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Dipeptidyl peptidase IV (CD26/DPP-IV) inhibitory and free radical scavenging potential of *W. somnifera* and *T. foenum-graecum* extract

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Abstract

The incretin hormone glucagon-like peptide 1 (GLP-1), proposed as a new target for the treatment of type 2 diabetes, is rapidly cleaved by dipeptidyl peptidase-IV enzyme (DPP-IV). DPP-IV inhibitors enhance circulating GLP-1 level, which in turn resulted into improved glucose tolerance and insulin secretion. The present study was designed to evaluate DPP-IV inhibitory activities *vis a vis* anti-peroxidative potential, if any, in two antidiabetic plants. We studied *in vitro* DPP-IV inhibition; DPPH radical scavenging potential; ß-carotene bleaching; reducing power and total phenolics content at the varying concentrations (0.1 – 1 mg/ml) in the extracts of *W. somnifera* root and *T. foenum-graecum* seeds. Methanolic extract (1 mg/ml) of *W. somnifera* inhibited DPP-IV activities (69.7 ±0.56%) at greater extent than that of *T. foenum-graecum* (51.8±1.24%), as compared to control. *W. somnifera* extract contains relatively higher amount of total phenolics, elevated DPPH free radical scavenging potential and pronounced reducing power efficacy than *T. foenum-graecum*. The result of present study ravel that *W. somnifera* and *T. foenum-graecum* extracts contain some novel DPP-IV inhibitors with antiperoxidative potential and could be developed as therapeutic molecules for type 2 diabetes mellitus.

Keywords: Antidiabetic plants; Antioxidant; DPP-IV inhibitors; In vitro; Type 2 diabetes mellitus, free radical-scavenging.

Introduction

Type 2 diabetes mellitus (DM) is initially managed by exercise and dietary modification. If blood glucose levels are not adequately lowered by these measures, medications may be needed. In the treatment of type 2 DM oral hypoglycaemic agents are currently in use. However, existing therapies for the treatment of type 2 DM is not sufficient and having various adverse effects [1,2]. Because of their complex mechanism of actions; these therapies antidiabetic molecules are associated with one or the other side effects such as weight gain, hypoglycaemia, hypothyroidism, abdominal pain, obesity, insulin resistance, atherosclerosis etc.[2-5]. Therefore, a novel approach in the treatment of type 2 DM based on the incretin hormone glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) was developed. While after secretion from gut; GLP-1 and GIP are no longer available in the active form due to their rapid inactivation by dipeptidyl peptidase-IV enzyme (CD26/DPP-IV) [6]. GLP-1 and GIP have a significant role on the beta cells, such as increased mass of the beta-cell and beta-cell survival [7] and GIP-dependent reduction of the insulin clearance that helps to promote the peripheral insulin levels to maintain normal blood glucose level [8]. Therefore, preventing the cleavage of GLP-1 and GIP became an attractive therapeutic target for type 2 DM [9].

Plethora of scientific literature is available on the regulation of type 2 diabetes mellitus by herbal extracts. In literature, some reports are there claiming presence of some novel enzyme inhibitors including that of DPP-IV in plants extracts [9-11]. Botanicals have been the major source of drugs in the treatment of DM in Indian medicine as well as in other ancient systems in the world [12,2]. In literature, there is no report available on describing correlation between DPP-IV inhibition and antioxidant properties of antidiabetic plants. Antioxidant molecules have a property to inhibit the production of free radicals generated by metabolic disorder and its supplements might promote a good health [13-15].

The objective of the present study was to evaluate DPP-IV inhibition activity *vis a vis* antioxidant potential, if any, in the methanolic extracts of *W. somnifera* root and *T. foenum-graecum* seed. *Withania somnifera* (family Solanaceae) and *T. foenum graecum* (family Leguminoceae) commonly known as Ashwagandha and fenugreek, respectively have been widely used as home remedy for several decades. Furthermore, these plants are being used as folklore medicine by Indian and African people for the treatment of diabetes, cholera, dysentery and other metabolic disorders.

Material and Methods

Chemicals

Dipeptidyl peptidase-IV enzyme from porcine kidney, Gly-Pro-p-nitroanilide and Tris-HCl were obtained from Sigma-Aldrich(R), St. Louis, USA. All other chemicals were of reagent grade and procured from Loba Chemie, Mumbai, India.

Plant extracts

Methanolic extract of *W. somnifera* root powder (herb: extract 5:1 w/w) and *T. foenum-graecum* seed (seed: extract 15:1 w/w) were donated as gift by Amasar Pvt. Ltd, Indore, India. The voucher specimens, WS-1142 and TFG-1144, for *W. somnifera* and *T. foenum-graecum*, respectively were deposited in departmental herbarium.

DPP-IV enzyme assay

DPP-IV assay was performed following the protocol originally described by earlier workers [9,11] as routinely followed in our laboratory [16]. In a 96-well Microtiter plate chromogenic substrate is cleaved by the serine protease DPP-IV resulting into release of a yellow coloured product. In brief, DPP-IV inhibition activities of plant extracts were determined by measuring the release of 4-nitroaniline from an assay mixture containing 0.1 M Tris-HCl (pH 8.0) and 50µl of DPP-IV (0.05 U/ml) enzyme with varying concentrations (0.1, 0.2, 0.3, 0.4, 0.5 mg/ml) of test samples. Then the plate was incubated for 15 min at 37 C followed by the addition of 50µl of 0.5 mM Gly-Pro p-nitroanilide (substrate). After gentle mixing the plate was incubated for 30 min at 37 C. Finally the reaction was stopped by the addition of 25µl of sodium acetate buffer (pH 4.5) and absorbance was measured at 405nm on a Microtiter plate reader. While Sitagliptin monophosphate was used as a reference drug and control was prepared without inhibitor/plant extract. A decrease in DPP-IV activity is a measure for the inhibition. The % inhibition was calculated using following formula.

$$\% \text{ inhibition} = \left[\frac{\text{Absorbance of control Absorbance of inhibitor}}{\text{Absorbance of control}} \times 100\right]$$

DPPH scavenging Assay

The free radical scavenging activity of the plant extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH•) using the method described by Gyamfi et al [17]. In brief, 200 µl of sample extract (0.1 – 1 mg/ml in 80% methanol) or ascorbic acid (standard; 0.1 – 1 mg/ml) was mixed with 800 µl of 100 mM Tris–HCl buffer (pH 7.4). Then 1 ml of 500 µM DPPH freshly prepared in 80% methanol was added to it. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm on a UV-VIS Spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. DPPH radical concentration was calculated using the following formula.

$$\text{DPPH• scavenging effect } (\%) = \left[\frac{A_0 - A_1}{A_O}\right] 100$$

Total phenolics content

The amount of total phenolics in plant extracts was determined by the Folin-Ciocalteau reagent method following the protocol described by Spanos and Wrolstad [18] as modified by Lister and Wilson [19]. In brief, 100µl of extract dissolved in 1500 µl (1/10 dilution) was added with Folin–Ciocalteu reagent. After 1 minute incubation at room temperature; sodium carbonate (Na_2CO_3) solution was added to this. The mixture was shaken and incubated for one and a half hour in dark at room temperature. Finally absorbance of all the samples was measured at 725 nm using UV-Vis Spectrophotometer. Gallic acid was used as standard. Total phenolics content was expressed as mg Gallic acid equivalents (GAE/g dry matter).

Determination of antioxidant activity

Determination of antioxidant activity by ß-carotene bleaching method was carried out according to a protocol described elsewhere [20] as routinely done in our laboratory [16]. One ml of ß-carotene solution (0.2 mg/ml in chloroform) was added to a 50 ml round-bottom flask containing 20 µl of linoleic acid and 200µl of Tween 20. Then added 200 µl of test samples. For control, samples were replaced by 200µl methanol (80% v/v) whereas for standard, samples were replaced by 200 µl BHT (40 g/l). After evaporation to dryness under vacuum at 40 C for 30 minutes, 100 ml of distilled water was added and the mixture was shaken to form a liposome solution. The samples were then subjected to thermal autoxidation using water bath at 45 C. The absorbance of the solution at 470 nm was monitored on an UV-Vis Spectrophotometer by taking measurements at 20 minutes intervals for 120 minutes in dark room to avoid ß-carotene oxidation.

Determination of reducing power

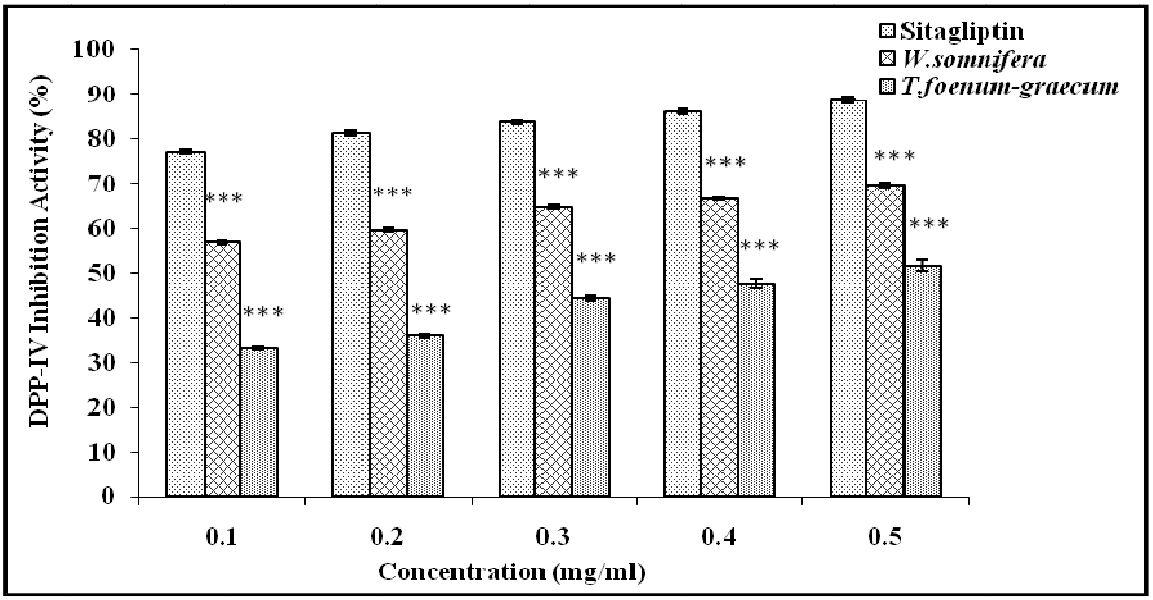
The reducing power of plant extracts was determined according to the method described by Wu et al [21]. In brief, the samples (1 ml) were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Then mixture was incubated at 50^0C for 30 min, followed by addition of 2.5 ml of 10% trichloroacetic acid. Then centrifuged at 2000 rpm followed by an aliquot (2.5 ml) of reaction mixture 2.5 ml of distilled water and 0.5 ml of 1% $FeCl_3$ were added. The absorbance of the solution was measured at 700 nm on a UV-Vis Spectrophotometer after 10 min incubation at room temperature. The blank of each sample was prepared by adding the distilled water instead of $FeCl_3$. Ascorbic acid was used as standard reference. Increased absorbance of the reaction mixture indicates pronounced reducing power.

Statistical analyses

Data are expressed as mean ± S.E.M. For statistical evaluation of the data, analysis of variance (ANOVA), followed by the *post hoc* Newman–Keuls multiple comparison test by using the trial version of Graph Pad Software Prism version 5.01 was used. The % inhibition was calculated using the formula, O. D. of Control- O. D. of Sample/ O. D. of Control x 100.

Results

Result of DPP-IV assay revealed that methanolic extract of *W. somnifera* inhibited enzyme activity at greater extent (69.7 ±0.56%) than that of *T. foenum-graecum* (51.8±1.24%) as compared to standard; Sitagliptin at concentration (0.5mg/ml) (Figure. 1; Table-1).



Figurer.1-Percentage inhibition in DPP-IV activity at varying concentrations (0.1,0.2,0.3,0.4 and 0.5 mg/ml) of *W. somnifera* and *T. foenum-graecum*) extract as compared to control; Sitagliptin. Each vertical bar represents the mean ± S.E.M. (n=3). ***p< 0.001 and **p<0.01 as compared to respective control values.

Table-1: Comparative assessment of W. *somnifera* and *T. foenum-graecum* extract in DPP-IV inhibition, DPPH, antioxidant activity and reducing power; all in percentage (%).

	Control	*W. somnifera*	*T. foenum-graecum*
DPP-IV Inhibition Concentration (0.5 mg/ml)	88.7±0.49	69.7 ±0.56***	51.8±1.24***
DPPH Concentration (0.5 mg/ml)	96.86±0.9	61.1±10.83***	19.06±0.54***
Antioxidant Activity Concentration (0.5 mg/ml)	73.06±1.58	47±1.23***	32.6±0.72 ***
Reducing Power (Absorbance at 700 nm) Concentration (0.8 mg/ml)	0.236±0.004	0.191±0.007 **	0.148±0.003***

Data are mean ± SEM. (n = 3). *** p< 0.001 and **p<0.01 as compared to respective values of control.

Data of DPPH scavenging assay suggested that varying concentrations of *W. somnifera* extract inhibited the free radicals at greater extent than *T. foenum-graecum* (Figure. 2). Furthermore, it was observed that W. somnifera contains considerable higher amount of total phenolics (63±0.81) mg GAE/g of dry extract) then that of T. foenum-graecum (49.06±1.56) mg GAE/g of dry extract) at 1 mg/ml concentration (Figure. 3). W. somnifera extract showed better antioxidant activity (47±1.23%) than T. foenum-graecum (32.6±0.72%) (Figure. 4). The reducing ability of methanolic extract of W. somnifera and T. foenum-graecum showed dose dependence increase, as compared to control. W. somnifera extract showed higher reducing power ability as compared to T. foenum-graecum at all the studied doses (0.1 – 0.8 mg/ml in methanol) (Figure. 5; Table-1).

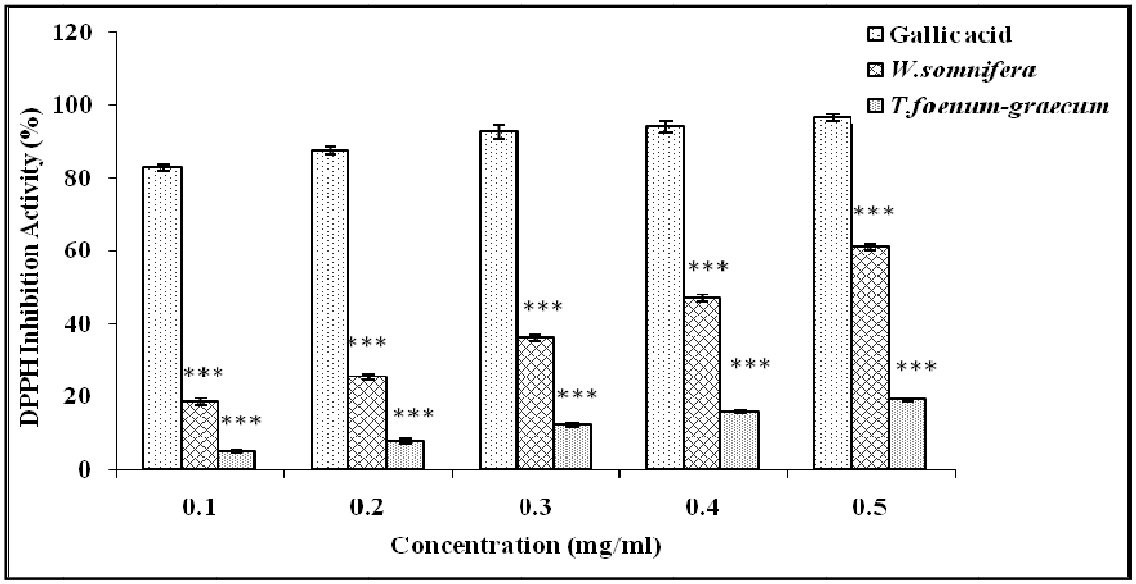
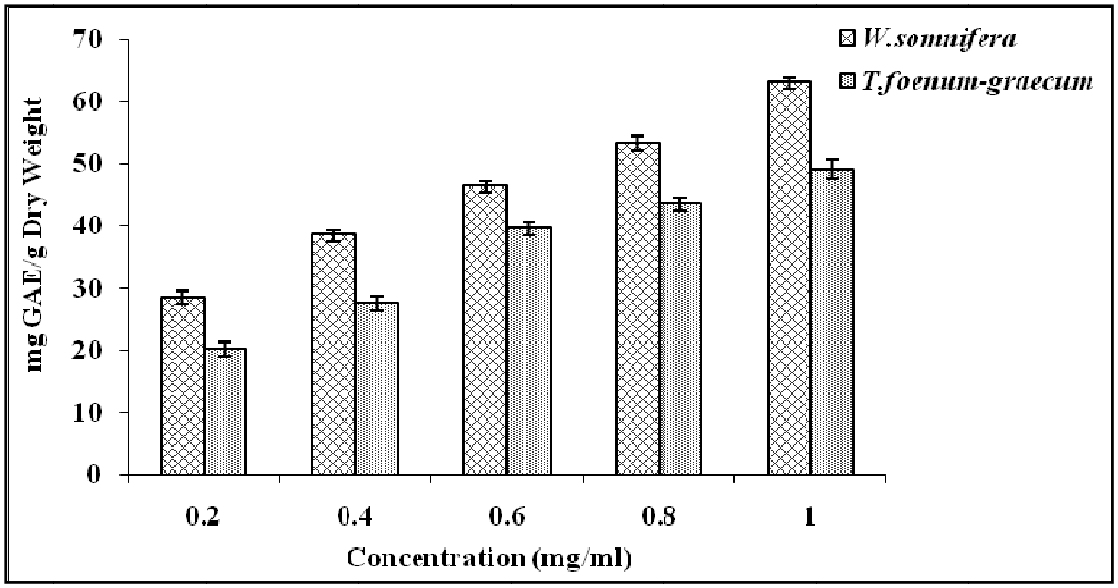


Figure. 2-Comparative potential of DPPH free radical scavenging (%) at varying concentrations (0.1,0.2,0.3,0.4 and 0.5 mg/ml) of *W. somnifera* and *T. foenum-graecum* extracts as compared to standard; Gallic acid. Each vertical bar represents the mean ± S.E.M. (n=3). ***p< 0.001 and **p<0.01 as compared to respective control values.



. **Figure.3-**Total phenolic content in the extracts of *W. somnifera* and *T. foenum-graecum* at varying concentrations (0.1,0.2,0.5,0.8 and 1 mg/ml). Each vertical bar represents the mean ± S.E.M. (n=3). ***p< 0.001 and **p<0.01 as compared to respective control values.

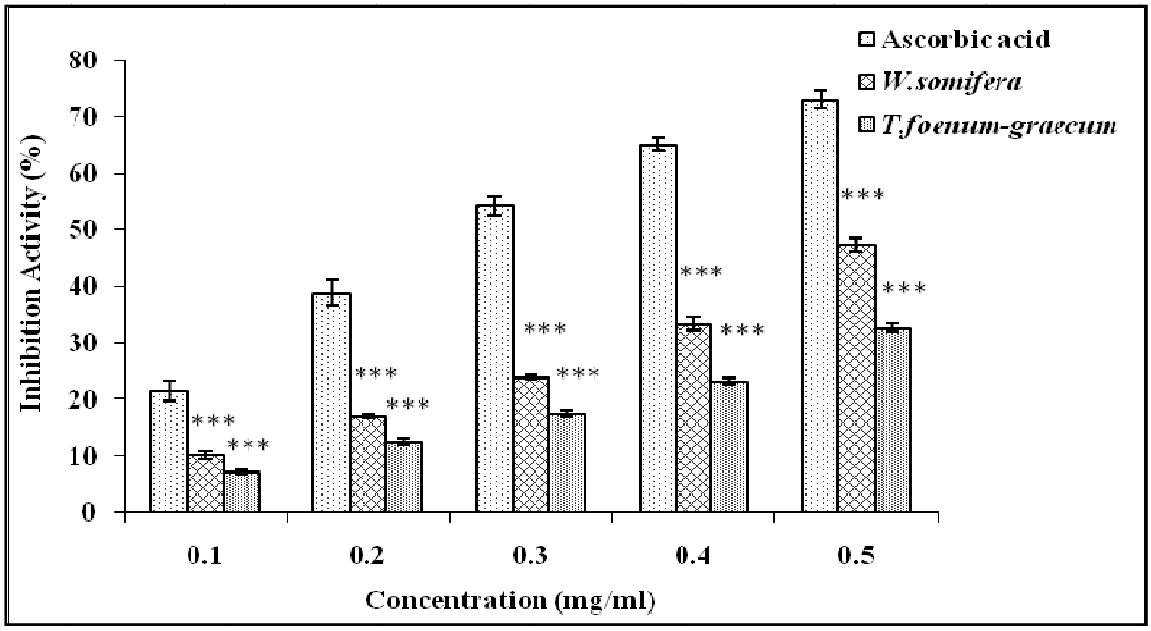


Figure.4-Antioxidant activity (%) at varying concentrations (0.1,0.2,0.3,0.4 and 0.5 mg/ml) of *W. somnifera* and *T. foenum-graecum* extract as compared to control: BHA. Each vertical bar represents the mean ± S.E.M. (n=3).***p< 0.001 and **p<0.01 as compared to respective control values.

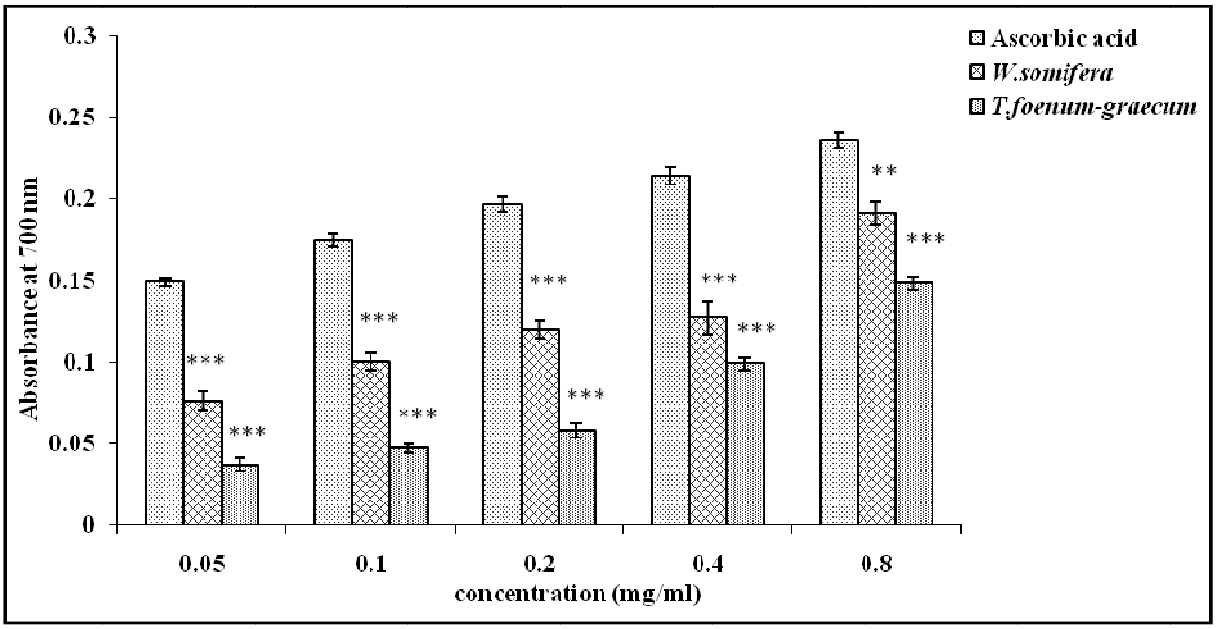


Figure.5-Reducing activity at varying concentrations (0.05,0.1,0.2,0.4 and 0.8 mg/ml) of *W. somnifera* and *T. foenum-graecum* extract as compared to control; Ascorbic Acid. Each vertical bar represents the mean ± S.E.M. (n=3).***p< 0.001 and **p<0.01 as compared to respective control values.

Discussion

The findings of the present study clearly demonstrate that methanolic extracts of *W. somnifera* and *T. foenum-graecum* contains novel DPP-IV inhibitors. Interestingly, *W. somnifera* extracts have relatively higher content of phenolics, reducing power and DPPH radicals scavenging efficacies, as compared to *T. foenum-graecum* indicating peroxidative and antidiabetic potential.

The results of the present study are corroborating our earlier findings where some other antidiabetic plants were reported to contain novel DPP-IV inhibitors with antiperoxidative efficacies [16]. The gut hormones GLP-1 and GIP are secreted following intake of meal from the L and K cells of the in the intestinal mucosa, respectively. The gut hormones contribute to approximately 60% of the insulin secretion postprandially and are responsible for the incretin effect [22]. However, DPP-IV rapidly inactivates GLP-1 and

GIP. Therefore, DPP-IV inhibitors represent a unique approach in the treatment of type 2 diabetes without any known side effect. Inhibition of DPP- IV prolong and also enhances the activity of endogenous GLP-1 and GIP, which serves as important prandial stimulator of insulin secretion and blood glucose regulator. The findings of the present study revealed that varying concentration of methanolic extract of *W. somnifera* and *T. foenum-graecum* inhibited DPP-IV activity, reflecting the therapeutic potential for type 2 DM. Interestingly, our findings corroborate the reports where authors claimed to posses novel DPP-IV inhibitors in some indigenous plants earlier [9,11,16,23].

Oxidative stress arises due to imbalance between the levels of free radicals in cells and its antioxidant defenses in favor of former. Diabetes mellitus is a disease of oxidative stress; therefore antidiabetic pharmacologicals additionally contains antiperoxidative potential [7,16,24]. Phenols and antioxidant molecules are important constituent of various officinal plant parts of the plants due to their antioxidant properties [18,19,25]. Antioxidant activity of phenolic compound is basically due to their redox properties which play an important role in free radicals scavenging potential [25,26]. Observations made on DPPH scavenging efficacy of plant extracts revealed that both *W. somnifera* and *T. foenum-graecum* extracts inhibited free radicals, however the later showed better activity over former one. In literature, reports are there suggesting the positive correlation between total phenol and free radical scavenging activities by herbal extracts [16,27,28]. Observations made on the total phenolic content in the extracts of *W. somnifera* and *T. foenum-graecum* reflecting the presence of noticeable amount of natural antioxidants. Interestingly, *W. somnifera* extract scavenge DPPH radicals at greater extent than that of *T. foenum-graecum*. The pronounced radical scavenging potential of *W. somnifera* could be an outcome of relatively higher amount of total phenolics content in the plant extract.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [29, 30]. In contrast to DPPH radical scavenging activity, the *W. somnifera* extract showed the better reducing activity as compared the data of *T. foenum-graecum*. Indeed, relatively higher reducing capacity and the antioxidant activity of the *W. somnifera* extract is associated with its antioxidant constituents. This fact was further supported by the findings made on total phenolics contents in studied plant extracts.

Conclusion

The results of the present study exhibited that *W. somnifera* root and *T. foenum-graecum* seed extract contain novel DPP-IV inhibitors with additional anti-peroxidative potencies. However, *W. somnifera* root extract could be better lead in the development of DPP-IV based antidaibetic therapy, as reflected by the observations made on various antioxidant parameters. Therefore, studies on pharmaceutical preparations would be of great importance as a therapeutic treatment for type 2 DM.

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