rats. It was noted that this activity returned almost to normal when applying treatment with RO-EA fraction extract and also the same was observed with glibenclamide drug. No significant difference was noticed in normal rats treated with RO-EA compared to normal rats.

Table 4 Effect of RO-EA fraction extract on activities of Hexokinase, Glucose-6 phosphate Dehydrogenase, Glucose-6-phosphatase, Fructose-1, 6-bisphosphatase, in the liver of control and experimental animals

Groups	Hexokinase (unit/g protein)	Glucose-6 phosphate Dehydrogenase ($\times 10^{-4}$mL U/mg protein)	Glucose-6-phosphatase (unit/min/mg protein)	Fructose-1, 6-bisphosphatase (unit/h/mg protein)
Normal Control	163,3±6,05	3,870±0,18	0,2054±0,012	5,095±0,69
N+RO-EA 300 mg/kg b. w	159,7±5,09	3,950±0,14	0,2210±0,016	5,149±0,78
Diabetic control	111,1±3,81[b]	1,300±0,45[b]	0,5432±0,019[b]	11,27±0,81[b]
D+RO-EA 300 mg/kg b. w	149,5±5,41[c]	3,040±0,24[c]	0,3274±0,024[c]	7,778±0,71[c]
D+Gliben 5 mg/kg b. w	153,2±2,23	3,360±0,14	0,3072±0,017	7,048±0,95

Values were expressed as means ± SEM(n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control vs N+RO-EA, [b] in respect to N.control vs Diabetic control, [c] in respect to Diabetic control vs D+RO-EA, [d] in respect to Diabetic control vs D+Gliben, (ANOVA followed with Tukey test), N; normal, D; diabetic, Gliben; glibenclamide, RO; *Rosmarinus Officinalis*, EA; ethyl acetate fraction.

Effect of RO-EA fraction extract on glycogen content, glycogen synthase, glycogen phosphorylase, and liver weight

Table 5 summarizes the variations in liver weight, glycogen content, and the enzymatic activity of glycogen synthase and glycogen phosphorylase in control and experimental rats, a significant decrease in liver weight, glycogen level, and glycogen synthase activity was observed in diabetic rats, while glycogen phosphorylase activity was increased significantly compared to normal rats, so these altered parameters were restored almost to normal upon treatment with RO-EA fraction extract and the same thing was observed with the drug glibenclamide, no significant difference was observed in normal rats compared to normal rats treated.

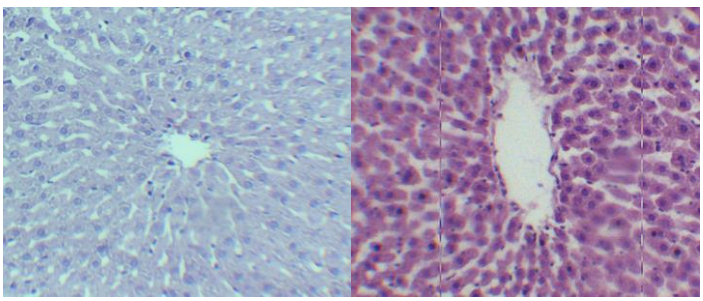
Table 5 Effect of RO-EA fraction extract on glycogen content, glycogen synthase, glycogen phosphorylase, and liver weight

Groups	Glycogen synthase (µ moles of UDP formed/ h/mg protein)	Glycogen phosphorylase (µ moles Pi liberated/h/mg protein)	Liver glycogen (mg/g tissue)	Liver weight (g)
Normal Control	694,6± 26,36	535,6± 20,94	65,37± 2,46	14,45± 2,61
N+RO-EA 300 mg/kg b. w	700,5± 27,17	543,0± 12,28	66,45±3,47	13,59± 1,68
Diabetic control	352,4± 21,25 [b]	791,7± 12,05 [b]	33,75± 1,66 [b]	6,504± 1,69 [b]
D+RO-EA 300 mg/kg b. w	612,0± 19,38 [c]	601,7± 16,08 [c]	58,66± 3,49 [c]	11,48± 2,56 [c]
D+Gliben 5 mg/kg b. w	622,2± 23,57	595,6± 11,11	60,50± 2,68	12,74± 2,57

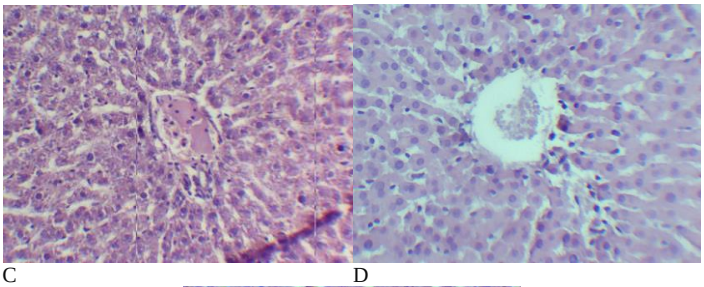
Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to N.Control vs N+RO-EA, [b] in respect to N.control vs Diabetic control , [c] in respect to Diabetic control vs D+RO-EA, [d] in respect to Diabetic control vs D+Gliben, (ANOVA followed with Tukey test), N; normal, D; diabetic, Gliben; glibenclamide, RO; *Rosmarinus officinalis*, EA; ethyl acetate fraction.

Liver histomorphometric study

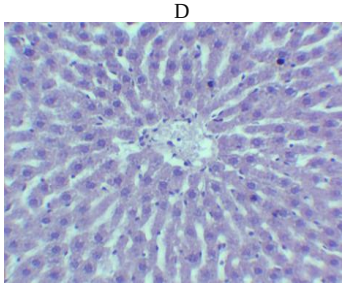
Fig. 5 represents histological sections of the H & E staining of liver tissue, after 21 days of treatment in control and experimental rats, the normal rats represent a normal architecture of the hepatocyte, nucleus, sinusoids, and central veins (A), diabetic rats showed an alteration in the distribution of hepatocytes in radial form, the disappearance of the nucleus, development of necrotic zones around the centrilobular vein with infiltration of inflammatory cells, degeneration of the sinusoids, on the other hand, it was observed that these changes are significantly attenuated in diabetic rats treated with RO-EA or glibenclamide.



A B



C D



E

Figure 5 Histological section of the liver of different groups after 21 days of treatment (40×), (A); normal control, (B); normal rats + R0-EA 300 mg/kg b. w, (C); diabetic control, (D); diabetic rats+R0-EA 300 mg/kg b. w, (D); diabetic rats+ glibenclamide 5 mg/kg b. w

DISCUSSION

Diabetes mellitus is now a serious metabolic disease threatening most people around the world therefore we used an experimental model of diabetes induced by Alloxan **(Ajiboye *et al*., 2020)**, this model is well known to study different hypoglycemic agents **(Sekar *et al*., 2020)**, which have a crucial role in the prevention of complications related to diabetes through the good control of blood glucose **(Preshaw et Bissett, 2019).** On the basis of this statement, we used normal rats loaded with glucose to obtain a hyperglycemic model which will allow us to reveal the anti-hyperglycemic activity of certain plant extracts **(Ogar *et al*., 2018)**. To determine the ability of an extract to prevent hyperglycemia, we used a hyperglycemic model loaded with glucose **(Krishnan *et al*., 2021)**, from this model the 2 fractions of the extract RO-EA and TN -EA revealed an anti-hyperglycemic activity, and more significant at a dose of 300 mg/kg b. w of the RO-EA fraction, we know that a high blood glucose level will stimulate the secretion of insulin which will promote the entry of glucose to the peripheral tissue and are controlled through several mechanisms **(Campbell et Newgard, 2021)**. So from our study (control glucose), it seems that insulin requires 2 hours to reach the fasting glycemia level. On the other hand, in the RO-EA fraction and glibenclamide only 1 hour, which gives an idea of the strong implication of the activity of RO-EA and glibenclamide in the use of glucose **(Mendes *et al*., 2021)**. During a state of glucose tolerance, glibenclamide plays a crucial role in the stimulation of the β cells of the pancreas for the high release of insulin **(Stožer *et al*., 2021)**, therefore it seems that the anti-hyperglycemic activity of the flavonoid fraction RO-EA involves an insulin-like effect **(Etsassala *et al*., 2021)**, probably either through the improvement of peripheral glucose consumption, or enhanced the sensitivity of β cells to glucose, which will promotes the release of insulin **(Pereira *et al*., 2019)**, and also either by inhibition of gluconeogenesis enzymes **(Hasanpour *et al*., 2020).** Several different plant extracts with anti-hyperglycemic activity have been described in several literatures **(Jacob et Narendhirakannan, 2019)**, yet the RO-EA fraction exhibited hypoglycemic activity. This activity probably due to the involvement of the RO-EA fraction in the increased release of insulin. This effect seems similar to the mechanism of action of the drug sulphonylureas **(Chinsembu, 2019; Blahova *et al*., 2021)**. On the other hand, the extract of the TN-EA fraction did not exhibit any hypoglycemic activity in normal rats or treated diabetic ones and although it has previously exhibited anti-hyperglycemic activity in the glucose-loaded model **(Honari *et al*., 2018)**, it is proposed that this mechanism is similar to the action of biguanide drug **(Kifle *et al*., 2022)** which does not promote insulin release, it is involved in the enhancement of glucose uptake by peripheral tissues, reduce hepatic gluconeogenesis **(Herrera-Balandrano *et al*., 2021)**, moreover it has antihyperglycemic and non-hypoglycemic activity **(Haile *et al*., 2021)**.

Alloxan induces diabetes mellitus through selective necrosis of β cells of the pancreatic islets, therefore the amount of insulin decreases an imbalance in glucose control **(Papuc *et al*., 2021)**. The persistence of high glucose levels will trigger a glycation reaction between plasma proteins and glucose **(Toma *et al*., 2020)**, Hb is one of these proteins, a high level of its glycated form HbA1c is a reliable index in the control of glycemia and diagnosis of diabetes. The high amount of HbA1c is proportional to the fasting glucose level in diabetic patients **(Lundholm *et al*., 2020)**, the administration of the RO-EA fraction and glibenclamide for 21 days significantly reversed the insulin and glucose levels and restored the levels of Hb and HbA1c to almost normal in treated diabetic rats **(Gerges *et al*., 2021)**. We can conclude that the restoration of insulin level is produced by the effect of the RO-EA fraction that leads to an improvement in glucose control **(Rodrigues *et al*., 2019)**, probably by inhibition gluconeogenesis or improvement of peripheral glucose consumption **(He *et al*., 2019; Momtaz *et al*., 2019)**. Several studies have cited the side effects of diabetes induced by Alloxan in rats, among these effects are polyphagia, polydipsia, wasting, and muscle loss **(Elangovan *et al*., 2019)**, the latter due to insulin deficiency which leads to increased protein catabolism, the increase in proteolysis aims to compensate for the role of carbohydrates in the production of energy **(Arcaro *et al*., 2021)**. The administration of the RO-EA fraction and glibenclamide improves the recovery of body weight, consumption of water and food, the restoration body weight in treated diabetic rats probably due to increased glycemic control which in turn will lead to decreased proteolysis **(Rodrigues *et al*., 2019; Salles *et al*., 2021; Saravanakumar *et al*., 2020)**. Diabetes mellitus has a direct or indirect impact on glucose control, generally by the decrease in insulin release, insulin-dependent enzymes such as hexokinase, glucose-6-phosphate dehydrogenase, and glycogen synthase will be inhibited **(Kalaivani et Sankaranarayanan, 2021; Mabate *et al*., 2021)**, which will promote the activity of enzymes of gluconeogenesis and glycogenolysis (glucose-6-phosphatase, fructose-1,6-bisphosphate, glycogen phosphorylase) **(Sundaram *et al*., 2019)**, and consequently, hepatic glycogen will be depleted, and proteolysis increases, which will lead to a decrease in liver weight **(Balakrishnan *et al*., 2019)**. In our study, we recorded all these alterations in diabetic rats induced by Alloxan, the administration of the RO-EA fraction and glibenclamide reversed all these alterations, the weight of the liver was recovered following the increase in the enzymatic activity of hexokinase, and glucose-6-dehydrogenase and glycogen synthase **(Zangeneh *et al*., 2018; Vinayagam *et al*., 2018; Krishnan *et al*., 2020)**, which are insulin-dependent enzymes, play a role in the control and metabolism of glucose, therefore the enzymatic activity of glucose-6-phosphatase, fructose-1,6-bisphosphatase and glycogen phosphorylase was decreased compared to untreated diabetic rats **(Amadi *et al*., 2021)**, which in turn leads to an increase in hepatic glycogen levels, this improvement in glucose control probably due to the action of the RO-EA fraction and glibenclamide on the regeneration of pancreatic β cells which increases the insulin level **(Sasikala *et al*., 2019; Jugran *et al*., 2021)**,several studies have been described that the RO-EA fraction rich in carnosic acid molecule and carnosol **(Lefebvre *et al*., 2021)**, these molecules directly involved in the activation of AMPK (AMP-activated protein kinase) this protein inhibits gluconeogenesis and promotes glycogenesis, glycolysis, and glucose uptake **(Hasei *et al*., 2021; Roghani-Shahraki *et al*., 2021)**.

The histological study in diabetic rats induced by Alloxan has denied different alterations at the level of hepatic tissue **(Ansari *et al*., 2019)** disappearance of the nuclei, areas of necrosis around the central vein, infiltration of the inflammatory cells **(Rašeta *et al*., 2020)**. These hepatic alterations involve the oxidative stress in their development, and this leads to the progression of complications of diabetes mellitus. Our results showed that the treatment of diabetic rats with the RO-EA fraction and glibenclamide attenuate these alterations on the hepatic tissue almost to normal, this improvement shown by the RO-EA fraction could be due to the action of the acid carnosic and carnosol on hepatoprotection **(Al-Sharafi *et al*., 2020; Singh *et al*., 2022)**.

CONCLUSION

In conclusion, the administration of the extract of the RO-EA fraction (300 mg/kg b.w) to diabetic rats induced by Alloxan has an appreciable effect, it significantly modulates plasma glucose and insulin levels, body weight, food consumption, Hb and HbA1c levels, and hepatic glycogen levels, thus this fraction significantly restored the altered activity of key carbohydrate metabolism enzymes almost to normal, this fraction of the RO extract -EA is considered a therapeutic virtue and can be developed as a treatment for the complication of diabetes mellitus. Further studies are needed to reveal the exact mechanism of action of the RO-EA fraction on key carbohydrate metabolism enzymes.

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