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PHYTOCHEMICAL, ANTIHYPERGLYCEMIC AND ANTIHYPERLIPIDEMIC STUDY OF CRUDE HYDROALCOHOLIC EXTRACT OF AERIAL PARTS OF *MARRUBIUM VULGARE L.* IN NORMAL AND STREPTOZOTOCIN INDUCED-DIABETIC WISTAR RATS

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ABSTRACT: The phytochemical screening, the antihyperglycemic and antihyperlipidemic effects of crude hydroalcoholic extract of aerial parts of *Marrubium vulgare L.* were evaluated in normal and Streptozotocin- induced diabetic in Wistar rats. First, the preliminary phytochemical test to identify the chemical constituents of crude hydroalcoholic extract of aerial parts of *M. vulgare* was carried. Next, the rats were treated with a single intraperitoneal injection dose of 300 mg/kg body weight. The changes in fasting blood glucose level were measured, in the short term, before (0, 60, 120 and 180min) after the treatments. After 14 days experimental period, rats were weighed and blood was collected for measurement of serum glucose, total cholesterol, and triglycerides. The phytochemical investigation of *M. vulgare* led to the characterization of several families of secondary metabolites: flavonoids, coumarins, saponins, tannins, terpenes and/or sterols. The statistical data indicated the very significant ($p<0.0001$) decrease in the fasting blood glucose, total cholesterol and triglycerides, for diabetic rats, 2 weeks after injection of extract studied, compared to control diabetic rat. This decrease is about 61%, 26% and 15%, respectively, compared to initial value day in which the treatment commenced. The hydroalcoholic extract of aerial parts of *M. vulgare L.* has had beneficial effects in reducing the elevated blood glucose level and lipid profile of STZ-induced-diabetic rats.

INTRODUCTION: Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1].

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Over 90% of patients with diabetes have type 2 diabetes; the remainder has type 1 diabetes.

According to the recent data from World Health Organization (WHO) and International Diabetes Federation (IDF), the number of people affected with diabetes worldwide has increased dramatically over recent years. Currently there are over 366 million diabetics worldwide and this is likely to increase to 552 million more by the year 2030 [2].

The disease becomes a real problem of public health in developing countries, where its prevalence is increasing steadily and adequate.

Where its prevalence is increasing steadily and adequate treatment is often expensive or unavailable [3].

In modern medicine insulin therapy is used for the management of diabetes mellitus but there are several side effects such as insulin resistance, anorexia nervosa, brain atrophy and fatty liver [4, 5]. Also, chronic treatment with sulfonylurea and biguanides is associated with some side effects [6]. Therefore, herbal medicine development against the non-communicable disease like diabetes is one of the trust areas of research for finding natural drugs with hypoglycemic activity.

For a long time, diabetics have been treated with medicinal plants based on traditional medicine information. Traditional herbal remedies have increased in popularity everywhere in the world in the recent years. There are several species of medicinal plants popularly used in the treatment of diabetes mellitus.

Ethnopharmacological surveys indicate that more than 1200 plants are used worldwide in traditional medicine for their alleged hypoglycemic activity [7, 8, 9, 10].

Algeria has a rich heritage of medicinal plants of wide diversity, which are used by the local population and the traditional healers for the treatment of several diseases including diabetes[11, 12].

Marrubium vulgare L. (Lamiaceae), commonly known as "Marrîwa, merrîwut" is a widespread Mediterranean plant used in folk medicine to cure a variety of diseases. The plant is reported to possess vasorelaxant [13], antihypertensive [14], analgesic [15], anti-inflammatory [16], and antioxidant properties [17].

M. vulgare is one of medicinal plant used for the treatment of diabetes mellitus in the Maghreb, Algeria [11, 12] and Morocco [18, 19, 20].

The main objective of our study is to elucidate the effect of crude hydroalcoholic extract of aerial parts of *M. vulgare* (EMv), with a single intraperitoneal injection dose of 300 mg/kg body weight (b.w), on hyperglycemia and dyslipidemia in normal and Streptozotocin (STZ)-induced diabetes in rats.

MATERIALS AND METHODS:

Plant materials and extraction: The plant, *M. vulgare* was collected in December 2013 from Mansourah Wilaya of Tlemcen, North Western Algeria.

The aerial parts (leaves and stems) of the plant were washed, dried at room temperature in the dark and then finely ground to a powder.

Fifty grams (50g) of the aerial parts powder was suspended in a solvent mixture of methanol: water (7:3) (500 mL) and heated to boil under reflux for 30 min. The decoction obtained was filtered through Whatman filter paper No.1.

The filtrate was concentrated to dryness in a rotary vacuum evaporator at 45°C, which yielded a sticky material (yield: 9.47%, w/w)

For assuring stability, the residue was stored at −20^{o}C until used.

The fraction (EMv) was dissolved in distilled water just prior to experimentation.

Phytochemical analysis: A preliminary phyto-chemical test to identify the chemical constituents (alkaloids, tannins, saponins, flavonoids, sterols and terpenes, coumarins and reducing sugar) of crude hydroalcoholic extract of aerial parts of *M. vulgare* (EMv) was carried out according to the method described by Trease and Evans (1989) and Harborne (1998) [21, 22].

1. **Test for alkaloids (Mayer's and Wagner's tests):** 2 ml of organic extract and 0.2 ml of dilute hydrochloric acid (1% HCl) were taken in a test tube. Then 1 ml of Mayer's reagent or Wagner's reagent was added. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

2. **Tests for tannins (Ferric Chloride test):** 5 ml of organic extract was taken in a test tube. Then 1 ml of 5% Ferric chloride solution was added.

Greenish black precipitate was indicated the presence of tannins.

3. **Test for saponins (Foam test):** 5 ml of organic extract was shaken vigorously with 5 ml of distilled water in a test tube. The appearance of foam that persisted for at least 15 min confirmed the presence of saponins.

4. **Test for Flavonoids (Shinoda test):** To 2 ml of organic extract, added four pieces of magnesium fillings followed by few drops of concentrated hydrochloric acid. A pink or red color indicates the presence of flavonoid.

5. **Test for steroids and terpenoids (Liebermann's test):** 2 ml of the organic extract was dissolved in 2 ml of chloroform and 2 ml of acetic acid was added and the solution cooled well in ice. Sulphuric acid was then added carefully. A color change from violet to blue to green indicates the presence of a terpenoidal or steroidal nucleus.

6. **Tests for coumarins (Fluorescence response):** Put the organic extract on silica gel chromatography plate, dry under UV light observation, and then spray 1% potassium hydroxide reagent. If there is present blue - green fluorescence, and fluorescence enhancement, then it may contain coumarins.

7. **Tests for anthraquinones:** 1ml of the organic extract was stirred and placed in aqueous ammonia. A color red, by adding acid, indicates the presence of anthraquinones.

8. **Tests for reducing sugar (Fehling's test):** 2 ml of organic extract was added to 1 ml of a mixture of equal volumes of Fehling's solutions A and B, and was boiled for few minutes. A brick red color precipitate was formed in the presence of a reducing sugar.

Animals: Male Wistar rats (*Rattus norvegicus* var. albinus) (aged 2-3 months, weighing 150–250 g) were obtained from the Department of Biology , faculty of Natural Sciences and Life Sciences of the Earth and the Universe, University Abou bekr Belkaid Tlemcen.

Animals were kept under standard environmental conditions (22 ± 2^{o}C); 12:12 h dark/light cycle.

The animals were fed on standard laboratory chew with food and tap water *ad libitum*.

Induction of diabetes mellitus: Diabetes was induced by a single intraperitoneal (i.p) injection of a freshly prepared streptozotocin (STZ) solution (Sigma, No. 242-646-8) (55 mg/kg b.w. in ice-cold acetate buffer 0.1 M, pH 4.5) to overnight-fasted rats [23]. After 72h of STZ injection, diabetic rats (fasting blood glucose level, >250 mg/dl) were selected for the study.

Experimental animals: In the experiment, 20 animals (10 diabetic rats and 10 normal rats) were randomly divided into 4 groups (n = 5/group).

Group 1: Normal control rats (RTN);

Group 2: Normal rats were administrated EMv (RNE);

Groups 3: Diabetic control rats (RTD);

Groups 4: Diabetic rats were administrated EMv (RDE).

The initial bodyweight and blood glucose level of the experimental rats were measured and noted.

Evaluation of anti-hyperglycemic activity: Animals in the control group (RTN and RTD) received a single (i.p) injection of normal saline (NaCl 0, 9%). The test group of animals (RNE, RDE) was treated by crude hydroalcoholic extract of aerial parts of *M. vulgare* (EMv) with a single i.p injection dose of 300 mg/kg b.w. in the short term, blood was collected by a puncture of tail- vein, before (0, 60, 120 and 180 min) after the treatments for estimation of blood glucose with a rapid glucose analyser (elegance glucometer: Accu-Chek).

Anti-hyperglycemic effects of EMv, were sought during two weeks, after i.p injection of 300 mg/ kg b.w., for one dose per week, in normal rats and diabetic rats described previously.

The rodents were weighed and blood was collected for measurement of glucose, total cholesterol, and triglycerides.

After 14 days of treatments, blood samples, collected by retro-orbital sinus puncture using capillary tubes, were centrifuged at 3000 rev/min for 15 min, and the serum obtained was stored (−20 ◦C) until analyzed.

Glucose levels are expressed in mg/dl and changes in blood glucose levels are expressed in percentage (%) compared to 'G_0' indicates the initial glycaemia in which the treatment commenced.

Percentage glycaemia change was then calculated using the following formula:

% decrease glycaemia= [(final glycaemia−initial glycaemia) /initial glycaemia)]×100

Serum levels of total cholesterol and triglycerides were measured enzymatic methods **Fasce (1982) and Fossati et Prencipe (1982)** [24, 25], respectively.

Parameters levels in all groups were determined after 18 h of fasting.

Parameters levels are expressed in mg/dl and changes in blood levels are expressed in percentage (%) compared to '0 day' indicates the initial day in which the treatment commenced.

Statistical analysis: Data were expressed as mean ± SEM, $n = 5$. Statistical analyses were performed using Student's t test. The values were considered significantly different when the *P*-value was less than *0.05* in comparison to baseline values (starting values).

RESULTS:

Phytochemical screening: Table 1 shows the result of the phytochemical screening of crude hydroalcoholic extract of aerial parts of *M. vulgare* (EMv). The result shows the presence of flavonoids, coumarins, saponins, tannins, terpenes and/or sterols, and reducing sugar. Alkaloids and anthraquinones were found to be absent **(Table 1).**

Effect of EMv on blood glucose level: The effect of intraperitoneal injection of 300mg/kg b.w of EMv on blood glucose levels in normal and STZ-induced diabetic rats are shown in Table 2

In the short term, we noted no significant differences in blood glucose for 3 h after intraperitoneal injection of 300mg/kg b.w of EMv in normal rats and STZ-induced diabetic rats (RNE and RDE), compared to controls rats (RTN and RTD).

After 14 days, we recorded a very significant decrease in the fasting blood glucose in STZ-induced diabetic rats (RDE) *(P <0.0001)* compared with control diabetic rats (RTD). This reduction is about 61% compared to the basal blood glucose time '0-day'. By against, we have not noted significant differences in blood glucose levels in rats treated with EMv compared to normal control rats **(Table 2)**.

Effects of EMv extract on lipids parameters: In normal and STZ-induced controls diabetic rats (RTN and RTD), no significant changes of both serum total cholesterol and triglycerides levels were noted after a single administration (i.p) of 300mg/kg b.w of EMv **(Table 3)**.

However, we noted a very significant decrease *(P <0.0001)* in total cholesterol in normal and STZ-induced diabetic rats (RNE and EDE) treated with EMv, compared with '0-day'of the order of 21% and 26%, respectively.

The same, a very significant decrease *(P <0.0001)* in serum triglycerides levels was recorded in STZ-induced diabetic rats (EDE) of about 15%, compared with '0-day' (Table3).

Effects of EMv extract on body weight: After 14 days, body weight of STZ-induced diabetic rats (RTD) was found to be significantly ($p < 0.01$) less compared to body weight in initial day (0 day) with about 13%. This decrease was lower in STZ-induced diabetic rats (EDE) treated with EMV (4%) in the same period **(Table 3)**.

TABLE 1: PHYTOCHEMICAL SCREENING OF THE CRUDE HYDRO ALCOHOLIC EXTRACT OF THE AERIAL PART OF *M VULGARE*

Chemical constituent	Tests	Composition
Alkaloids	Mayer's test	-
	Wagner's tests	-
Tannins	Ferric Chloride test	+
Saponins	Foam test	++
Flavonoids	Shinoda test	+++
Steroids and terpenoids	Liebermann's test	+
Coumarins	Fluorescence response	+++
Anthraquinones	Aqueous ammonia	-
Reducing sugar	Fehling's test	++

+++ means present in large quantity, ++ means present in moderate quantity and + means present in trace or small amount, - Absent.

TABLE 2: EFFECT OF INTRAPERITONEAL ADMINISTRATION OF EMV (300mg/kg b.w) ON BLOOD GLUCOSE LEVEL, IN NORMAL AND STZ-INDUCED DIABETIC RATS ON 14TH DAY

Groups	Fasting serum glucose concentration (mg/dl) ± SEM					% decrease glycaemia[c]
	G_0 (0 day)	1h	2h	3h	14 day	
RTN: Vehicle control	93± 2.4	88± 3.3	91± 2.3	93± 5.2	87± 4.9	(-) 7.7%
RNE: Normal+EMv	94± 8.3	91± 8.4	95± 8.5	88± 9.4	93± 4.8	(-) 1.06%
RTD : Diabetic control	325± 28.5	346± 21.6	345± 31.4	363± 25.0	318± 18.4	(-) 2.22%
RDE: Diabetic+ EMv	350± 13.4	345± 22.9	311± 38.7	297± 36.6	137 ± 24.3 [a,b]	(-) 60.96%

Values were expressed as mean± SEM, n = 5 in each group;

[a] ($p < 0.0001$) by comparison with STZ-induced diabetic control rats RTD; [b] ($p < 0.0001$) by comparison with 0 day; [c] % decrease glycaemia= [(final glycaemia 14 day −initial glycaemia 0 day) /initial glycaemia)]×100, (−): indicates a decrease in blood glucose. '0 day' indicates the initial day in which the treatment commenced; Samples were collected after 18 h of fasting.

TABLE 3: EFFECT OF INTRAPERITONEAL ADMINISTRATION EMV (300mg/kg b.w) ON BODY WEIGHT, TOTAL CHOLESTEROL AND TRIGLYCERIDE IN NORMAL AND STZ-INDUCED DIABETIC RATS ON 14TH DAY

Groups	Body weight (g)		Total cholesterol (mg/dl)		Triglyceride (mg/dl)	
	0 day	14 th day	0 day	14 th day	0 day	14 th day
RTN	234± 21	251± 26	73± 6.6	74± 8.8	95± 2.9	99± 5.2
RNE	252± 04	260± 04	77± 6.5	61± 2.9 [b]	96± 4.5	91± 7.9
RTD	201± 11	174± 23 [d]	98 ± 2.7	93± 3.1	113± 10.4	124± 7.9
RDE	157± 13	150± 20	92± 5.7	68± 4.7[a,b]	117± 11.1	100± 1.31[c,b]

Values were expressed as mean± SEM, n = 5 in each group; [a] ($p < 0.0001$), [c] ($p < 0.01$) by comparison with STZ-induced diabetic control rats RTD; [b] ($p < 0.0001$), [d]($p < 0.01$) by comparison with 0 day; '0 day' indicates the initial day in which the treatment commenced. Samples were collected after 18 h of fasting.

DISCUSSION: This study was carried out in order to elucidate the influence of single intraperitoneal injection for 14 days of 300mg/kg b.w of EMv of crude hydroalcoholic extract of aerial parts of *M. vulgare* (EMv) on Fasting serum glucose, triglycerides, total cholesterol levels and body weight in normal and STZ- induced diabetic rats.

Earlier phytochemical investigation of *M. vulgare* led to the characterization of several families of secondary metabolites: flavonoids, coumarins, saponins, tannins, terpenes and/or sterols.

This result confirms the presence of constituents that are known for their medicinal and biological activity [26].

In this study, methanol was used which has a wide range of solubility in both polar and non-polar metabolites.

The intraperitoneal injection of streptozotocin at the dose of 55 mg/kg into rats was characterized by polydipsia, polyuria, weight loss and hyper-glycemia.

These symptoms agree with the previous findings of Shenoy and Ramesh (2002) [27]. The elevated level of blood glucose observed after 72h of streptozotocin induction confirmed the diabetic state in rats which may be attributed to the selective cytotoxicity effect of streptozotocin on the beta cells [28, 29, 23].

In this study significant hyperglycemia was achieved within 72 hours after STZ (55mg/kg b.w. (i.p)) injection.

Treatment with of EMv at a dose of 300mg/kg b.w for 2 weeks exhibited a very significant *(p<0.0001)* decrease in the fasting blood glucose in STZ-induced diabetic rats as compared to diabetic control, with a decrease in blood glucose of about 61%, compared to the basal glycaemia at time '0 day'.

This result confirms the work of Boudjelal et al., 2012 [30]. They found that oral administration of 200 and 300 mg/kg b.w. of aqueous extract the *M. vulg*are induced an significant effect antidiabetic (dose-dependent effect). A decrease in blood glucose by 50% for the dose 100mg/kg and more than 60% for doses 200 and 300 mg/kg, compared with alloxane induced diabetic controls rats.

Marrubium vulgare is used also in Mexican traditional medicine for the treatment of diabetes mellitus. The hypoglycemic effects produced by the acute administration of various ethanolic extracts (root, leaf and stem) from *M. vulgare* on normoglycemic rats were investigated by Vergara-Galicia *et al* (2012) [31.] Both extracts (root and stem) resulted in significant reductions of glycemia in healthy rat after intragastric administration at a dose of 100 mg/kg.

Roghani et al., (2012) [32] concluded that a long-term oral administration of aerial part of EMv is effective in reducing contractile response of vascular system and probably prevents hypertension in diabetic rats.

The antihyperglycemia effect of EMv could be linked to more than one mechanism. One might involve modulation of insulin secretion and/or insulin action probably related with extrapancreatic and pancreatic effects.

It has been suggested that insulin deficiency in diabetes is associated with a variety of abnormalities in metabolic and regulatory processes, which causes the accumulation of lipids [33].

The most commonly observed lipid abnormalities are hypertriglyceridemia and hypercholesterolemia [34, 35, 36]. Insulin deficiency results in failure to activate lipoprotein lipase thereby causing hypertriglyceridemia [35, 36].

In our present study, there was a very significant decrease in total cholesterol and triglycerides levels, for diabetic rats, 2 weeks after intraperitoneal injection of 300mg/kg of extract studied, compared to control diabetic rat. This decrease is about 26% and 15% for both lipid parameters: total cholesterol and triglycerides, respectively, compared to initial value '0 day' indicates the initial day in which the treatment commenced.

EMv can act by decreasing the cholesterol biosynthesis especially by decreasing the 3-ydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCoA reductase) activity, a key enzyme of cholesterol biosynthesis [37] and/or by reducing the NADPH required for fatty acids and cholesterol synthesis [36, 39].

In addition, EMv may improve hyper-cholesterolemia by modifying lipoprotein metabolism: enhanced uptake of LDL by increasing LDL receptors and/or by increasing the lecithin: cholesterol acyl transferase (LCAT) activity which may contribute to the regulation of blood lipids [40, 41].

The observed correction hypertriglyceridemia may be due to a decrease of fatty acids synthesis, enhanced catabolism of LDL, activation of LCAT and tissues lipases [41] and/or inhibition of acetyl-CoA carboxylase [42] and production of triglycerides precursors such acetyl-CoA and glycerol phosphate [40]

This antihyperglycemic and dyslipidemia activity may be from the presence of the identified phytochemicals which have been previously reported to possess antidiabetic effect [43].

CONCLUSION: These results indicate the presence of antidiabetic and dyslipidemia active principles in the crude hydroalcoholic extract of aerial parts of *M. vulgare*.. The phytochemical screening shows the presence of flavonoids, coumarins and saponins.

Finally, our results give support to the traditional use of *M. vulgare* as an antidiabetic herbal medicine.

However, further studies should be carried out to confirm its antidiabetic activity, determine its toxicity, mechanism of action and identification and quantification phytochemicals present in this extract.

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