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Original Research

Studies on the Constituents of *Rosmarinus officinalis* and Their Synergistic Effect in Experimental Diabetic Rats

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ABSTRACT

Aim and Background: Antioxidant effects of aqueous extracts of Rosmarinus officinalis (RO) leaves on kidneys of streptozotocin (STZ)-induced experimental diabetic rats were assessed. **Methods:** The experiment was carried out on 4 groups with 6 rats in each group and extracts were administered orally at a dose of 200 mg/ Kg, body weight for 45 consecutive days. Blood samples drawn from all rats and analysed for glucose, reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) as well as kidney function test. **Results:** The present study, revealed a significant decrease in blood glucose level in the animals given herb extract as compared with diabetic rats. In addition, results also revealed that antioxidant markers, GSH and CAT were significantly reduced in diabetic rats, while MDA, uric acid, urea and creatinine have increased as compared with control rats. On the other hand, both the diabetic group and the treated with extract showed significant improvement in the antioxidant markers and renal function towards the control group. Petra/ Osiris/ Molinspiration (POM) analyses of principal bioactive constituents of rosemary showed that these compounds have no side effect. **Conclusions:** Thus, Rosemary extract could be a promising therapeutic agent for the treatment of diabetic-related diseases.

KEY WORDS: *Rosmarinus officinalis,* antioxidant, renoprotective, Petra/ Osiris/ Molinspiration (POM) analyses.

INTRODUCTION

Diabetes Mellitus (DM) is a common and possibly the world's fastest growing metabolic disease. It can be defined as a group of metabolic diseases characterized by chronic hyperglycemia, which is caused by reduced entry/utilization of glucose into/ by various tissues and increased release of glucose into the circulation by hepatic gluconeogenesis[1]. It results from defects in insulin secretion, insulin action or both, causing impaired carbohydrate, lipid and protein metabolism and an increased risk of cardiovascular diseases [2]. DM is recognized as one of the leading causes of morbidity and mortality in the world; about 2.5-7% of the world's population are diagnosed with *Diabetes Mellitus* (DM) [3]. Despite the significant effect of anti-hyperglycemic drugs and insulin sensitizers, there remain side effects that necessitate the search for other alternatives. Medicinal plants may provide such valuable therapeutic alternatives.

Although insulin has become one of the most important therapeutic drugs for treatment of diabetes, efforts are ongoing to find insulin substitutes from other sources. In fact, aside from classical chemically prepared antihyperglycemics, the use of traditional medicinal plants with hypoglycemia effect has recently gained popularity worldwide. More than 400 traditional plant treatments for DM have been reported, but only a small number of these have received scientific and medical evaluation [4].Many plant species are known in folk medicine of different cultures for their hypoglycaemic properties and therefore, are used for treatment of DM

[5], however, very few traditional antidiabetic plants have received proper scientific screening.

Rosemary, *Rosmarinus officinalis* L. (*Labiatae*), is an evergreen perennial shrub grown in many parts of the world [6,7] . It has been reported to possess a number of therapeutic applications in folk medicine in curing or managing of a wide range of diseases such as DM, respiratory and gastrointestinal disorders, and inflammatory diseases [8,9]. The water decoction of rosemary leaves has been traditionally used to treat diabetic patients without much scientific evidence of its utility. Rosemary has been widely accepted as one of the species with the highest antioxidant [10]. It has long been recognised as having antioxidant molecules, such as rosmarinic acid, carnasol, and rosmaridiphenol which are found in ethanol-soluble fraction [10-12]. Hypoglycemic and hepatoprotective properties of the *Rosemainus officinalis* have been reported by different research groups [13].

Rosemary contains a number of biologically active compounds, including antioxidants carnosic acid (1) and rosmarinic acid (2) in addition to other compounds such as ursolic acid (3), betulinic acid (4), rosmaridiphenol (5), and rosmanol (6) (Figure 1). In view of the wide continued interest in the biological activity profile of *Rosmarinus officinalis*, the present work is undertaken to investigate the antioxidant effects of aqueous extract of *Rosmarinus officinalis* leaves on kidneys of STZ-induced experimental diabetic rats along with other antidiabetic parameters. In addition, the principal constituents of *Rosmarinus officinalis* were subjected to Petra/Osiris/ Molinspiration (POM) analysis to get insights on the degree of their toxicity.

METHODS

Animals

Twenty four Sprague–Dawley *albino* male rats (160–200 g) were used in this study. All rats were fed the control diet for 10 consecutive days to adapt to laboratory condition. The basal diet consisted of casein (12.5%), corn oil (10%), choline chloride (0.2%), vitamin mixture (1%), cellulose (5%), salt mixture (4%), sucrose (22%), and corn starch (up to 100%) [14]. Each rat was housed in an individual stainless steel cage under optimal controlled condition in the Animal House of the Faculty of Medicine (*Umm al Qura University*). The experimental protocol was approved by institutional animal ethical committee.

Extract Preparation

Cleaned and sun-dried leaves of Rosemary (*Rosmarinus officinalis*) were crushed in a porcelain grinder to a fine powder to pass through sieve mesh pores of 1mm in diameter. A mixture of 400 g of powder sample and 1000 mL of distilled water was placed in a conical flask equipped with a condenser and was refluxed for one hour. The mixture was then cooled and filtered and the solvent was removed under reduced pressure at 70 °C to afford a dried powder which was then dissolved in distilled water [14].

Induction of diabetes in rats

Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, Missouri, USA) at a dose of 50 mg/ kg body weight. STZ was dissolved

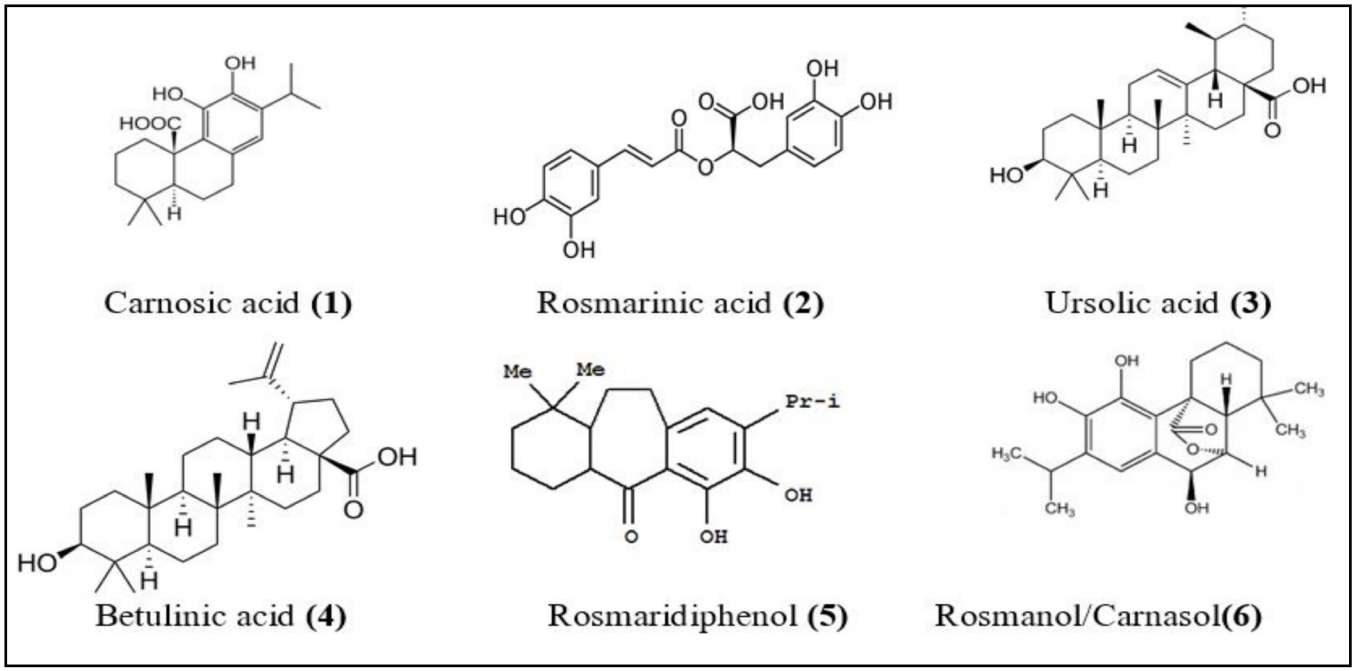

Figure 1. Effect of thiamine and pyridoxine singly and in combination on aspartate transaminase concentration.

immediately before use in 0.05 mol/L sodium citrate at pH 4.5 [15]. Two days after STZ injection, fasting blood samples were obtained by retro-orbital method to estimate fasting serum glucose; rats with fasting serum glucose level of more than 200 mg /dL were considered diabetic for this study.

Experimental Design

Twenty four rats were randomly collected and divided into 4 equal groups (6 animals in each group). In group 1, rats were fed on basal diet for 45 days and served as negative control. Group 2, (DM) diabetic, rats were fed on basal diet for 45 days. Group 3 (DM and RO), diabetic rats and treated orally with 200 mg / kg bw/d of aqueous extract of RO for 45 days. Group 4, (RO) negative control, rats were given, orally, aqueous rosemary extract at a dose of 200 mg/ Kg bw/d [13]. Throughout the investigation period, the total body weight of the experimental rats was monitored.

Blood sampling

Animals were starved overnight and blood samples were collected using the retro-orbital method by means of microcapillary glass heparinized tubes. Blood was collected into a clean dry centrifuge tube and left to clot in a water bath at room temperature for half an hour and then centrifuged for 10 min at 3000 rpm to separate the serum. Serum was carefully aspirated and transferred into clean quick-fit plastic tubes and kept frozen at -20°C until needed for analysis.

Biochemical analysis

The following determinations were carried out for all serum samples: Antioxidant markers, reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA), were determined using commercially available ELISA Kits (Cayman Chemical Co.). Plates were read at 450 nm and a correction wavelength of 550 nm on a computerized automated microplate ELISA reader. Glucose, uric acid, urea, and creatinine were estimated spectrophotometrically (BM Co. Germany, 5010), (Crescent Diagnostic Co. KSA) according to the manufacturer's instructions.

Histological studies

Kidney tissues were processed for histological observation by staining with haematoxylin and eosin stain. Light microscope (CarlZeiss Axiostarpluz model 440950 CP ACHROMAT) coupled with a digital camera (Olympus, model C'7070Wide Zoom) was employed to get photos for observations.

Statistical analyses

Statistical analyses were performed by using SPSS statistical package. Data are expressed as the mean ± standard deviation (SD); differences were considered significant at $P \leq 0.05$.

RESULTS

Body weight

Diabetic rats had significantly lower body weight, compared with healthy control (160.14±4.21 g vs 182.1±5.16 g) ($P < 0.05$), while the diabetic rats treated with RO extract showed a significant increase in body weight (175.0±4.25 g) as compared with diabetic rats.

Glucose levels

Shown in Table 1 are results obtained pertaining to blood glucose of treated and untreated rats. Results revealed a highly significant increase of blood glucose in the diabetic rats (DM) as compared with the control ($P < 0.001$) and a significant decrease in DM + RO group when compared with the diabetic group.

Table 1. Effects of Rosmarinus officinalis aqueous extract on glucose, antioxidant and oxidative stress markers (mean ± SD) in control and diabetic rats.

Parameters	Experiment Groups			
	Control	DM	DM+RO	RO
Glucose (mg/dL)	84.4±2.1^{c}	245.2± 11.2^{a}	142.9±2.8^{b}	81.8±3.1^{c}
GSH (µmol/mL)	14.52±1.45^{b}	8.92±1.25^{c}	12.14±1.15^{b}	19.92±1.68^{a}
CAT (U/mL)	29.25±0188^{b}	14.89±1.92^{d}	21.41±1.72^{c}	39.08±1.96^{a}
SOD (U/mL)	2.34±0.20^{a}	1.78±0.28^{b}	1.92±0.44^{b}	2.49±0.39^{a}
MDA (µmol/mL)	8.95±0.72^{c}	14.10±0.61^{a}	12.12±0.49^{b}	9.11±0.61^{c}

Means in the same raw not followed by the same letter differ significantly ($P < 0.05$)

Antioxidant markers and lipidperoxidation

Levels of the antioxidant markers, GSH, CAT, and SOD have significantly decreased in the diabetic group, while MDA has significantly increased when compared with the control group. On the other hand, treatment with *Rosmarinus officinalis* extract alone caused a significant increase in GSH, and CAT as compared to the control group. Similarly, there was a significant increase in GSH and CAT levels and a decrease in MDA in diabetic and *Rosmarinus officinalis* treated group when compared with diabetic group as displayed in Table 1.

Renal Markers

Listed in Table 2 are results that show the effect of the herb's extract on kidney function of rats. *Rosmarinus officinalis* extract has reduced the concentrations of uric acid, creatinine, and urea in comparison with diabetic rats; this improves kidney function. On the other hand, those parameters uric acid, creatinine, and urea in rats treated with *Rosmarinus officinalis* extract alone did not significantly differ from those of the control group.

Histological Observations

Normal renal architecture was observed in animals of negative control group, however, congestion of blood vessels and eosinophilic proteinaceous casts in the lumen of renal tubules were observed in the diabetic group as shown in Figure 2. On the other hand, group 3 (diabetic animals treated with 200 mg/ Kg bw rosemary extract) displayed interesting observations that included granularity of the cytoplasm of epithelial lining of the renal tubules, pyknosis of some nuclei of epithelial cells lining some renal tubules, and focal area of tubular necrosis associated with mononuclear cells infiltration as depicted in Figure 2. On the other hand, an apparent normal renal parenchyma was noticed in kidneys of animals from group 4 treated with 200 mg/kg bw, aqueous extract of *Rosmarinus officinalis*.

In this investigation, the histological observations, although basic and minimal, reveal that while the renal architecture of the diabetic control animals was heavily damaged, animals treated with the herb seemed to be well protected; this area needs further detailed study.

Collectively, results of the present work indicate that regular daily intake of *Rosmarinus officinalis* dried leaf powder or aqueous extract can have an overall theraputic effect not only on the hyperglycemia and other related parameters but also on the renal architecture of diabetic rats. The herb, therefore, can be utilized as a preferable food adjuvant for hyperglycemic patients. Moreover, our experimental results indicate that the chemical constituents of plants with interesting and biologically important functional groups that may have excellent biological properties, deserve to be further investigated by slightly modifying their structures. For this purpose, we have employed POM analyses which are unique platforms to find pharmacophoric sites responsible for various biological properties and to get an idea about sites of structural modifications for exploring pharmacologically more useful molecules as compared to their precursors.

Molecular properties calculations

For the development of binding approaches for compounds 1-6 of Rosemary, identification of active phamacophoric site structures present is important. The objective of this study is to investigate the potential pharmacophoric sites of potential species. To verify these structures, further Petra/Osiris/Molinspiration (POM) analyses were carried out for example in the calculation of net atomic charges, bond polarity, atomic valence, electron delocalization and lipophicity.

Various investigators have used computational methods to understand differences between natural products and other sources of drug leads. Modern drug discovery is based, in large part, on high throughput screening of small molecules against macromolecular disease targets requiring that molecular screening libraries that contain drug-like or lead-like compounds. We have analyzed known molecules of Rosemary for drug-like and lead-like properties. With this information in hand, we could establish a strategy to design specific drug-like or lead-like bioactive compounds 1–6.

Table 2. Effects of Rosmarinus officinalis aqueous extract on some renal markers (mean ± SD) in diabetic rats.

Parameters	Experiment Groups			
	Control	DM	DM+RO	RO
Creatinine mg/dL	0.81±0.16^{b}	1.52±0.21^{a}	0.95±0.15^{b}	0.74±0.18^{b}
Urea mg/dL	26.31±4.11^{c}	50.11±3.11^{a}	36.35±6.51^{b}	24.92±5.18^{c}
Uric acid mg/dL	1.12±0.22^{b}	1.87±0.18^{a}	1.35±0.25^{b}	1.08±0.16^{b}

Means in the same raw not followed by the same letter differ significantly ($P < 0.05$)

Table 3. Osiris calculations of toxicity risks and drug-score of compounds 1-6

Compd.	MW	Toxicity risks [a]				Drug-score [b]			
		MUT	TUMO	IRRI	REP	CLP	S	DL	DS
1	332					3.52	-4.24	-5.43	0.36
2	360					1.65	-2.23	-2.07	0.49
3	456					5.40	-6.11	-3.66	0.18
4	456					5.44	-6.28	-21.40	0.05
5	316					4.67	-4.72	-4.27	0.30
6	360					2.67	-3.88	-5.19	0.39

: not toxic ; : slightly toxic; : highly toxic. [a] MUT: mutagenic; TUMO: tumorigenic; IRRI: irritant; REP: reproductive effective. [b] CLP: cLogP, S: Solubility, DL: Drug-Likness, DS: Drug-Score.

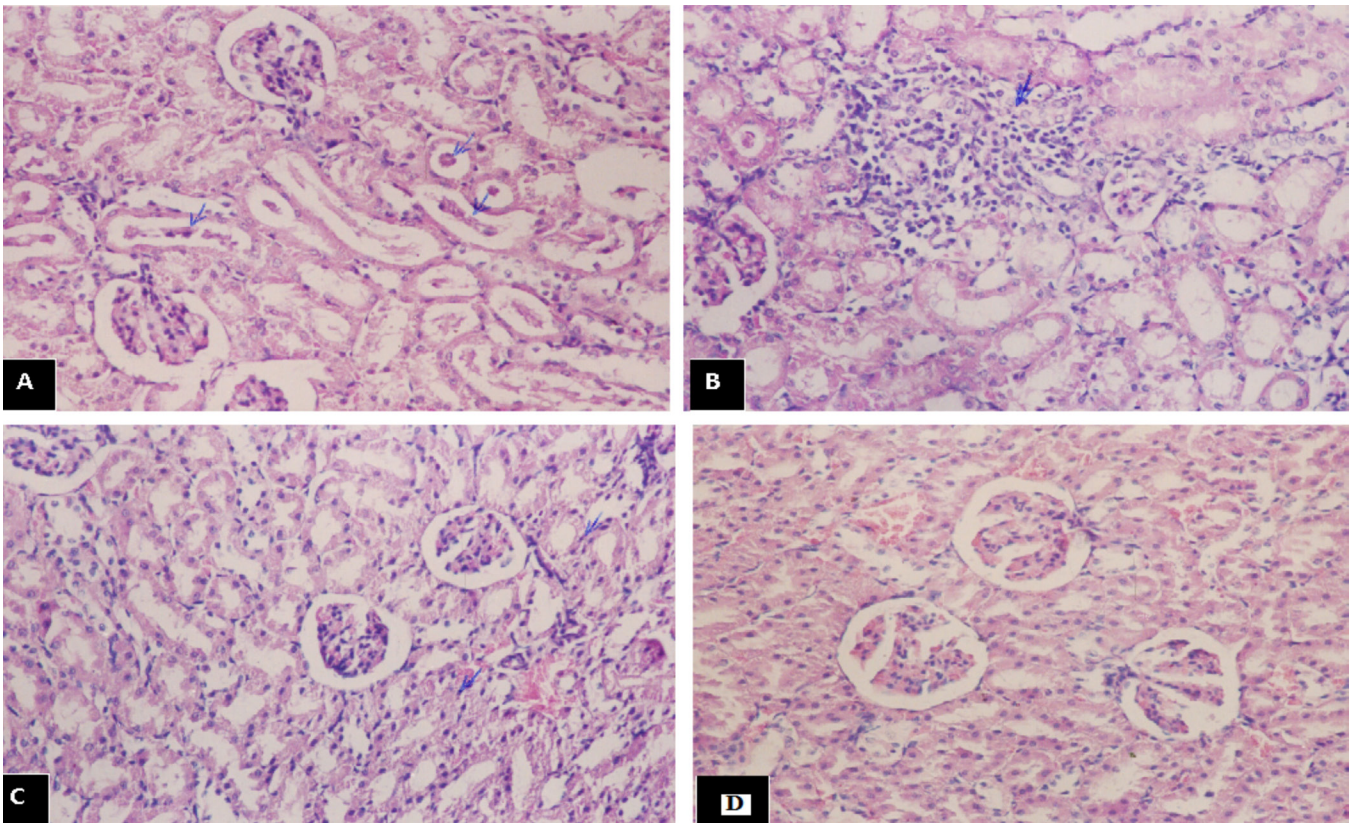

Figure 2. Diabetic animals treated with Rosmarinus extract, group 3: A: Shows proteinaeous casts in the lumen of some renal tubules, B: Shows focal area of tubular necrosis associated with mononuclear cell infiltration. C: Shows atrophy of glomerular tufts and pyknosis of nuclei of renal tubular epithelium (H and E X 200). D: animals treated with Rosmarinus aqueous extract, group RO: Shows a normal renal architecture with no histopathological abnormalities (H and E X 200).

Osiris calculations

Structure based design is now fairly routine but many potential drugs fail to reach the clinic because of ADME-Tox liabilities (Absorption, Distribution, Drug-Drug Interaction, Enzyme Kinetics, Metabolism, Stability and Toxicology) as it is described online (http://pharmacadence.com/services/adme/).

An important class of enzymes, responsible for many ADMET problems, is cytochromes P450. Inhibition of these or production of undesired metabolites can result in many adverse drug reactions. Recently, Bennani et al., [16] have reported combination of various pharmacophoric sites by using a spiro-heterocyclic structure; using this approach, it is now possible to predict activity and/or inhibition with increasing success. This is done using a combined electronic/

structure docking procedure and an example will be given here (Table 3). The remarkably well behaved mutagenicity of divers synthetic molecules classified in data base of CELERON Company of Swiss can be used to quantify the role played by various organic groups in promoting or interfering with the way a drug can associate with DNA.

Data given in Table 3 indicate that, compounds 1-6 are supposed to be non-mutagenic, non-irritating with no reproductive effects when run through the mutagenicity assessment system except for compound 4. The cLogP value of a compound, which is the logarithm of its partition coefficient between n-octanol and water, is a well-established measure of the compound's hydrophilicity. Low hydrophilicities and therefore, high cLogP values may cause poor absorption or permeation. It has been shown that for compounds to have a reasonable probability of being well absorbed, their cLogP values must not be greater than 5.0. On this basis, four compounds have cLogP values under the acceptable criteria. Compounds 1-6 showed moderate to good drug score.

Molinspiration calculations

cLogP (n-octanol/water partition coefficient) is calculated by the method developed by Molinspiration as a sum of fragment based contributions and correction factors (Table 4). The method is very robust and is able to process practically all organic and most organometallic molecules. Molecular Polar Surface Area TPSA is calculated based on the method outlined by Ertl, et al. [17] as a sum of fragment contributions. O– and N– centered polar fragments are considered. PSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability and blood–brain barrier penetration. Prediction results of compounds 1–6 molecular properties (TPSA, GPCR ligand and ICM) are given in Table 4.

Polar surface area (PSA) values are important properties for the prediction of per oral bioavailability of drug molecules. The polar surface area (PSA) is calculated from the surface areas that are occupied by oxygen and nitrogen atoms and by hydrogen atoms attached to them. Thus, PSA is closely related to the hydrogen bonding potential of a compound. Molecules with PSA values of 140 Å or more are expected to exhibit poor intestinal absorption. Table 4 shows that compounds 1-6 are within this limit. These compounds have zero or only one violation of the Rule of 5. Two or more violations of the Rule of 5 suggest possibility of problems in bioavailability. Drug likeness values of compounds 1-6 are listed in Table 4. Drug likeness may be defined as a complex balance of various molecular properties and structure features which determine whether a particular molecule is similar to known drugs. These properties, mainly hydrophobicity, electronic distribution, hydrogen bonding characteristics, molecular size and flexibility and presence of various pharmacophoric features, influence the behaviour of molecule in a living organism, including bioavailability, transport properties, affinity to proteins, reactivity, toxicity, metabolic stability and many others. Activity of all six compounds were rigorously analyzed under four criteria of known successful drug activity in the areas of GPCR ligand activity, ion channel modulation, kinase inhibition activity, and nuclear receptor ligand activity. Results pertaining to compounds 1-6 are shown in Table 4 by means of numerical assignment. Most of these compounds have consistent positive values in most categories and conforming numerical values. Therefore, all of these compounds are expected to have promoting activity against some enzymes and should be used in further enzymatic studies to compare their bioactivity to standard drugs used based upon these four rigorous criteria (GPCR ligand, ion channel modulator, (kinase inhibitor, and nuclear receptor ligand).

Table 4. Molinspiration calculations and drug-score of compounds 1-6.

Compd.	Physico-chemical properties [a]					Drug likeness [b]					
	TPSA	O/NH	VIOL	ROTB	VOL	GPC	ICM	KI	NRL	PI	EI
1	78	3	0	2	322	0.41	0.24	-0.24	0.72	-0.02	0.32
2	145	5	0	7	304	0.17	-0.08	-0.18	0.57	0.15	0.24
3	58	2	1	1	478	0.23	0.08	-0.41	0.73	0.17	0.54
4	58	2	1	2	472	0.31	-0.03	-0.50	0.93	0.14	0.55
5	58	2	1	1	315	0.16	0.01	-0.42	0.41	-0.11	0.25
6	87	3	0	1	321	0.36	0.11	-0.19	0.54	-0.04	0.32

DISCUSSION

A variety of herbs and herbal extracts contain different phytochemicals with biological activity that can produce therapeutic effects. Regarding to the total body weight, our result revealed, an increase in the total body weight in the diabetic rats treated with RO extract when compared with diabetic group This in agreement with results obtained by other researchers [12-13], who found an improved total body weight in diabetic rats treated with RO extracts for 21 days. A significant decrease in body weight, in diabetic rats, may be due to increased muscle wasting and loss of tissue proteins [18]. On the other hand, the improvement in body weights in diabetic rats treated with rosemary extract compared with untreated diabetic rats may be due to the protective effect of the extract in controlling muscle-wasting which may be attributed to the increase of glucose metabolism [12].

A few reports dealing with the anti-diabetic potential of various ethanol extracts of *Rosmarinus officinalis* have been published; these reports have revealed that the plant has a hypoglycaemic effect [8]. The anti-hyperglycemic effect of RO extract was documented in our work, with a significant decrease in fasting glucose blood level of diabetic rats and treated with RO extract. Rosemary stimulates insulin secretion from the remnant β-cells or regenerated β-cells [12]; this may be due to the increase in betatrophin hormone secretion which increases the number of insulin-producing cells in the pancreas [19,20]. Najla [13], on the other hand, suggested that hypoglycaemic effect of the aqueous extract of *Rosmarinus officinalis* is a result of induced-insulin secretion from β –cell of the pancreas in diabetic rats.

The present study, showed that the depletion in the antioxidant system in diabetic rats were improved in diabetic treated group with RO extract. Cheung, and Tai [12] reported an elevation in antioxidant system and reduction in lipid peroxidation serum levels in diabetic rabbits treated with *Rosmarinus officinalis* extract. Diabetics and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycaemia, which depletes the activity of antioxidative defence system and thus promotes de novo free radicals generation [21, 22]. Oxygen free radicals react with all biological substances. However, the most susceptible ones are polyunsaturated fatty acids and reactions with these cell membrane constituents lead to lipid peroxidation [23]. Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors [24]. Any compound, natural or synthetic, with antioxidant activity might totally or partly alleviate this damage. In this context, several research groups have reported on the in vitro antioxidant activity of *Rosmarinus officinalis* extracts [10, 12].

Serum creatinine , urea and uric acid as a marker of kidney function was significantly increased in diabetic rats. Our results, agreed histopathologically by congestion of blood vessels and eosinophilic proteinaeous casts in the lumen of renal tubules in diabetic rats, while the lesions were reduced in diabetic rats treated with *Rosmarinus officinalis* extract. Our results are in excellent agreement with those obtained by Najla [13], who found a decrease in uric acid, creatinine, and urea serum levels in diabetic rats treated with *Rosmarinus officinalis* extract for 21 days. In addition, an investigation conducted by Tavafi et al. [18], concluded that rosmarinic acid, at a dose of 200 mg/Kg bw/d, for 8 weeks in diabetic rats, could significantly reduce glomerular hypertrophy, loss of glomerular number, glomerulosclerosis and attenuated serum urea and serum creatinine. The renal damage in diabetic rat, could be attributed to hyperglycaemic and diuresism which associated with inflammatory process [25]. In conclusion, the renoprotective effect of *Rosmarinus officinalis* extract could be attributed to its antioxidant action and or anti-inflammatory properties. Feeding the animals for longer periods of time may lead to greater improvement in reducing the concentrations of creatinine, urea, and uric acid, and ultimately to an improvement in kidney function.

CONCLUSIONS

In conclusion, the aqueous extract of *Rosmarinus officinalis* exhibited hypoglycaemic, anti-oxidant effects, in addition to improving renal function and reducing renal damage in diabetic rats. The dietary supplementation of *Rosmarinus officinalis* is recommended to reduce the nephropathy damage in diabetic patients. Additionally, results from this investigation prompted several pertinent observations: (i) This type of Rosemary can furnish an interesting model for studying the interaction of natural steroids and antioxidants with bio-targets because of the possible charge modification of substituents and O/N of pharmacophore group (ii) The flexible pharmacophoric site(s) geometric conformation will enable us in the future to prepare molecules with multi-therapeutic potentials and high selectivity.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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