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Research Article
Protective Effect of *Rosmarinus officinalis* Flavonoids Rich Fractions Against Testicular and Reproductive Dysfunction in Streptozotocin-induced Diabetic Mice

[1,2]Messaoud Belmouhoub, [1]Alla-Eddine Khellouf, [1]Zakia Ouatah, [1]Ouassila Belkacem, [1]Souhila Djermoune, [1]Silya Khanouche and [1]Mokrane Iguer-Ouada

[1]Laboratoire Associé en Ecosystèmes Marins et Aquacoles, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algérie
[2]Département de Médecine, Faculté de Médecine, Université de Constantine 3, Ali Mendjeli, Algérie

Abstract
Background and Objective: Impotence and infertility are secondary complications of diabetes that affect a large number of diabetic men in the world. *Rosmarinus officinalis* is widely used in traditional treatment of diabetes and several recent researches have shown this action. However, no previous studies investigated the impact of its flavonoids rich fractions on reproductive biology including animal libido and sperm motility in association to antidiabetic action. Therefore, the aim of the present study was particularly to investigate whether flavonoids rich fractions of *Rosmarinus officinalis* could protect the reproductive function of diabetics against the degenerative action of chronic hyperglycemia. **Materials and Methods:** This study was carried out in September-November, 2015, at animal and marine ecosystem laboratories of Bejaia University. The *Rosmarinus officinalis* total extract was obtained by maceration of the powdered leaves in 85% methanol (1:10, w/v) at 50°C. The total extract was fractionated into diethyl ether (DE) and n-butanol (BUT) fractions which are subsequently used in the present study. The different experiments were carried out on streptozotocin-induced diabetic male mice. The experiment mice were divided into 4 groups and treated by tested extracts for 14 days as follow: Normal group, non treated diabetic group, diabetic group treated with 400 mg kg^{-1} of DE fraction and diabetic group treated with 400 mg kg^{-1} of BUT fraction. **Results:** Both fractions showed a significant ($p<0.05$) decrease in glucose level after 14 days of treatment with 37.44 and 44.44%, respectively for DE and BUT fraction. In parallel with antidiabetic action, DE fraction showed a real improving effect on reproductive function and sexual behavior in diabetic male mice. The results demonstrated that DE fraction improved significantly sperm motility (18.22%) compared to the untreated diabetic mice (10.66%). The sexual behavior analyzed trough mount frequency and the number of orientation of male toward females was also maintained in a significant manner in DE treated group. **Conclusion:** These findings revealed that *Rosmarinus officinalis* flavonoids rich fractions, especially DE fraction presented a potential protective effect against the deleterious impacts of diabetes on reproductive function.

Key words: Sexual behavior, streptozotocin, diabetic mice, reproductive function, rosmarinus officinalis, flavonoids

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Corresponding Author: Messaoud Belmouhoub, Laboratoire Associé en Ecosystèmes Marins et Aquacoles, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algérie Tel: +2130551077899

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action or both[1]. Currently, diabetes mellitus is a real public health problem and its prevalence in the world is constantly increasing. In 2013, more than 382 million diabetics are registered and this number is expected[2] to reach 592 million in 2035.

In the long term, chronic hyperglycemia can alter the functioning of the vessels, leading to the development of severe and disabling complications such as nephropathy, retinopathy, cardiovascular disease and limb amputations[3]. In diabetic men, chronic hyperglycemia can also impair the reproductive system, leading to the development of disorders in reproductive function[4].

It is well established that diabetes mellitus is associated with sexual and reproductive dysfunction in males and many diabetic patients present a severe problem in their sexual function[4].

The dysfunction of reproductive system caused by diabetes which can be at multiple levels such as alteration of erectile function[5], disturbances of ejaculation[6], perturbation of spermatogenesis[7], decrease in mobility and concentration of spermatozoa[5] and perturbation in secretion of sexual hormones[8-10].

In this context, many scientific reports have demonstrated that plants extracts can significantly improve reproductive function in diabetic animals models; such as *Sesamum indicum*[11], *Zingiber officinalis*[12], *Rhus coriaria*[13], *Panax ginseng*[14] and other various plants.

The mechanisms action of this plants were largely investigated and several actions have been proposed, such as the decreasing of oxidative stress level[9,15], the enhancement of insulin, gonadotropins and testosterone secretion[13].

Rosmarinus officinalis L. (Lamiaceae) is a small evergreen plant which grows wild in most Mediterranean countries[16]. It is an important medicinal plant with several therapeutic effects. Various pharmacological studies have demonstrated the efficacy of this plant as anti-diabetic[17,18], anti-inflammatory, anti-oxidant[19] anti-bacterial, anti-cancer[20] and as hepatoprotector agent[21]. On reproductive function, Sebai *et al.*[22] demonstrated that the essential oil of *Rosmarinus officinalis* afforded a significant protective effect against reproductive damage in alloxan-induced diabetic rats, by protecting male reproductive organs against oxidative damage. Farzadi *et al.*[23] showed that rosmarinic acid ameliorate the libido and the sexual behavior in diabetic male rats by increasing testosterone level.

However, to our knowledge, no previous studies investigated, in an integrative manner, the impact of flavonoids rich fraction of *Rosmarinus officinalis* on reproductive biology including animal libido and sperm motility in association to anti-diabetic action. Therefore, the aim of the present study was particularly to investigate whether flavonoids rich fractions of *Rosmarinus officinalis* could reduce the impact of diabetes on reproductive function in streptozotocin-induced diabetic mice.

MATERIALS AND METHODS

Chemicals: Streptozotocin, gallic acid and quercetin were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Plant materials: Fresh *Rosmarinus officinalis* L. was collected in Tazmalt-Bejaia locality of Algeria and was authenticated by Pr. M. Sahnoune, Laboratory of Ecology and Environment, Department of Ecology in Bejaia University. Voucher specimen is deposited at the herbarium of Bejaia University. The plant was dried under shade at 25°C and the dried leaves were grounded with a blender.

Extraction and preparation of the flavonoid rich fractions: The flavonoid rich fraction from *Rosmarinus officinalis* leaves was obtained as described by Wang *et al.*[20]. Briefly, the powdered leaves (100 g) were extracted twice by maceration with 85% methanol (1:10, w/v) during 24 h for each time at a temperature of 50°C. After filtration, a last extraction was realized with 50% methanol. The extracts were filtered through a filter paper and dried with a freeze drier. The freeze dried methanol extract was dissolved in distilled water (300 mL) and fractionated with 100 mL of petroleum ether, 100 mL of diethyl ether and 100 mL of n-butanol, respectively. The fractionated samples were then concentrated in a rotating vacuum evaporator at 40-45°C. Diethyl ether (DE) and n-butanol (BUT) fractions were selected to be used in the present study.

Determination of total phenolic compounds: Total phenolic content of the extract was determined using Folin-Ciocalteu reagent as described by Owen and Johns[24]. Each sample (200 mL) was mixed with 1 mL of Folin-Ciocalteu reagent (0.1 N) and incubated at room temperature (25°C) for 5 min before the addition of 800 μL of Na_2CO_3 (7.5%, m/v). The mixture was incubated at 25°C for 60 min and the absorbance was measured at 765 nm using a spectrophotometer (Multiskan Spectrophotometer, Thermo Electron (Thermo

Fisher Scientific, USA). Gallic acid was used for constructing the standard curve and the results were expressed in terms of mg gallic acid equivalents (GAE)/g of extract.

Determination of total flavonoid compounds: Total flavonoid content was determined using aluminum chloride as described by Kumar *et al.*[25], the results were expressed in terms of mg quercetin equivalents (QE)/g of extract. Briefly, 1 mL of the appropriate diluted extract (BUT or DE) in methanol was mixed with 1 mL of $AlCl_3$ (2%) and the mixture was incubate at room temperature for 15 min. The absorbance of the reaction mixture was subsequently measured at 410 nm and the total flavonoid content was calculated as quercetin equivalent.

Experimental animals: Healthy adult males Swiss albino mice of 3 months in age, weighing 25-35 g were obtained from Medicine Faculty of Constantine University, Algeria. Mice were housed under controlled conditions with temperature maintained from 20-25°C and 12 h dark/light cycle. The animals were acclimatized for a 2 week period before starting the experiment and had *ad libitum* access to food and water.

The all procedures used in the present study were carried out in accordance with the care of laboratory animals and the ethical guidelines for investigations of experimental and pain in conscious animals, following the directive number 2010/63/EU of 22 September 2010.

Induction of experimental diabetes: The experimental diabetes was induced according to the method described by Tian *et al.*[26]. After overnight fasting, diabetes was induced by intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, MO) dissolved in 0.1 M cold citrate buffer (pH 4.5) at a dose of 110 mg kg^{-1} b.wt. After 72 h of STZ treatment, development of diabetes was confirmed by measuring fasting blood glucose levels in a tail vein blood sample using a blood glucometer (One Touch Ultra, LIFESCAN, Milpitas, CA 95035 USA). Mice with fasting blood glucose (FBG) level higher than 200 mg dL^{-1} were considered diabetic and used for further tests.

Animal treatment: Twenty mice were divided equally into 4 groups and the duration of experiment was of 14 days. The treatment was administered orally as follows:

- **Group I: (normal mice):** Mice of this group received daily vehicle solution (NaCl 0.9%)
- **Group II: (No treated diabetic mice):** Received daily vehicle solution (Nacl 0.9%)
- **Group III:** Diabetic mice treated daily with 400 mg kg^{-1} of DE extract solution
- **Group IV:** Diabetic mice treated daily with 400 mg kg^{-1} of BUT extract solution

Measurement of blood glucose level: At the time of experiment, fasting blood glucose level was measured at 1, 7 and 14 days of treatment. Blood was collected from the tail vein and glucose level was measured using a commercial glucometer (One Touch Ultra, LIFESCAN, Milpitas, CA 95035 USA).

Effect of tested extract on sexual behavior: This parameter is measured every 3 days during the experiment. Mice from each experimental group are individually placed in observation cage for 10 min for acclimation, an adult female is then introduced into the cage and the sexual behavior of each male mouse is followed for 15 min. The behavioral parameters recorded are the number of mount and the frequency of orientation of males towards females.

Epididymal sperm preparation: After 14 days of treatment, mice were sacrificed; testis of each mouse were collected and each epididymis was thoroughly isolated, lacerated and incubated at 37°C in 1 mL of isotonic NaCl solution. Then, gentle tearing of the epididymal tissue was realized to make spermatozoa swim out into the NaCl solution for later analysis.

Sperm motility analysis: Sperm motility (%) was analyzed 1 h after incubation at 37°C using a computer-assisted semen analysis (Sperm Class Analyzer, SCA Microptic, S.L., Version 3.2.0, Barcelona, Spain).

Statistical analysis: Statistical analysis was carried out using graphPad.Prism.v6.01. Test of significance was evaluated by Student's 't-test' at the level of $p<0.05$. The results were expressed as mean±SEM.

RESULTS

Determination of total polyphénols and total flavonoids: The content of total polyphenols and flavonoids of DE and BUT fractions is shown in Fig. 1. The results showed that, BUT fraction contained 260±7 mg EAG g^{-1} of polyphenols and 113.33±2 mg EQ g^{-1} of flavonoids. The DE fraction contained

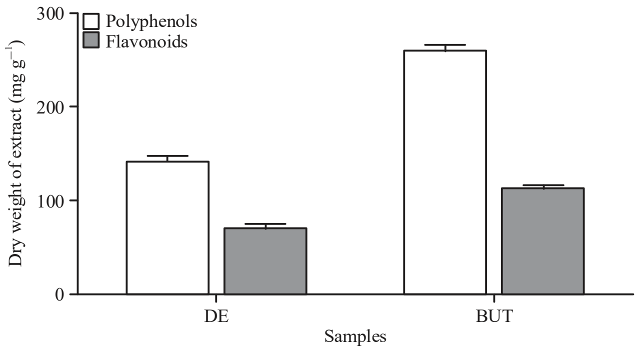

Fig. 1: Total flavonoid and polyphenol content of *Rosmarinus officinalis* leaves

DE: Diethyl ether fraction, BUT: n-butanol fraction, Values are expressed as Mean±SEM

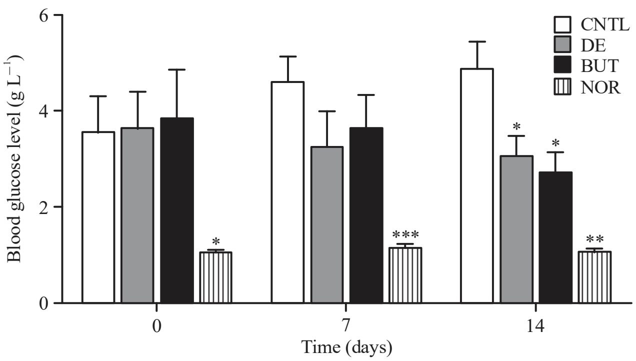

Fig. 2: Fasting blood glucose levels in diabetic mice

CNTL: Diabetic mice, DE: Diabetic mice treated with diethyl fraction at dose of 400 mg kg^{-1}, BUT: Diabetic mice treated with n-butanol fraction at dose of 400 mg kg^{-1}, NOR: Non-diabetic mice, Values are expressed as Mean±SEM, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

Table 1: Number of mounts in diabetic mice during 15 min

Groups	Day 3	Day 6	Day 9	Day 12
CNTL	0	0	3	3
DE	1	3	5	7
BUT	1	0	3	6
NOR	2	4	6	6

CNTL: Untreated mice, DE: Diabetic mice treated with diethyl ether and BUT: Butanol fractions at a dose of 400 mg kg^{-1}, NOR: Normal mice

less polyphenols and flavonoids with 141.66±2 and 70.33±2 mg EQ g^{-1}, respectively. Flavonoids accounted for 50 and 44% of total phenolic compounds in the DE and BUT fractions, respectively.

Effect of DE and BUT fractions on plasma glucose level in STZ-induced diabetic mice: The effect of DE and BUT fractions on fasting plasma glucose levels in STZ-diabetic

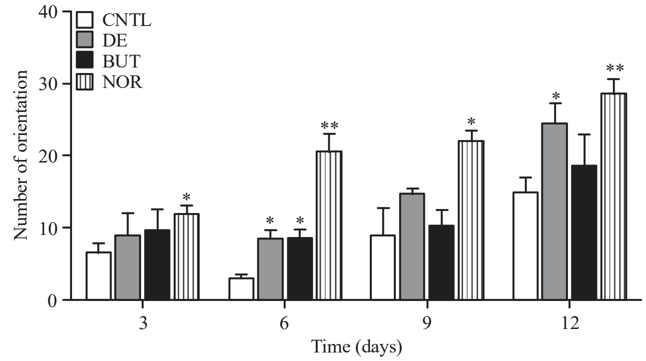

Fig. 3: Male orientation number towards females during 15 min of observation

CNTL: Diabetic mice, DE: Diabetic mice treated with diethyl fraction at dose of 400 mg kg^{-1}, BUT: Diabetic mice treated with n-butanol fraction at dose of 400 mg kg^{-1}, NOR: Non-diabetic mice, Values are expressed as Mean±SEM. $^*p<0.05$, $^{**}p<0.01$

mice is represented in Fig. 2. After 7 days of treatment, the hypoglycemic effect was not observed again, where the glycemia decreased slightly in both groups treated mice. After 14 days of treatment, the glucose level decreased significantly ($p<0.05$) in diabetic mice treated from 3.65-3 g L^{-1} in group treated with DE and from 3.84-2.7 g L^{-1} in group treated with BUT. However, in untreated group mice the glycemia increased from 3.55-4.87 g L^{-1}.

Number of male orientation towards female during 15 min of observation: The number of male mice orientation towards females during 15 min of observation is showed in Fig. 3. After 3 days of treatment, no significant difference was observed in number of orientation between treated diabetic mice (DE and BUT) and untreated diabetic mice. However, after 6 days of treatment, both treated diabetic mice (DE and BUT) presented a significant elevated number of orientation ($p<0.05$) with 9 times during 15 min for each group versus 3 times only in untreated diabetic mice. After 9 days of treatment no significant effect has been noted. At day 12 of treatment, the number of orientation increased significantly in group treated with DE fraction with 25 times compared to the control group (CNTL) with 15 times.

Number of mounts: Table 1 shows the number of mounts measured in diabetic mice during 15 min every 3 days. The results showed that after 6 and 9 days of treatment, the diabetic mice treated with DE fraction presented, respectively 5 and 7 mounts versus 0 and 3 mounts in untreated diabetic mice (CNTL). After 12 days of treatment, both diabetic mice

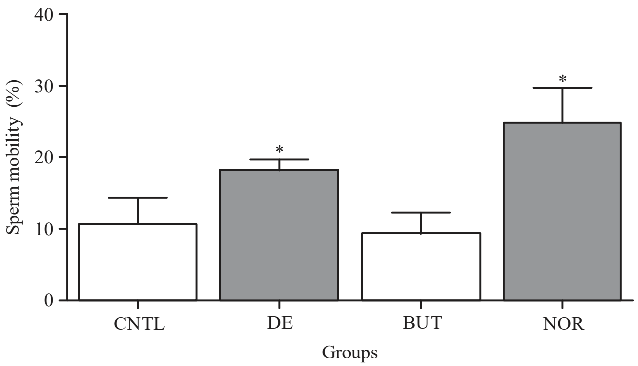

Fig. 4: Sperm mobility (%) after 14 days of treatment
CNTL: Untreated diabetic mice, DE: Group of diabetic mice treated with a 400 mg kg^{-1} of the diethyl ether fraction, BUT: Group of diabetic mice treated with a 400 mg kg^{-1} of the n-butanol fraction, NOR: Group of untreated non-diabetic mice, Values are expressed on average±SEM (*p<0.05)

groups treated with flavonoids rich fractions showed an important frequency of mounts, with 6 times in group treated with BUT and 7 times in group treated with DE.

Effect of DE and BUT on sperm motility: The results of the effect of DE and BUT fractions on sperm mobility are shown in Fig. 4. Sperm mobility was significantly improved in diabetic mice treated with DE fraction with 18.22% of versus 10.66% in the CNTL group. The BUT fraction did not show a positive effect on the mobility of the spermatozoa (9.33%). The best sperm mobility is recorded in the NOR group (24.66%).

DISCUSSION

Diabetes mellitus is one of the main causes of the sexual behavior and reproductive dysfunction in men[27]. It may affect male reproductive function at multiple levels: impairment in semen quality[28], impairment in ejaculation[6], disruption of spermatogenesis[7], decreasing of sperm motility[4] and disturbance in sexual hormones[8-10].

These dysfunctions are the result of severe changes in the vascular system, mainly caused by chronic hyperglycemia[29]. Several studies have shown that diabetes induces an overproduction of reactive oxygen species (ROS) in several cell types, including Leydig and Sertoli cells[4], which consequently affects spermatogenesis and testosterone synthesis[11,27]. The ROS produced by excess glucose concentration are also involved in lipid peroxidation altering consequently sperm morphology and spermiogenesis causing impairment of sperm motility and concentration[29,30].

It has been reported also in some experimental studies that diabetes causes a significant disturbances in sexual behavior[31]. These disturbances are caused mainly by oxidative stress and non-enzymatic glycation of proteins that affect the erectile function at different levels as: damage of genital nerve which reduces the passage of the nerve signal[32], damage at the smooth muscles and endothelial cells of cavernous vessels, reduces thus the synthesis, release and action of nitric oxide (NO), which negatively influence the erection phenomenon[29].

In this respect, plant extracts have been reported to be more effective and potent therapeutic factors against sexual behavior and reproductive system dysfunction[11,13,33].

In this work, the extraction was based essentially on gradient of polarity; diethyl ether solvent was used to recuperate non-polar compounds such as flavonoid aglycones, whereas butanol solvent was used to recuperate the polar compounds such as flavonoid glycosides[34].

The obtained results showed that both flavonoids rich fractions of *Rosmarinus officinalis* (DE and BUT) at the dose of 400 mg kg^{-1} decreased significantly the fasting blood glucose concentration in STZ-induced diabetic mice (Fig. 2). In parallel to antidiabetic effect, the DE fraction (flavonoids non glycosylated) ameliorates also the sexual behavior (Table 1, Fig. 3) and the sperm motility (Fig. 4). It has been reported by some scientific studies that flavonoids of *Rosmarinus officinalis* ameliorate significantly the hyperglycemia in diabetic animal model[17,35].

The anti-diabetic action has been attributed in part to the enhancement of insulin secretion as reported by Bakirel *et al.*[18]. In this respect, insulin could act as an effective factor in the amelioration of reproductive function. In fact, previous reports have shown that insulin enhances testosterone synthesis[4,9,36], FSH and LH secretion and action[37].

Together with insulin action, the high antioxidant activity of rosemary's flavonoids[18] may be the main cause of improvement of the reproductive function, the sexual behavior and the sperm motility in diabetic mice (Fig. 3, 4 and Table 1).

Indeed, *Rosmarinus officinalis* extract is known to contain potent antioxidant components[38,39], such as flavonoids[18,40], which can ameliorate the reproductive function by protecting of the sexual cells against oxidative stress.

It has been shown in some studies that flavonoids, either glycosylated or non-glycosylated, can act directly on the reproductive function[41] or indirectly by their anti-diabetic effect, particularly by improving the secretion of insulin[9,37].

Similarly, various experimental studies have shown that the flavonoids of several medicinal plants can correct erectile dysfunction in animal models by several mechanisms of action such as: inhibition of the RhoA/Rho-kinase signaling pathway

(Ras homolog gene family, member A) an enzyme implicated in vasoconstriction[41,42], protection of testis cells against oxidative stress caused by chronic hyperglycemia[15], improvement of NO synthesis by endothelial cells of cavernous vessels[41] and improvement of spermatogenesis and testosterone secretion[43].

CONCLUSION

On the basis of the current results, DE fraction or rich fraction of non-glycosylated flavonoids of *Rosmarinus officinalis* showed an effective protective effect against the deleterious impacts of diabetes on reproductive performances, while no evident adverse symptoms were observed. The protective effects were revealed at two levels including sperm motility and libido. The current results open up interesting research prospects; they could concern both the plant and the exploration of *in vitro* and *in vivo* effects. In this sense, further investigations could lead to a better identification of the molecules and mechanisms of action involved in the therapeutic effects. Similarly, the protective effect expressed on the reproductive function in the male deserves to be explored in the female while investigating the actual impact on fertility after mating.

SIGNIFICANCE STATEMENT

This study discovers the protective effect of flavonoids rich fraction of *Rosmarinus officinalis* against degenerative action of diabetes on male reproductive system. In this respect, rosemary extracts by its anti-oxidant action can protect the body against other chronic diabetic complications such as retinopathy, nephropathy, neuropathy and cardiovascular complications. Therefore, we can suggest that flavonoids of rosemary can be beneficial in the treatment of diabetic by slowing down the appearance of complications. In the current therapy of diabetes, all antidiabetic drugs used are only meant to reduce chronic hyperglycemia, but none has a double therapeutic effect, anti-diabetic and anti-oxidant. This double action that can be shown by rosemary compounds will probably be the key therapeutic for diabetics.

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