



DIPEPTIDYL PEPTIDASE-IV INHIBITION AND *EX-VIVO* ANTIOXIDANT POTENTIAL OF PHYTOCOMPOUNDS OF *WITHANIA SOMNIFERA*, *TRIGONELLA FOENUM* AND *BAUHINIA PURPUREA*

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ABSTRACT

Indigenous medicinal plants possess peculiar kinds of phytochemicals used for various kinds of ailments as well as particularly a remedy for diabetes by local people through utilizing traditional knowledge and are a source of new drug discovery. Phytochemicals have been found to possess a wide range of activities which may help in protection against chronic disease. The efficiency of certain antidiabetic plants contains some bioactive compounds of natural origin which could be, at least moderately, explained by the inhibition of DPP-IV. In the present study, we isolated and quantified some of the phytochemicals such as alkaloids, flavonoids, phenolic and phytosteroids etc, which act as DPP-IV inhibitors, which is a novel potential for the treatment of type 2 diabetes mellitus; we isolated the different bioactive fractions from *Withania somnifera*, *Trigonella foenum*, *Bauhinia purpurea* and investigated their potential to serve as a natural source of DPP-IV inhibitors. Methanolic extract (1 mg/ml) of *W. somnifera* inhibited DPP-IV activities (76.3±1.45%) to a greater extent than that of *T. foenum* (65.6±0.98%) and *B. purpurea* (63.5±0.78%) as compared to standard drug i.e. sitagliptin (96.3±1.26%). *W. somnifera* and *B. purpurea* extracts were reported to inhibit DPPH free radicals and inhibit erythrocyte haemolysis in higher concentrations than *T. foenum*. The results of the present study reveal that *W. somnifera*, *T. foenum* and *B. purpurea* plant extracts possess some novel DPP-IV inhibitors like phytochemicals and can be developed as therapeutic molecules for type 2 diabetes mellitus. The results of the present study reveal that extracts of WS and TFG contain novel DPP-IV inhibitors with antiperoxidative potential and can be developed as therapeutic molecules for type 2 diabetes.

KEYWORDS: Dipeptidyl Peptidase-IV inhibition, Erythrocytes hemolysis, Phytochemicals, Antioxidant, Type 2 diabetes mellitus.

INTRODUCTION

Medicinal plants play an imperative role in the therapeutics of type 2 diabetes mellitus (T2DM) by adjuvating the development of diabetic problems and amending metabolic irregularities. Similarly, the conventional herbal remedies have been used by T2DM patients around the world and many scientific studies have established the assistance of medicinal plants. Additionally, through the previous few years, some of the new bioactive drugs isolated from hypoglycemic floras have been confirmed to have antidiabetic activity as particularly to DPP-IV inhibition with comparative efficacy to synthetic oral hypoglycemic agents used in clinical therapy regimens. Dipeptidyl peptidase IV (DPP-IV) inhibitors are potent mediators for the therapeutics of diabetes and the effectiveness of certain antidiabetic extracts of natural origin could be, at least partially, explained by the inhibition of DPP-IV.^[1-3]

Plants have a vast ability to synthesize aromatic substances mainly secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total.^[4] The synthesized aromatic substances (metabolites) are used by plants as defensive molecules against predation by microorganisms, insects and herbivores. However, some of which may involve in plant odour (terpenoids), pigmentation (tannins and quinones), and flavour (capsaicin).^[5] However, these defensive molecules give plants their medicinal value which is valued by human beings because of their great importance in health care of individuals and communities. In India, the use of different parts of several medicinal plants to cure specific ailments has been in vogue from ancient times. The indigenous system of medicine namely Ayurvedic, Siddha and Unani have been in existence for several centuries.^[6]

The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the

therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contain complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans.^[5,7] In order to be used as a modern drug, an extract may be further processed through various techniques of fractionation to isolate individual chemical entities. For the isolation of dipeptidyl peptidase IV (DPP-IV) inhibitors plants were selected on the basis of their efficacies in regulating type 2 diabetes mellitus. The anti-diabetic plants which were selected for the proposed research include *Withania somnifera*, *Bauhinia purpurea* and *Trigonella foenum*.

MATERIALS AND METHODS

Sample preparation for phytochemical analysis

The officinal parts of these plants (*W. somnifera*, *T. foenum* and *B. purpurea*) were collected from in and around of new campus, Jai Narain Vyas University, Jodhpur, India and also provided by AMSAR Private Ltd (Indore, India). Before proceeding for the isolation; DPP-IV inhibition assay was performed in all selected plant extracts to ascertain the inactivation of inhibitors in the process of extraction using solvent. All the animal studies safety evaluation for *ex-vivo* experiment after taking ethical clearance from Institutional Animal Ethics Committee (IAEC), Department of zoology, Jai Narain Vyas University, Jodhpur, registration no 1646/GO/Ere/S//12CPCSEA., IAEC of our University is constituted as per the guidelines laid down by the Committee for the Purpose of Control and Supervision on Experiments in Animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi.

Extractions of plant derived active principles essentially involve in usage of selective solvents following by standard procedures. The extracts obtained, after standardization were used for the isolation of active components for further uses. The extracts may contain complex mixture of many medicinal plant metabolites such as alkaloids, flavonoids, tannins, saponins, glycosides, phenols, terpenoids, and steroids.

Qualitative and Quantitative estimation of bioactive constituents

Alkaloids

For the qualitative test of Alkaloids, the plant extract was evaporated to dryness and the residue was heated on a boiling water bath with 2N HCl. After cooling at room temperature, the mixture was filtered and reacted with few drops of Mayer's reagent. The presence of turbidity or precipitates confirms the presence of Alkaloids in the sample.^[8]

Flavonoids

In Flavonoids test, the plant extract was treated with a few drops of concentrated HCl and magnesium turnings (0.5 g). The presence of flavonoids was indicating if pink or magenta-red colour developed within 3 min.^[9]

Tannins

For the tannins estimation, the plant extracts were evaporated and the residues were extracted by 0.9% NaCl solution, filtered and divided into 3 aliquots. Then sodium chloride solution was added to one portion of the test extract, 1% Gelatin solution to a second portion and the gelatin-salt reagent to a third aliquot. The formation of blue-black, green or blue green colour precipitates by following FeCl₃ addition, confirm Tannins in samples.^[10]

Steroids

The Liebermann Burchard reaction was performed for the qualitative estimation of steroids by following standard protocol.^[11]

Terpenoids

The solvent extract of plant material was taken in a test tube and then added few pieces of tin plus 3 drops of thionyl chloride, violet or purple colour developed indicated the presence of Terpenoids.^[5]

Glycosides

The qualitative estimation of glycosides was performed by using glycosides acid test. In brief, 0.5 g of solvent extract was dissolved in 2.0 ml of glacial acetic acid containing one drop of FeCl₃ Solution. This was then under laid with 1.0 ml of concentrated H₂SO₄. A brown ring obtained at the interface indicated the presence of Glycosides.^[12] They were developed in chromatographic jar. The plates could develop till the solvent run about 5/6 of the plate. Developed plates removed by chromatographic jar and allowed to dry in open air. And viewed under UV fluorescence light at 254 and 365nm; spread with suitable visualized reagents.^[5,13,14]

DPP-IV inhibition assay

The assay is based on the cleavage of Gly-Pro-p-nitroanilide by DPP-IV enzyme resulting into generation of a stable chromophore.^[15,16] 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 2 mM Gly-Pro p-nitroanilide (substrate). After incubation at 37°C, the reaction was stopped by the addition of sodium acetate buffer (pH 4.5) and absorbance at 405 nm was measured using a UV-VIS Spectrophotometer. A percentage decrease in DPP-IV activity is a measure for the inhibition.

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

DPPH scavenging Assay

This assay was performed to study the relative efficacies in inhibiting the free radicals by the plant extracts in *in vitro* condition, as described by earlier workers (Gyamfi *et al.*, 2002).^[1,17] In brief, 200 μ l of sample extract (0.05 –

0.8 mg/ml in 80% methanol) or ascorbic acid (standard) (0.1 – 1 mg/ml) was mixed with 800 μ l of 100 mM Tris–HCl buffer (pH 7.4). Then 1 ml of 500 μ M DPPH freshly prepared in 80% methanol was added.

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

Lipid per-oxidation inhibition activity

Lipid peroxidation inhibition efficacies of plant extracts were determined according to the method described by Malterud *et al.*^[18] A male Wistar rat was anaesthetized and sacrificed to obtain the liver tissues. After washing the tissues with phosphate buffered saline (PBS; 0.1 M; pH 7.4) 10% (w/v) liver homogenate was prepared by simple chopping. Different concentrations (20 to 200 μ g/ml) of the analyses and Ascorbic Acid were incubated with 1 ml of homogenate and the reaction initiated by the addition of 0.1 ml of FeSO₄ (25 μ M), 0.1 ml of ascorbate (100 μ M), and 0.1 ml of KH₂PO₄ (10 mM), and the volume was made up to 3 ml with distilled water and incubated at 37°C for 1 h. Then, 1 ml of 5% trichloroacetic acid (TCA) and 1 ml of 1% thiobarbituric acid (TBA) was added to this reaction mixture and the tubes were boiled for 30 min in a boiling water bath. This was then centrifuged at 3500 rpm for 10 minutes. The extent of lipid peroxidation was evaluated by the estimation of thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532 nm % inhibition of LPO was calculated using the formula.

$$\% \text{ LPO Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Erythrocyte haemolysis inhibition

The procedure described by Barreira *et al.*^[19] was adopted with minor modification (eg. the type of haemolysis inducer) to evaluate the inhibition of erythrocyte haemolysis by varying concentrations of plants extracts. A male wistar rat was sacrificed to obtain the erythrocyte. The erythrocyte haemolysis was performed with H₂O₂ as free radical initiator. The erythrocytes were added with different concentration of plants extracts and 100 M H₂O₂ (in PBS, pH7.4). The reaction mixture was incubated at 37°C for 3 h with occasional and gentle shaking during incubation and absorbance was measured at 540 nm by spectrophotometer The haemolysis caused by 100 M H₂O₂ was taken as 100% haemolysis; and the percentage haemolysis inhibition was calculated by the equation.

$$\% \text{ haemolysis inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Statistical analyses

Data are expressed as mean \pm S.E.M. For statistical evaluation of the data, analysis of variance (ANOVA) followed by the *post hoc* Newman–Keuls multiple comparison test using a trial version of Prism 4 software for Windows (Graph Pad Software, Inc., La Jolla, CA, USA) was used.^[23] The % inhibition was calculated using the formula, O. D. of Control- O. D. of Sample/ O. D. of Control x 100.

RESULTS

Qualitative estimation of phytoconstituents

Among the plants tested for the presence of phytocompounds, the Alkaloids contain in *Withania somnifera* extract (60% methanol) was found to be rich as compared to *Trigonella foenum* showed very less amount in 60% methanol solvent. Tannin found noticeable amount in all the plant extract. Terpinoids suggested that extract of *Bauhinia purpurea* contains significant amount as compared to *Withania somnifera*. Interestingly, *Trigonella foenum* extract were reported to contain higher amount of flavonoids and phenols as respected to *Withania somnifera* but *Bauhinia purpurea* found significant higher amount of flavonoid present..

Table 1: Qualitative estimation of Alkaloids, Flavonoids, Tannins, Phenolic and Terpenoids in the plant extracts with DPP-IV enzyme inhibitory activities (60% and above) viz. *W. somnifera*, *B. purpurea* and *T. foenum*.

S.NO	Plant Extract	Secondary Metabolite	Aqueous	60% Methanol	Methanol	Chloroform
1	<i>W. somnifera</i>	Alkaloids	+++	+++	+	-
		Tanins	-	-	-	-
		Terpenoids	+	+	+	-
		Flavonoids	++	++	+	-
		Phenols	-	-	-	-
2	<i>B. purpurea</i>	Alkaloids	-	-	+	-
		Tanins	+	+	+	-
		Terpenoids	++	+++	++	-
		Flavonoids	+++	+++	+	-
		Phenols	+++	+++	+++	+
3	<i>T. foenum</i>	Alkaloids	-	++	-	-
		Tanins	+	+	++	-
		Terpenoids	++	++	++	-
		Flavonoids	+++	+++	+	-
		Phenols	+++	++	++	-

Symbol (+) show the extent of presence of phytochemical and symbol (-) reflect the trace/ undetectable amount of phytochemical in the respective plant extract

Quantitative estimation of phytoconstituents

In the qualitative estimation in the different solvent for test of alkaloids, tannins, terpenoids, flavonoids, phenols suggested that aqueous-methanolic (40%-60%) extract showed maximum bioactive compounds and chloroform showed list active bioactive compounds. *Withania*

somnifera contains significant amount of alkaloids and some amount of flavonoids and terpenoids while *Bauhinia purpuria* and *Trigonella foenum* has noteworthy amount of terpenoids, phenols and some amount of alkaloids.(Table:2).

Table 2: Quantitative estimation of Alkaloids, Flavonoids, Tannins, Steroids, Terpenoids and Glycosides in the plant extracts with DPP-IV enzyme inhibitory activities (60% and above) viz. *W. somnifera*, *B. purpurea*, *T. foenum*.

Antidiabetic Plants	Alkaloid (mg/g dry weight)	Flavonoid (mg Quer./g dry weight)	Phenolic (mg GAE/g dry weight)	Tannin (mg GAE/dry weight)	Terpenoid (mg/camphor/g dry weight)
<i>W. somnifera</i>	243±1.75	91±1.91	78±0.63	---	109±0.95
<i>T. foenum</i>	174±2.78	145±2.58	165±2.42	79±0.79	162±1.67
<i>B. purpurea</i>	26±0.96	287±4.06	254±3.68	183±1.97	10.9±0.71

Data are expressed as S.E.M. (n=3).

DPP-IV inhibition potential of phytoconstituents

DPP-IV inhibition activity showed that *Withania somnifera* content was higher inhibition activity as

compared to *Bauhinia purpuria* and *Trigonella foenum* with compared to sitagliptin as a positive control. (Table: 3).

Table 3. DPP-IV Inhibition Activity of plants extract.

S.No	Plant	Plant product / Rich Fraction	Inhibition Activity (%)
1	Sitagliptin	Sitagliptin	96.3±1.26
2	<i>W. somnifera</i>	Alkaloids	76.3±1.45
3	<i>T. foenum</i>	Flavonoids/Phenolic	65.6±0.98
4	<i>B. purpurea</i>	Flavonoid/Phenolic/	63.5±0.78

Data are expressed as S.E.M. (n=3).

DPPH scavenging activity, Erythrocytes haemolysis inhibition and hepatic lipid per-oxidation

DPPH scavenging assay suggested that varying concentrations was observed that *Bauhinia purpuria* and *Withania somnifera* contains of good amount of

scavenging properties that inhibited the free radicals at greater extent than *Trigonella foenum*. *Bauhinia purpuria* and *Withania somnifera* showed better hemolytic inhibition activity and hepatic lipid per-oxidation. (Fig: 1,2,3)

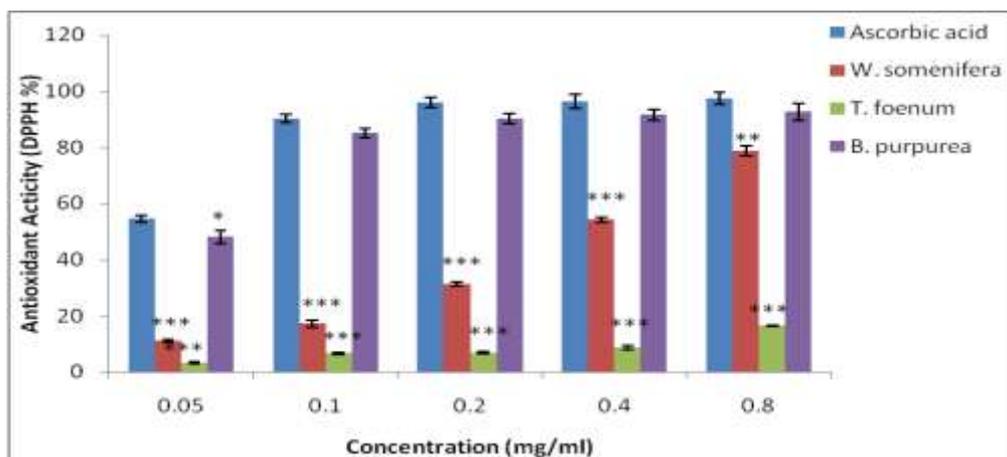


Figure 1: DPPH free radical scavenging activity (%) of *W. somnifera*, *B. purpurea*, *T. foenum* extract as compared to control; Gallic acid.

Each vertical bar represents the mean \pm S.E.M. (n=3). ***p< 0.001, **p<0.01 and *p<0.05 as compared to respective control values.

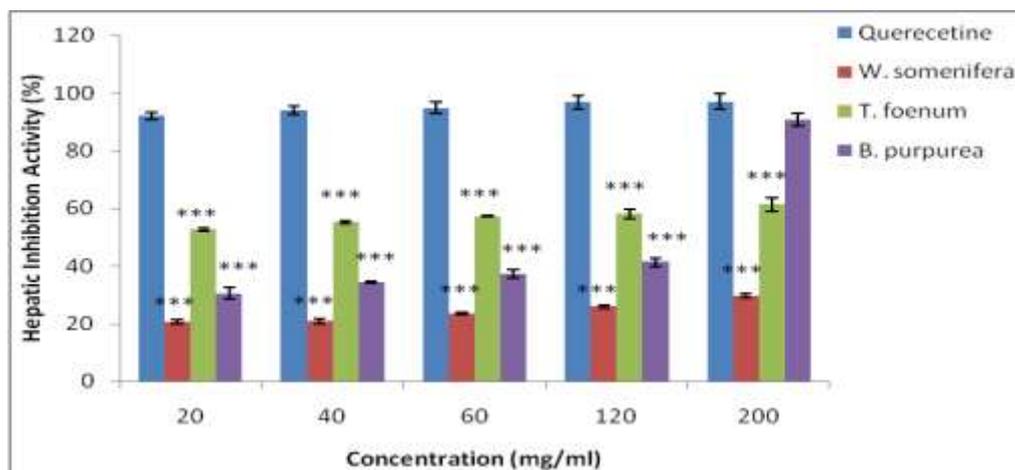


Figure 2: Hepatic per-oxidation inhibition activities of *W. somnifera*, *B. purpurea*, *T. foenum* extracts as compared to control; Ascorbic Acid.

Each vertical bar represents the mean \pm S.E.M. (n=3). ***p< 0.001, **p<0.01 and *p<0.05 as compared to respective control values.

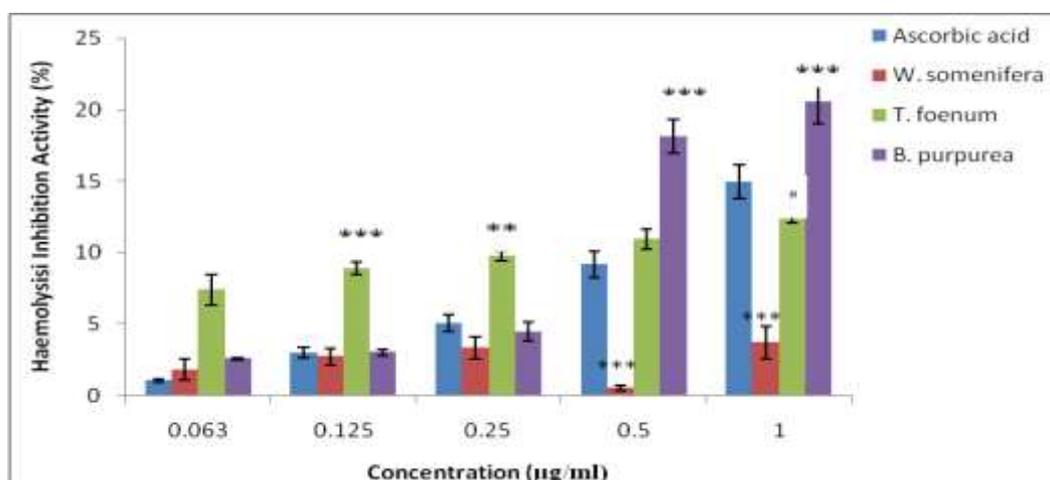


Figure 3: Erythrocyte haemolysis inhibition efficacies of *W. somnifera*, *B. purpurea*, *T. foenum* extracts as compared to control; Ascorbic Acid.

Each vertical bar represents the mean \pm S.E.M. (n=3). ***p< 0.001, **p<0.01 and *p<0.05 as compared to respective control values.

DISCUSSION

The photochemical often play an important role in plant defence against prey, microorganism, stress as well as interspecies protection. Hence photochemical screening serves as the initial step in predicting the type of potential active compounds from plants.^[20] The medicinal value of plants uses in some chemicals substance that have definite physiological actions on the human body. Different photochemical have been found to possess a wide range of activates, which may help in protection against chronic disease. The traditional healers or practitioners make use of water primarily as a solvents but there are many reports where organic solvents showed better activity as a compared with aqueous solvents.^[21,22] The photochemical and quantitative estimation of the percentage crude extract yield of chemicals constituent of the plants studied showed that the leaves and stems were rich in alkaloids.^[23] They were known to show medicinal activity as well as exhibiting physiological activity.^[24] The absence of saponin in the present study is in contrast with the opinion of gill,^[25] who noted that saponin is the active constituents. Steroids and tannin were found to be present all the plants .it has been found hat some of their investigated plants contained steroids compounds. It should be note that steroids compounds are of importance and interest in a pharmaceuticals due to their relationship with such compounds as a sex hormones.^[23,26] The presence of terpenoids in ws has also been reported by other researcher and this plants is widely used in herbal medicine.^[27,28] They are also used in the treatment of cough ,asthma, and fever.^[29] The plants studied here can be seen as a potential source of used full drugs further studies are going on as their plant in order to isolation, identification and characterization. Ascorbic acid plays a central role in pulmonary functions, immune responses, prevention of heart disease, cancer. It also acts as a scavenger of oxygen radicals and inhibitions of lipid per oxidation. Phenolic compounds have been shown to exhibit cellular defence mechanism in antherogenesis, cancer and antioxidant activates. They are often protection against LDL oxidation and inhibition of platelets aggregation. Recently increased evidence support the hypothesis that the phenolic compounds could play an essential health promoting roles. Flavonoids compounds have shown to lipid lowering, antiulcer, hepatoprotective, anti-inflammation, antioxidant, and hypoglycaemic activity. They exhibit insulin like activity acting as a glucose transport action of fat cells.^[25,30-32] It is very promising in reducing the level of free radicals and severity of diabetic complications and many chronic metabolic diseases. Flavonoids in human diet may reduced the risk of various cancer and as well as preventing menopausal symptoms.^[33]

Herbs that have tannins as their compounds are astringent in nature and are used for treating intestinal disordered such as diarrhoea and dysentery.^[34] Thus these plants can be a potent source of useful drugs;

further studies are going on isolation, identification and characterization the structure of the bioactive compounds.^[14,35] Inhibitors of the enzyme DPP-IV provide a strategy for the treatment of type 2 diabetes. DPP-IV rapidly inactivates the incretin gut hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Inhibition of DPP IV prolongs and enhances the activity of endogenous GLP-1 and GIP, which serve as important prandial stimulators of insulin secretion and regulators of blood glucose control.^[24,36-39] The herbal plants product have DPP-IV inhibitory activity and have less side effect than a chemically synthesis.^[40]

Oxidative stress arises due to disproportion 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 ant-diabetic pharmacological must additionally contain antiperoxidative /cytoprotective potentials.^[1,41] Phenols and other antioxidant molecules are important constituent of various officinal plant parts of the plants due to their antioxidant properties.^[42,43] Observations made on DPPH radicals scavenging efficacies revealed that both *W. somenifera* and *B. purpurea* extracts inhibited free radicals, however the later showed better activity over former one. In the literature, reports are there suggesting the positive correlation between total phenol and free radical scavenging activities by herbal extracts.^[25,44] Hydrogen peroxide, can cross the red blood cells (RBC) membrane and acts on the intracellular moieties, form ferryl radical or hydroxyl radical by interacting with hemoglobin and initiates a series of reactions, resulting in haemolysis^[45] and extent of Haemolysis is then determined by measuring released hemoglobin into the supernatant,^[46] Interestingly, *B. pupuria* and *T. foenum* as comparison to *W. somnifera* extract protect the RBC membrane more pronounced. In biological systems, increase in hepatic lipid per-oxidation indicates increased in oxidative stress. The methanolic extracts of both the plants extracts showed almost same efficient to inhibit the lipid per-oxidation as compared to standard. The reduction of lipid per- oxidation might be attributed to the antioxidant activities of plant extracts.^[47,48] Thus, further studies are suggested to provide additional evidence that strengthens the claim that the plant can be a potential source of antioxidant based therapies as well as inhibitory activities on DPP-IV and may have therapeutic potential for type 2 diabetes mellitus. Interestingly, findings of the present study are in consistent previous studies where authors reported novel bioactive DPP-IV inhibitors from some indigenous plants.

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