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In Vitro α–Glucosidase Inhibition and Antioxidant Activity of *Walsura trifoliata* (A. Juss.) Harms

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Abstract

Walsura trifoliata is being used in traditional system of medicine with scant scientific documentation. This report is an attempt to provide laboratory findings in support of its traditional use. The crude extract of the root prepared with the solvent systems hexane, ethyl acetate and methanol were used separately for its α–glucosidase inhibition, phenolic contents, reducing ability assay and antioxidant activities. The antioxidant property of the crude extracts were tested using DPPH Radical Scavenging Assay, Hydroxyl Radical Scavenging Assay, Nitric Oxide Radical Inhibition Assay, Superoxide Scavenging Activity, Inhibition of Lipid Peroxidation and Ferric Reducing Antioxidant Power. Among the solvent preparations, the methanol extract of the root of *Walsura trifoliata* showed the maximum inhibitory activity for α–glucosidase inhibition with the IC_{50} value 690 ± 1.44 µg/ml. The IC_{50} value for the methanol extract in DPPH Radical Scavenging Assay was 620 ± 1.99 µg/ml. Among all the parameters studied, methanol extract showed the maximum activity while minimum activity was recorded in hexane extract.

Keywords: *Walsura trifoliata,* α–Glucosidase Inhibition, Antioxidant, Medicinal Plant.

1. Introduction

Dietary measures and traditional plant therapies as prescribed by ayurvedic and other indigenous systems of medicine are used commonly in India. Aromatic plants such as herbs and spices are especially rich in their phenolic content and have been widely used in traditional medicine as treatment for many diseases[1]. The phenolic compounds in plants are found to be effective antioxidants. The antioxidant potential is found to occur in wide range of plants[2–4].

Free radicals have been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes, etc. The compounds that can scavenge free radicals have great potential in ameliorating these disease processes. Antioxidants, vitamins C and E have been shown to reduce the oxidative stress in experimental diabetes[5, 6]. Many synthetic antioxidant components have shown toxic and/or mutagenic effects. Hence attention has been given to naturally occurring antioxidants[7]. An intensive search for novel types of antioxidants has been carried out from numerous plant materials[8–10].

Walsura trifoliata (Meliaceae) has been used in traditional system of medicine as emetic, emmenagogue, expectorant, pediculicide and stimulant. The bark is reported to possess saponin and much of tannin[11,12]. In the present investigation, hexane, ethyl acetate and methanol extracts of *W. trifoliata* were evaluated for *in vitro* α–glucosidase inhibition and antioxidant activity.

2. Materials and Methods

2.1 Chemicals and Reagents

DPPH (1, 1–Diphenyl, 2–Picrylhydrazyl), NBT (Nitro Blue Tetrazolium), NADH (Nicotinamide Adenine Dinucleotide Phosphate reduced), PMS (Phenazine Methosulphate), TCA (TriChloro Acetic Acid), ferric chloride and BHT (Butylated Hydroxyl Toluene) were obtained from Sigma Chemical Co., USA. Ascorbic acid was obtained from

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SD Fine Chem. Ltd., Biosar, India. β–Carotene, ferrozine, folin–phenol reagent and Tween 40 were purchased from Hi–Media Pvt. Ltd. Mumbai, India. All the other chemicals were of analytical grade.

2.2 Collection of *W. trifoliata*

The root of *Walsura trifoliata* was collected from Kambakkam, Nellore District of Andhra Pradesh, South India. The taxonomical identity of the plant was confirmed by Dr. D. Narasimhan, Department of Botany, Madras Christian College, Tambaram, South India.

2.3 Mode of Extraction

The root was shade dried and powdered. The powder (1 kg) was extracted three times by cold percolation method with 3 litres of hexane, ethyl acetate and methanol at room temperature for 72 h. The filtrates were concentrated under reduced pressure at 40˚C and stored in a refrigerator at 2–8˚C for use in subsequent experiments. The percent yield of the hexane, ethyl acetate and methanol extracts were 0.82%, 1.62% and 6.537% (w/w). The concentrations of extracts for *in vitro* α–Glucosidase inhibition and antioxidant assays were fixed based on the previous studies[7].

2.4 Determination of *in vitro* α-Glucosidase Inhibition and Antioxidant Assays

2.4.1 α–Glucosidase Inhibition of W. trifoliata

The small–intestine homogenate of mouse was used as α–Glucosidase solution. The inhibitory effect was measured using the slightly modified method from Dahlqvist[13]. After fasting for 20 h, the small intestine between the part immediately below duodenum and the part immediately above the cecum was cut, rinsed with ice–cold saline, and homogenized with 12 ml of Maleate Buffer (100 mM, pH 6.0). The homogenate was used as the α–Glucosidase solution. The assay mixture consisted of 100 mM Maleate Buffer (pH 6.0), 2% (w/v) each sugar substrate solution (100 µl), and the sample extract (200–1000 µg/ml). The mixture was preincubated for 5 min at 37˚C, and the reaction was initiated by adding the crude α–Glucosidase solution (50 µl) to it, followed by incubation for 10 min at 37˚C. The glucose released in the reaction mixture was determined by GOD–POD method and the absorbance was read at 505nm. The rate of carbohydrate decomposition was calculated as the percentage ratio to the amount of glucose obtained when the carbohydrate was completely digested. The rate of prevention was calculated by the following formula:

Inhibition (%) = [(amount of glucose produced by the positive control) _ (amount of glucose produced by the addition of sample) / (amount of glucose produced by the positive control)] _ 100.

2.4.2 Determination of Total Phenolic Content

Total phenolic content of *W. trifoliata* hexane, ethyl acetate and methanol extracts were assessed according to the Folin–Ciocalteau method[14] with some modifications. Briefly, 0.1 ml of extracts (200–1000 µg/ml), 1.9 ml distilled water and 1 ml of Folin–Ciocalteau's reagent were seeded in a tube, and then 1 ml of 100 g/l Na_2CO_3 was added. The reaction mixture was incubated at 25˚C for 2 h and the absorbance of the mixture was read at 765 nm. The sample was tested in triplicate and a calibration curve with six data points for catechol was obtained. The results were compared to a catechol calibration curve and the total phenolic content of *W. trifoliata* was expressed as mg of catechol equivalents per gram of extract.

2.4.3 Reducing Ability Assay of W. trifoliata

The reducing power of *W. trifoliata* hexane, ethyl acetate and methanol extracts were evaluated according to the method of Oyaizu[15]. Different concentrations of the extracts (200–1000 µg/ ml) were suspended in distilled water and mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50˚C for 20 min; 2.5 ml of 10% TCA was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated the ability of reducing power. BHT was used as standard.

2.4.4 DPPH Radical Scavenging Assay of W. trifoliata

DPPH quenching ability of *W. trifoliata* hexane, ethyl acetate and methanol extracts were measured according to Hanato *et al*[16]. The methanol DPPH solution (0.15%) was mixed with serial dilutions (200–1000 µg/ ml) of the extracts and after 10 min, the absorbance was read at 515 nm. The antiradical activity was expressed as IC_{50} (µg/ml), (the antiradical dose required to cause a 50%

inhibition). Vitamin C was used as standard. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = (A_0 - A_1) / A_0 \times 100 \quad (1)$$

where A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min. All samples were analysed in triplicate.

2.4.5 Hydroxyl Radical Scavenging Assay of W. trifoliata

The assay was performed as described by the method of Elizabeth and Rao[17] with minor changes. All solutions were prepared freshly. One millilitre of the reaction mixture contained 100 µl of 28 mM 2–deoxy–2–ribose (dissolved in phosphate buffer, pH 7.4), 500 µl solution of various concentrations of *W. trifoliata* hexane, ethyl acetate and methanol extracts (200–1000 µg/ml), 200 µl of 200 µM $FeCl_3$ and 1.04 mM EDTA (1:1 v/v),
100 µl H_2O_2 (1 mM) and 100 µl ascorbic acid (1 mM). After an incubation period of 1 h at 37˚C, the extent of deoxyribose degradation was measured by the TBA reaction. The absorbance was read at 532 nm against the blank solution. Vitamin C was used as a positive control. The scavenging activity was calculated using formula (1).

2.4.6 Nitric Oxide Radical Inhibition Assay of W. trifoliata

Sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide; it interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction[18].
In the present investigation, Griess Illosvoy reagent was modified using naphthylethylenediamine dihydrochloride (0.1% w/v) instead of 1-naphthylamin (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM*2 ml), phosphate buffer saline (0.5 ml) and different concentration of *W. trifoliata* hexane, ethyl acetate and methanol extracts (200–1000 µg/ml) or standard solution (0.5 ml) were incubated at 25˚C for 150 min.
After incubation, 0.5ml of the reaction mixture containing nitrite was pipetted and mixed with 1ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1ml of naphthylethylenediaminedihydrochloride (1%) was added, mixed and allowed to stand for 30 min. A pink coloured chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank. Vitamin C was used as positive control. The scavenging activity was calculated using formula (1).

2.4.7 Superoxide Scavenging Activity of W. trofoliata

Superoxide scavenging activities of *W. trifoliata* hexane, ethyl acetate and methanol extracts were determined by monitoring the competition of those with NBT for the superoxide anion generated by the PMS–NADH system[19]. Superoxide radicals were generated in 1 ml of 20 mM Tris–HCl buffer pH 8.0 containing 0.05 mM NitroBlueTetrazolium (NBT), 0.01 mM PhenazineMethoSulphate (PMS) and different concentration of extracts (200–1000 µg/ml) were preincubated for 2 min. The reaction was initiated by the addition of 0.078 mM NADH. Blue chromogen, formed due to NBT reduction was read at 560 nm. Results were expressed as percentage of inhibition of superoxide radicals. Vitamin C was used as a positive control. The scavenging activity was calculated using formula (1).

2.4.8 Inhibition of Lipid Peroxidation in Rat Liver Homogenate by W. trifoliata

The inhibition effect of *W. trofoliata* hexane, ethyl acetate and methanol extracts on lipid peroxidation was determined according to the thiobarbituric acid method
$FeCl_2$-H_2O_2 was used to induce liver homogenate peroxidation[20]. In this method, 0.2 ml of different concentration of extracts (200–1000 µg/ ml) was mixed with 1 ml of 1% liver homogenate (each 100 ml homo-genate solution contains 1 g rat liver); then 50 µl of $FeCl_2$ (0.5 mM) and H_2O_2 (0.5 mM) was added. The mixture was incubated at 37 ˚C for 60 min; then 1 ml of trichloroacetic acid (15%) with thiobarbituric acid (0.67%) was added and the mixture was heated in boiling water f r 15 min. The absorbance was recorded at 532 nm. Vitamin C was used as positive control. The percentage of inhibition was calculated using formula (1).

2.4.9 Ferric Reducing Antioxidant Power (FRAP)

A slightly modified method of[21] was adopted for the FRAP assay. A standard or sample extract (300 µg/ml) was mixed with 300 µl of ferric–TPTZ reagent (prepared by mixing 300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ at a ratio of 10:1:1

(v/v/v)). The mixture was incubated at 37°C and the absorbance readings were taken at 593 nm after 4 min. Results were expressed in mM Fe (II)/g dry mass.

2.5 Statistical Analysis

The data for biochemical and physiological parameters were analysed and expressed as means ± SD. The IC_{50} values were calculated from linear regression analysis. Results were processed by computer program, Microsoft Excel (2007).

3. Results

3.1 *in vitro* α–Glucosidase Inhibition and Antioxidant Assays of *W. trifoliata*

3.1.1 α–Glucosidase Inhibition

The results for α–Glucosidase inhibition assay of *W. trifoliata* root extracts (hexane, ethyl acetate and methanol) and acarbose were shown in Table 1. The concentration for 50% inhibition of methanol and acarbose were found to be 690.10 ± 1.44 and 290.90 ± 1.82 µg/ml, respectively. The hexane and ethyl acetate extracts showed less inhibition compared to methanol extract.

3.1.2 Total Phenolic Content

The total phenolic content of hexane ethyl acetate and methanol extracts of *W. trifoliata* was found to be 82.89 ± 0.32, 116.06 ± 0.57 and 202.51 ± 1.21 mg catechol equivalent/gram extract, respectively.

3.1.3 Reducing Power

Figure 1 shows the reductive capabilities of hexane, ethyl acetate and methanol extracts of *W. trifoliata* compared to BHT. The reducing power of *W. trifoliata* methanol root extract was very potent than that of hexane and ethyl acetate extracts and the activity of the extract increased with the sample quantity. The plant extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of BHT.

3.1.4 DPPH Radical Scavenging Activity

W. trifoliata root in methanol extract exhibited a significant dose dependent inhibition of DPPH activity compared to hexane and ethyl acetate extracts. The highest scavenging activity with 50% inhibition (IC_{50}) was found at a concentration of 620.02 ± 1.99 µg/ml of methanol extract when compared with hexane and ethyl acetate. The results are presented in Figure 2. The IC_{50} value of vitamin C was 330.10 ± 1.14 µg/ml.

3.1.5 Hydroxyl Radical Scavenging Assay

To attack the substrate deoxyribose, hydroxyl radicals were generated by reaction of Ferric–EDTA together with H_2O_2 and ascorbic acid. When the plant extracts were incubated with the above reaction mixture, it could prevent the damage against sugar. The results for hydroxyl scavenging assay are shown in Figure 3. The concentrations for 50% inhibition of ethyl acetate and methanol were found to be 710.50 ± 1.69 and 240.45 ± 1.42 µg/ml.

Table 1. α-Glucosidase inhibition of *Walsura trifoliata* hexane, ethyl acetate, methanol root extracts and acarbose

Sample	Concentration (µg/ml)	% of Inhibition	IC_{50}(µg/ml)
Hexane extract	200	7.00±1.80	
	400	11.62±2.07	
	600	20.00±2.71	50
	800	27.52±2.91	
	1000	31.45±1.56	
Ethyl acetate extract	200	14.70±1.56	
	400	25.47±1.94	
	600	32.13±2.31	50
	800	36.23±1.29	
	1000	43.41±2.07	
Methanol extract	200	24.61±2.56	
	400	35.89±2.35	
	600	44.10±1.53	690.10±1.44
	800	56.92±1.35	
	1000	65.47±2.31	
Acarbose	200	46.49±1.29	
	400	54.87±2.35	
	600	65.98±2.52	290.90±1.82
	800	72.82±2.56	
	1000	81.19±2.58	

Each value represents the mean ± SEM of triplicate experiments.

The IC_{50} value of vitamin C was 240.90 ± 1.26 µg/ml. Hexane extracts showed less inhibition.

3.1.6 Nitric Oxide Radical Inhibition Assay

The scavenging effect of nitric oxide by *W. trifoliata* root extract of hexane, ethyl acetate and methanol were increased in a dose–dependent manner as illustrated in Figure 4. At concentration of 890.50 ± 1.55, 640.30 ± 1.84, 500.90 ± 1.67 µg/ml of extract, ethyl acetate, methanol and vitamin C respectively 50% of nitric oxide generated by incubation was scavenged.

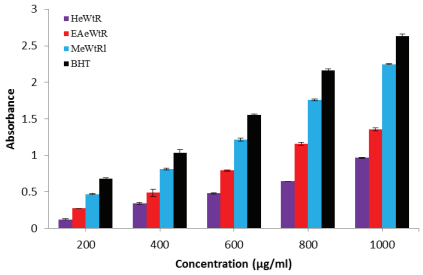

Figure 1. Reducing ability of *Walsura trifoliata* root extracts in different concentrations (200–1000 µg/ml) of hexane, ethyl acetate, methanol and vitamin C. Each value represents the mean ± SEM of triplicate experiments.

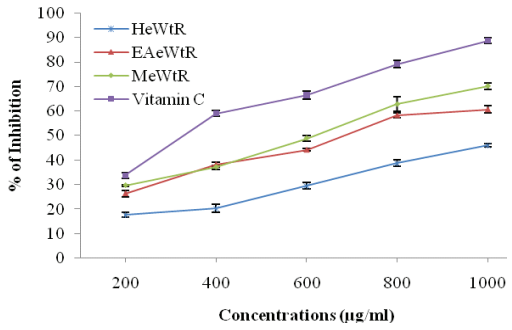

Figure 2. DPPH scavenging effect of *Walsura trifoliata* root extracts in different concentrations (200–1000 µg/ml) of hexane, ethyl acetate, methanol and vitamin C. Each value represents the mean ± SEM of triplicate experiments.

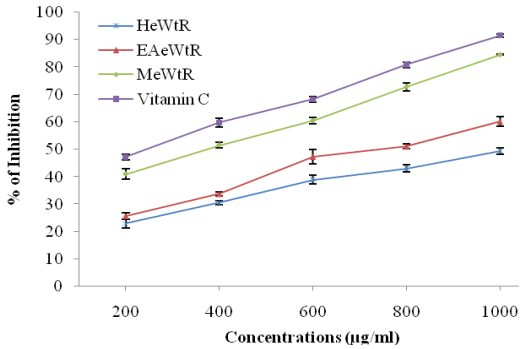

Figure 3. Hydroxyl radical scavenging effect of *Walsura trifoliata* root extracts in different concentrations (200–1000 µg/ml) of hexane, ethyl acetate, methanol and vitamin C. Each value represents the mean ± SEM of triplicate experiments.

3.1.7 Superoxide Scavenging Activity

The superoxide anion derived from dissolved oxygen by phenazinemethosulphate/NADH coupling reaction reduces nitro blue tetrazolium. When the absorbance decreases at 560 nm with the plant extract, it indicates the consumption of superoxide anion in the reaction mixture. As shown in Figure 5, the methanol extract from the root of *W. trifoliata* as well as vitamin C exhibited the scavenging activity; IC_{50} values, 710.50 ± 1.96 µg/ml and 560.10 ± 2.22 µg/ml respectively.

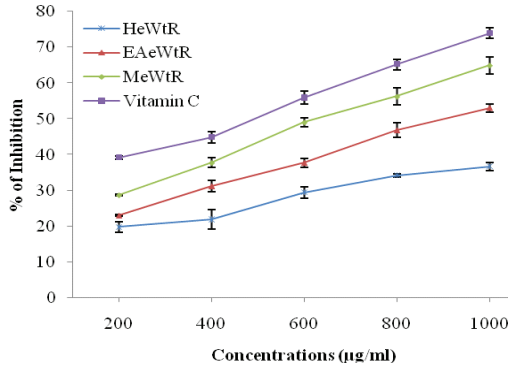

Figure 4. Nitric oxide scavenging effect of *Walsura trifoliata* root extracts in different concentrations (200–1000 µg/ml) of hexane, ethyl acetate, methanol and vitamin C. Each value represents the mean ± SEM of triplicate experiments.

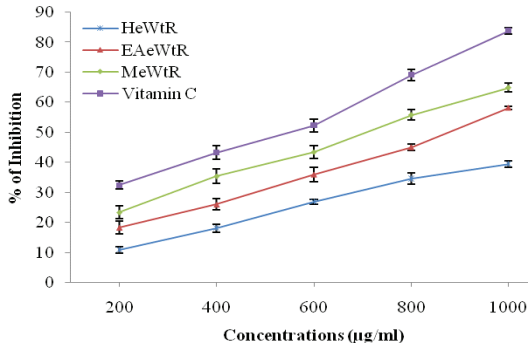

Figure 5. Superoxide scavenging effect of *Walsura trifoliata* root extracts in different concentrations (200–1000 µg/ml) of hexane, ethyl acetate, methanol and vitamin C. Each value represents the mean ± SEM of triplicate experiments.

3.1.8 *Lipid Peroxidation Assay*

Activity of extracts on lipid peroxidation was shown in Figure 6. Addition of Fe^{2+}/ascorbate to the liver microsomes cause increase in lipid peroxidation. Methanol extract from the root of *W. trifoliata* showed highest inhibition of peroxidation effect when compared to hexane and ethyl acetate, which showed 50% inhibition effect at 640.30 ± 1.84 µg/ml. The IC_{50} value of vitamin C was 500.90 ± 1.67 µg/ml.

3.1.9 *Ferric Reducing Antioxidant Power (FRAP)*

The root extract from *W. trifoliata* showed maximum ferric reducing antioxidant power in methanol extract (560 ± 1.35 µg/ml). Minimum reducing power was found in hexane extract. 50% of reducing power at 960.10 ± 2.31µg/ml was found in ethyl acetate extract (Figure 7).

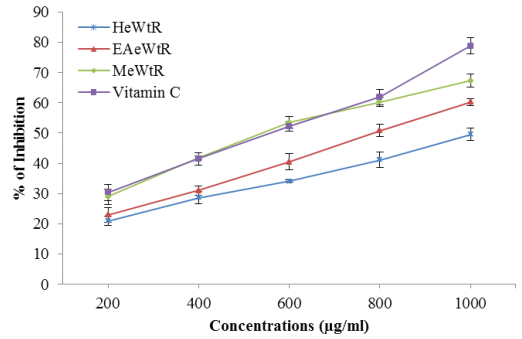

Figure 6. Lipid Peroxidation scavenging effect of *Walsura trifoliata* root extracts in different concentrations (200–1000 µg/ml) of hexane, ethyl acetate, methanol and vitamin C. Each value represents the mean ± SEM of triplicate experiments.

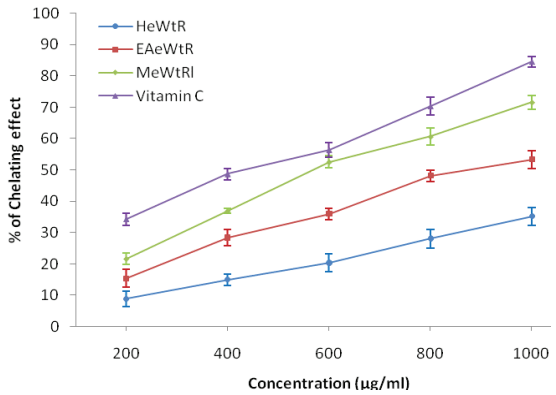

Figure 7. FRAP scavenging effect of *Walsura trifoliata* root extracts in different concentrations (200–1000 µg/ml) of hexane, ethyl acetate, methanol and vitamin C. Each value represents the mean ± SEM of triplicate experiments.

HeWtR = hexane extract of *W. trifoliata* root; EaeWtR = ethyl acetate extract of *W. trifoliata* root; MeWtR = methanol extract of *W. trifoliata* root.

4. Discussion

There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases[22]] Many synthetic antioxidant components have shown toxic and/or mutagenic effects. Hence attention has been given to naturally occurring antioxidants. Numerous plant constituents have shown free radical scavenging or antioxidant activity[23]. The phytochemical compounds, such as the phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen–or electron–donating agents, and metal ion chelating properties[24]. Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties[25]. Flavonoids and other phenolic compounds (hydroxyl cinnamic derivatives, catechines, etc.) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation[26]. Agents with α–Glucosidase inhibitory activity have been useful as oral hypoglycemic agents for the control of hyperglycemia in patients with diabetes. There are many natural sources with α–Glucosidase inhibitory activity[27]. In the present study, methanol extract from the root of *W. trifoliata* effectively reduced the glucose level in α–Glucosidase inhibition assay. There is no significant activity in hexane and ethyl acetate extract of *W. trifoliata*. The present study suggest that preventing an excessive postprandial rise of blood glucose level by α–Glucosidase inhibition from natural resources is effective in real life as well. There are many natural sources with α–Glucosidase inhibitory activity.

The total phenolic content estimation showed high amount of polyphenols in methanol extract. Polyphenols are the major plant compounds with antioxidant activity, is believed to be mainly due to their redox properties[28] which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. In our study the antioxidant property of *W. trifoliata* root extracts were evaluated with varying parameters.

The reducing ability of *W. trifoliata* was studied, using the measurements of Fe^{3+} to Fe^{2+} transformation[15]. The reducing power increased with increasing concentration

of the extract. The reducing capacity of the extract may serve as a significant indicator of its potential antioxidant activity[29]. DPPH test is usually used as the substrate to evaluate antioxidative activity of antioxidants[15]. This method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non–radical form DPPH–H by the reaction[30]. MeWtR extract has the ability to reduce the stable radical DPPH to the yellow– coloured diphenyl picrylhydrazine. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells[31]. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity[32]. MeWtR inhibited free radical mediated deoxyribose damage remarkably.

Nitric oxide plays an important role in various types of inflammatory processes in the animal body. Nitric oxide radical inhibition study showed that the extract was a potent scavenger of nitric oxide. The extract inhibited nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. Scavengers of nitric oxide competed with oxygen leading to reduced production of nitric oxide[33]. In the PMS–NADH–NBT system, superoxide anion derived from the dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease in the absorbance at 560 nm with antioxidants thus indicates the consumption of the generated superoxide anion in the reaction. Superoxide, the one–electron reduced form of molecular oxygen, is a precursor of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen that have the potential of reacting with biological macromolecules and thereby inducing tissue damages[34]. These results clearly indicated that MeWtR is a potent scavenger of superoxide radicals in a dose–dependent manner. Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. MalonDiAldehyde (MDA), one of the products of lipid peroxidation, has been studied widely as an index of lipid peroxidation and as a marker of oxi-dative stress[35]. MeWtR showed a strong inhibition of lipid peroxidation. MeWtR extract was able to reduce ferric ions when assessed by FRAP assay[36], indicating that the results obtained may have partly been contributed by these phenolics. MeWtR methanol extract showed potent α–Glucosidase inhibition and antioxidant activity, this may be due to the presence of good amount of phenolic and flavonoids.

5. Conclusion

This study suggested that the methanol extract of *W. trifoliata* root possessed α–Glucosidase inhibition and antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress–related diseases. Further, the isolation of active compound from this extract may increase the α–Glucosidase activities and lead to chemical entities for clinical use.

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