

## CYTOPROTECTIVE AND DIPEPTIDYL PEPTIDASE IV (DPP-IV/CD26) INHIBITORY ROLES OF *OCIMUM SANCTUM* AND *MOMORDICA CHARANTIA* EXTRACT

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### ABSTRACT

**Objective-** The present work deals with investigation of DPP-IV inhibition activity *vis a vis* cytoprotective potential, if any, in methanolic extracts of two antidiabetic plants; *Ocimum sanctum* (Tulsi) leaves and *Momordica charantia* (Karela / bitter melon) fruit.

**Methods-** The DPP-IV activity and cytoprotective efficacy was studied using different antioxidant activity assays such as hepatic lipid peroxidation, erythrocytes haemolysis inhibition, *in vitro* DPP-IV activity; DPPH radical scavenging efficacy; ferric reducing potential; reducing power and total phenolic contents at the varying concentrations in the extracts of *O. sanctum* and *M. charantia*.

**Result-** Methanolic extract (0.5 mg/ml) of *O. sanctum* inhibited DPP-IV activities (66.81±0.05%) at greater extent than that of *M. charantia* (53.25±0.04%); *O. sanctum* extract contains relatively higher amount of total phenolic content, elevated DPPH free radical scavenging potential and pronounced reducing power efficacy than that of *M. charantia*. *O. sanctum* inhibited erythrocytes haemolysis at greater extent (13.1±0.188 µg/ml) as compared to *M. charantia* (10.3±0.41 µg/ml).

**Conclusion-** The result of present study reveal that *O. sanctum* and *M. charantia* 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

### INTRODUCTION

Type 2 Diabetes mellitus (T2DM) is rapidly growing metabolic syndrome of multiple aetiologies causing hyperglycaemia with insulin resistance at cellular level [1,2]. According to the World Health Organization (WHO), diabetes is the World's fifth leading cause of death and it is estimated that it will surpass 366 million population worldwide by the year 2030 [3]. In the treatment of T2DM oral hypoglycemic agents are currently in use [4]. However, existing therapies of T2DM are not sufficient [4,5]; because of their complex mechanism of actions, antidiabetic molecules are associated with one or the other side effects such as weight gain; hypoglycaemia; hypothyroidism; abdominal pain; obesity; insulin resistance; atherosclerosis etc. [5-8].

Therefore, a novel approach in the treatment of T2DM, based on the incretin hormone glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) was developed. Major glucose regulating incretin hormones are produced from the L-cells and K-cells of the intestinal mucosa, respectively [9]. In T2DM, there is a decrease in the incretin effect and rapid degradation of short lived GLP-I and GIP [9]. While due to their rapid inactivation by dipeptidyl peptidase-IV enzyme (DPP-IV/ CD26); GLP-1 and GIP are no longer available in the active form [10].

Administration with DPP-IV inhibitors inactivate the enzyme and thereby prolong the half-life and biological activity of GLP-I [10]. This is among one of the recent therapies available in the treatment of T2DM [11]. GLP-1 and GIP have a significant role on the beta cells, such as increased beta-cell survival and mass of the beta-cells [12] and GIP-dependent reduction of the insulin clearance that helps to promote the peripheral insulin levels to maintain normal blood glucose concentrations [13]. Therefore, preventing the cleavage of GLP-1 and GIP became an attractive therapeutic target for T2DM [14].

There have been renewed interest in botanicals as source of drugs and many plants have been used to treat hyperglycaemia [15,16].

Plethora of scientific literature is available on the regulation of T2DM by herbal extracts [5, 14, 17,18]. Interestingly, some reports are there claiming presence of novel DPP-IV inhibitor in plants [14,17,18]. Till date, there is no report available on describing correlation between DPP-IV inhibition and antioxidant properties of antidiabetic plants, if any. Indeed, antioxidant molecules have a property to inhibit the production of free radicals generated by metabolic disorder and its supplements might promote a good health [20-22].

The objective of the present study was to evaluate possible DPP-IV inhibition activity *vis a vis* cytoprotective potential, if any, in the methanolic extracts of *Ocimum sanctum* (Tulsi) leaves and *Momordica charantia* (Karela/ bitter melon) fruit. *O. sanctum* (family Labiateae) and *M. charantia* (family Cucurbitaceae) are important for their therapeutic potentials. 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 [5, 17-19].

### MATERIAL AND METHODS

#### Chemicals

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

#### Plant extracts

Methanolic extract of *O. sanctum* (Tulsi) leaves powder (leave: extract 5:1 w/w) and *M. charantia* (Fruit extract 5:1 w/w) were donated as gift by Amasar Pvt. Ltd, Indore, India. The voucher specimens, batch no-2827 & no-2444 for *O. sanctum* and *M. charantia*, respectively, were deposited in departmental herbarium.

### Sample preparation

For the analysis of DPP IV inhibition, DPPH radical scavenging, total phenolic contents, FRAP assay and reducing power the, the aliquots of different concentrations of the *O. sanctum* and *M. charantia* extracts were prepared in methanol and used further.

### DPP-IV enzyme inhibition assay

DPP-IV assay was performed following the protocol originally described by earlier workers [14,18], as routinely followed in our laboratory [23]. In a 96-well microtiter plate the chromogenic substrate is cleaved by the serine protease DPP-IV resulting in release of paranitroaniline (pNA), a yellow coloured product (measured at 405 nm). In brief, DPP-IV inhibition activities of plant extracts at various concentration (0.1 to 0.5 mg/ml) was 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 on a Microtiter plate reader. Diprotein A was used as a standard. A decrease in DPP-IV activity is a measure for the inhibition.

### Study of DPPH radical scavenging potential

Free radical scavenging activities of the plant extracts were measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH<sup>•</sup>) using the method described by Gyamfi et al [24]. In brief, 200 µl of sample extract (0.1 – 0.5 mg/ml in 80% methanol) or ascorbic acid (standard; 0.1 – 0.5 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.

$$\text{DPPH}^{\bullet} \text{ radical scavenging (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

### Assessment of total phenolic contents

The amount of total phenolics in plant extracts was determined by the Folin-Ciocalteu reagent method following the protocol described by Spanos and Wrolstad [25], as modified by Lister and Wilson [26]. 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<sub>2</sub>CO<sub>3</sub>) 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).

### Ferric reducing antioxidant potential (FRAP) assay

Determination of Ferric reducing antioxidant activity was carried out according to a protocol described elsewhere [27]. The working FRAP reagent was prepared ex tempore by mixing 10 volumes of 300 mmol/L acetate buffer, pH 3.6, with 10 mmol/L TPTZ in 40 mmol/L HCl, and 20 mmol/L FeCl<sub>3</sub>·6H<sub>2</sub>O at 10:1:1 (v/v/v). The samples/ standard solutions (0.5 ml) were allowed to react with 2.0 ml of the FRAP working solution for 10 min at 37° C. Absorbance readings of the blue colored product were then recorded at 593 nm on a Spectrophotometer. Citrate buffer was used for background subtraction. Control contained all the reagents as above except the analytes. The standard curve was linear between 2 and 20 microgram ascorbic acid. Results were expressed as µg ascorbic acid equivalent antioxidant capacity (AEAC)/ ml of Ascorbic Acid.

### Reducing power estimation

The reducing power of plant extracts was determined following the the method of Wu et. al.[28]. 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°C 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<sub>3</sub> were added. Absorbance of the solution was measured at 700 nm on a UV-Vis Spectrophotometer after 10 min incubation at room temperature. Blank of each sample was prepared by adding the distilled water instead of FeCl<sub>3</sub>. Ascorbic acid was used as standard reference. Increased absorbance of the reaction mixture indicates pronounced reducing power of plant extract.

### Determination of Lipid peroxidation

Lipid peroxidation inhibition efficacies of plant extracts were determined according to the method described by Malterud et. al. [29]. 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 (12.5 to 200 µg/ml) of the analytes and Ascorbic Acid were incubated with 1 ml of homogenate and the reaction initiated by the addition of 0.1 ml of FeSO<sub>4</sub> (25 µM), 0.1 ml of ascorbate (100 µM), and 0.1 ml of KH<sub>2</sub>PO<sub>4</sub> (10 mM), and the volume was made up to 3 ml with distilled water and incubated at 37°C for 1 h. Finally 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;

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

### Assessment of erythrocyte haemolysis inhibition of extracts

The procedure originally described by Barros et. al. [30] 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 rat was sacrificed to obtain the erythrocytes. The erythrocyte haemolysis was performed with H<sub>2</sub>O<sub>2</sub> as free radical initiator. The erythrocytes were added with different concentration of plants extracts and 100 M H<sub>2</sub>O<sub>2</sub> (in PBS, pH7.4). The reaction mixture was incubated at 37°C for 3 h with occasional and gentle shaking during incubation. The reaction mixture was diluted with 8 ml of PBS and centrifuged at 3000 rpm for 10 min. The absorbance of the resulting supernatant was measured at 540 nm on a UV-VIS Spectrophotometer to determine the extent of haemolysis inhibition. However, the erythrocytes were treated with 100 M H<sub>2</sub>O<sub>2</sub> and without inhibitors (plants extracts) to obtain a complete haemolysis and without inducer to observe if the extracts have any haemolysis effect. The haemolysis caused by 100 M H<sub>2</sub>O<sub>2</sub> was taken as 100% haemolysis; and the percentage haemolysis inhibition was calculated by the equation.

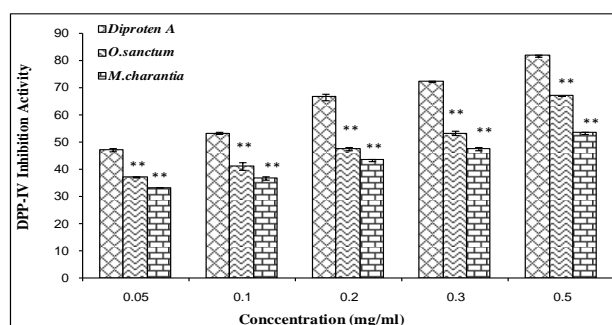
$$\% \text{ haemolysis inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

### Statistical analyses

Data are expressed as mean ± S.E.M. For statistical evaluation of the data, analysis of variance (ANOVA) followed by the *post hoc* Newman-Keuls multiple comparison tests 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.

## RESULT

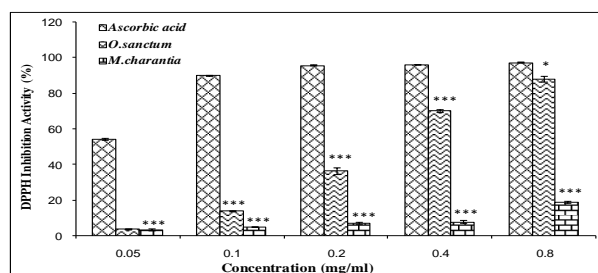
Results from DPP-IV inhibition assay revealed that methanolic extract of *O. sanctum* inhibited enzyme activity at greater extent (66.81±0.05%) than that of *M. charantia* (53.25±0.04%) as compared to standard; Diprotin A (Fig. 1).



**Fig 1: Inhibition of DPP-IV enzyme activity (%) by *O. sanctum* and *M. charantia* extract as compared to control; Diprotin A.**

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

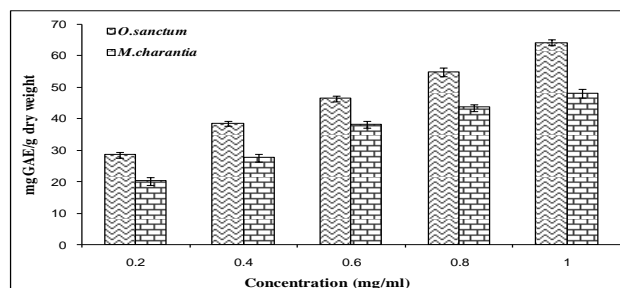
DPPH scavenging assay suggested that varying concentrations of *O. sanctum* extract (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) inhibited the free radicals at greater extent than *M. charantia* (Fig. 2).



**Fig 2: DPPH free radical scavenging activity (%) of *O. sanctum* and *M. charantia* extract as compared to control; Gallic acid.**

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

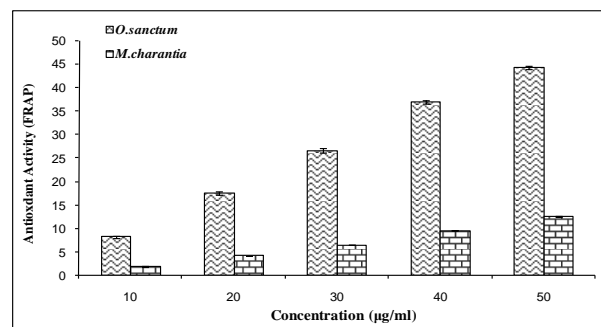
Furthermore, it was observed that *O. sanctum* contains considerable higher amount of total phenolics (64.1±0.93 mg GAE/g of dry extract) than that of *M. charantia* (48.1±1.4 mg GAE/g of dry extract) at 1 mg/ml concentration (Fig. 3).



**Fig 3: Total phenolic contents in *O. sanctum* and *M. charantia* extracts.**

Each vertical bar represents the mean ± S.E.M. (n=3).

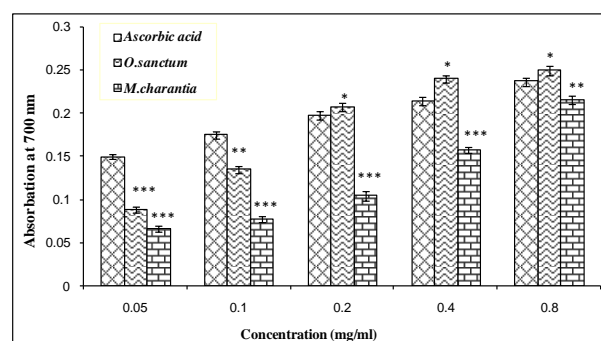
*O. sanctum* extract showed better antioxidant activity (44.2±0.25 AEAC) than *M. charantia* (12.36±0.14 AEAC) (Fig. 4).



**Fig 4: Ferric reducing antioxidant potential (%) in *O. sanctum* and *M. charantia* extract as compared to control; BHA.**

Each vertical bar represents the mean ± S.E.M. (n=3), as compared to respective control values.

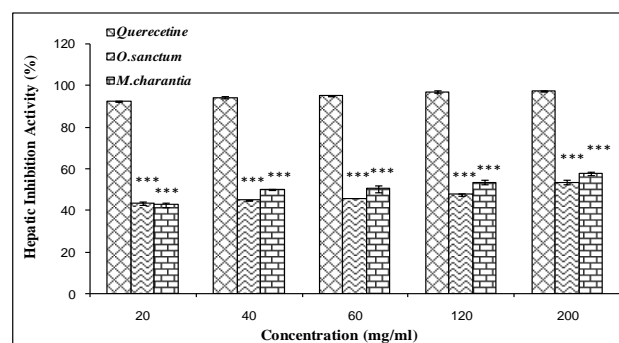
Reducing ability of *O. sanctum* and *M. charantia* extract showed dose dependent increase, as compared to control. *O. sanctum* extract showed pronounced reducing power ability as compared to *M. charantia* at all the studied doses (0.1 – 0.8 mg/ml) (Fig. 5).



**Fig 5: Reducing activity of *O. sanctum* and *M. charantia* extract as compared to control; Ascorbic Acid.**

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

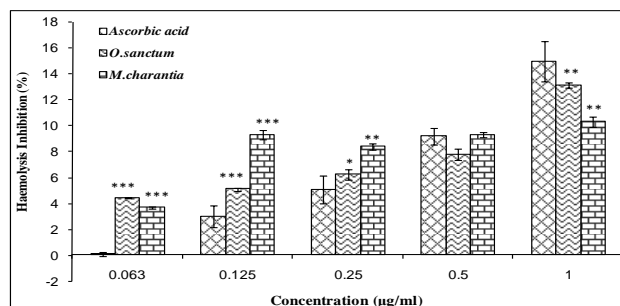
Similarly, studies on *ex vivo* lipid peroxidation in chopped rat liver ravel does dependent activities (Fig. 6).



**Fig 6: Hepatic lipid peroxidation inhibition activities of *O. sanctum* and *M. charantia* extracts as compared to control; Ascorbic Acid.**

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

Methanolic extract of *O. sanctum* inhibited erythrocytes haemolysis activity at greater extent (13.1±0.188%) than that of *M. charantia* (10.3±0.41%) as compared to Ascorbic Acid (Fig. 7).



**Fig 7: Erythrocyte haemolysis inhibition efficacies of *O. sanctum* and *M. charantia* 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

Results of the present study clearly demonstrate that methanolic extracts of *O. sanctum* and *M. charantia* contain novel DPP-IV inhibitors. Interestingly, higher content of phenolics, pronounced reducing power and DPPH radicals scavenging efficacies were exhibited by *O. sanctum* extract than that of *M. charantia*; indicating anti-peroxidative and anti-diabetic potential of the plants. Interestingly, plant extracts inhibited H<sub>2</sub>O<sub>2</sub> induced erythrocytes haemolysis reflecting the cytoprotective nature of *O. sanctum* and *M. charantia*. 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 benefits [23].

The gut hormones GLP-1 and GIP are secreted following intake of meal from the L and K cells of intestinal mucosa, respectively. The gut hormones contribute to approximately 60% of the insulin secretion postprandial and are responsible for the incretin effect [31]. While DPP-IV rapidly inactivates GLP-1 and GIP and DPP-IV inhibitors represent a unique approach in the treatment of type 2 diabetes mellitus 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 [31]. The findings of the present study revealed that varying concentration of methanolic extract of *O. sanctum* and *M. charantia* inhibited DPP-IV activities, reflecting the therapeutic potential for T2DM. Interestingly, our findings corroborate the reports where some other authors claimed to possess novel DPP-IV inhibitors in some indigenous plants earlier [14,18,23,32]. Indeed, antidiabetic potential [5, 19] of the studied plant extracts could be an outcome of DPP-IV inhibition.

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 pharmacological must additionally contain antiperoxidative /cytoprotective potentials [12,23,33]. Phenols and other antioxidant molecules are important constituent of various officinal plant parts of the plants due to their antioxidant properties [25,26,34]. Antioxidant activity of phenolic compound is basically due to their redox properties which play an important role in free radicals scavenging potential [34,35]. Observations made on DPPH radicals scavenging efficacies revealed that both *O. sanctum* and *M. charantia* 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 [23,36,37]. Observations made on the total phenolic content in the extracts of *O. sanctum* and *M. charantia* reflecting the presence of significant amount of natural antioxidants. Interestingly, *O. sanctum* extract scavenge DPPH radicals at greater extent than that of *M. charantia*. The pronounced radical scavenging potential of *O. sanctum* could be an outcome of relatively higher amount of total phenolics content in the plant extract, Ferric reducing antioxidant potential is based on

the capacity of the antioxidant to reduce the Fe<sup>3+</sup> to Fe<sup>2+</sup>, i.e. by donating the electron to the former. *O. sanctum* extract showed the better donation power than *M. charantia* due to higher redox potential of the former and hence higher reducing capacity.

Indeed, reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [38]. 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 [39] and extent of Haemolysis is then determined by measuring released hemoglobin into the supernatant [40,41]. Interestingly, *O. sanctum* extract protect the RBC membrane more pronounced than that of *M. charantia*. In biological systems, increase in hepatic lipid peroxidation indicates increased in oxidative stress. The methanolic extracts of both the plants extracts showed almost same efficiency in inhibiting hepatic lipid peroxidation as compared to standard. The reduction of lipid peroxidation might be attributed to the antioxidant activities of plant extracts.

However, in contrast to DPPH radical scavenging activity; *O. sanctum* extract showed better reducing power as compared to *M. charantia*. Indeed, relatively higher reducing capacity and the antioxidant activity of *O. sanctum* extract appeared to be associated with its active constituents. This fact was further supported by the findings made on total phenolics contents in studied plant extracts. 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 [42].

## CONCLUSION

Results of the present study clearly exhibited that *O. sanctum* and *M. charantia* extract contain novel DPP-IV inhibitors with additional cytoprotective potencies. However, *O. sanctum* extract could be a better lead in the development of DPP-IV based antidiabetic therapies, as reflected by the observations made on antioxidant parameters. Therefore, studies on pharmaceutical preparations considering studied plant extracts as lead would be of great importance especially for the patients where oxidative stresses along with tT2DM is a major concern.

**ABBREVIATIONS:** Dipeptidyl peptidase-IV (DPP-IV), 1,1-diphenyl-2-picryl-hydrazil (DPPH), Type 2 Diabetes mellitus (T2DM).

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