In-vitro antioxidant and antidiabetic activity of Tephrosia tinctoria PERS.: an endemic medicinal plant of South India

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ABSTRACT

The present study was carried out to explore the in vitro antioxidant and antidiabetic activity of the different parts (Leaf, Stem & Root) of Tephrosia tinctoria extracted with various solvents from non polar to polar basis (Hexane, Chloroform, Ethyl acetate & Ethanol). Among the various fractions tested, the Ethyl acetate fraction of stem of T. tinctoria exhibited maximum antioxidant (DPPH with IC50 value 33.54±4.2µg/ml) and antidiabetic (α-Glucosidase inhibition IC50 value 94.3±3.65µg/ml) activity. Further the ethyl acetate fraction of stem extract was assayed for antioxidative assays such as reducing power, lipid peroxidation inhibition activity, total phenols and total flavonoids. The ethyl acetate stem extract fraction of T. tinctoria was subjected to cytotoxicity studies by performing MTT assay on L6 muscle cell line, which showed "nil or low" toxicity effect at higher doses.

Key words: Antioxidant, DPPH, α-Glucosidase, Tephrosia tinctoria

INTRODUCTION

Diabetes mellitus is one of the world’s major public health burdens. In 2000, there were around 171 million diabetes causes and it is estimated that the number will double by 2030[10]. Numerous studies have been demonstrated that oxidative stress, mediated mainly by hyperglycemia induced generation of free radicals, contributes to the development and progression of diabetes and its complications[1-2]. Abnormally high levels of free radicals which cause membrane damage due to peroxidation of membrane lipids and protein glycation and simultaneous decline of antioxidant defense mechanisms leads to cell and tissue damage[3]. The increase of ROS leads to damage of β-cells through the induction of apoptosis and suppression of insulin biosynthesis[4-5]. As a new strategy for alleviating the oxidative damage in diabetes, interest has shown in the usage of natural antioxidants. It has been postulated that many of the negative effect of oxidative stress are diminished upon supplementation with certain dietary antioxidants such as Vitamin E, Vitamin C and other non-nutrient antioxidants such as flavonoids[6].

The genus Tephrosia (Fabaceae) with about 400 species is distributed chiefly in Asia, Africa, Australia and America[9-10]. About twenty-four species of Tephrosia were recorded in India[11-12]. Most of the Tephrosia’s are herbs to undershrubs and are grown as weeds. The genus is well known for its richness in prenylated flavonoids and is considered to possess insect repellent, larvicidal, pesticidal, antimicrobial and anticancer properties[13-14]. Tephrosia tinctoria (TT) is widely distributed in Andhra Pradesh, South India[13]. A new prenylated isoflavone 7-O-Geranylbiochanin together with three known compound, 7-O-methylglabranin, Flammachaparin and dehydrodegeulin was isolated from root[15]. The root methanolic extract of TT possess antimicrobial activity[16-18]. The objective of the present investigation is to explore the in vitro antioxidant and antidiabetic activity of bioactive guided fraction of TT herb. To our knowledge, this is the first report of antioxidant and antidiabetic activity in TT.

MATERIALS AND METHODS

Plant material

The herb TT was collected from Kolli hills of Namakkal (Dt), Tamil Nadu during the month of November 2009 and authenticated by the Botanical Survey of India (BSI) (Southern Circle), Coimbatore, Tamil Nadu. A voucher specimen (BSI/BRSC/523/09-10/tech-1569) was deposited in the Rappinart Herbarium, St.Joseph’s College, Tiruchirappalli, Tamil Nadu, India. The shade dried plant parts of TT (leaf, stem and root) were coarsely powdered with mechanical grinder and stored in dry place.

Chemicals

1.1 –diphenyl-2-pireyld-hydrazide(DPPH), butyalted hydroxyl tolune (BHT) were purchased from Hi media, Mumbai. All other chemicals and reagents were of analytical grade.

Preparation of plant extracts (Activity guided extraction)

100gm of powder (leaf, stem and root) was extracted with various organic solvents (Petroleum ether, Chloroform, Ethyl acetate and methanol) based on their polarity. All fractions were evaporated to dryness at 40°C in the rotary evaporator and stored. 100mg of each extract of different plant parts (Leaf, Stem & Root) of TT was dissolved in 1 ml of DMSO (Dimethyl sulfoxide) which was used for the following assays.

DPPH radical scavenging activity

The free radical scavenging activities of all extracts were measured by DPPH assay[27]. 0.9 ml of 1.5 x 10^{-5} M DPPH radical solution in methanol was prepared, and then mixed with 0.1ml of the sample dissolved in DMSO and kept in the dark for 30 min. The quantity of DPPH remaining in the mixed solution was measured at 517nm. The reduction in the absorbance of the DPPH solution indicated the free radical scavenging activities of the test extracts. DMSO without the sample was used as a control.

The DPPH radical scavenging activity was calculated according to the following formula: (%) inhibition ratio = [(Abs_0 – Abs_sample)/Abs_0] x 100.

α-Glucosidase inhibitory activity

The inhibitory effect was measured using the method slightly modified from Dahlqvist[28]. After fasting for 20h, 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 12ml of maleate buffer (100mM, pH 6.0). The homogenate was used as a-α-glucosidase solution. The assay mixture consisted of 100mM maleate buffer (pH 6.0), 2% (w/v) each maltose substrate solution (100µl), and the extracts (50-500µg/ml). The mixture was incubated at 37°C. The glucose released in the reaction mixture was determined with the kit (GOD-POD Method). The rate of carbohydrate decomposition was calculated as 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 rate (%) = [(Abs_0-A)/(A_B)] x 100

A0 – Amount of glucose produced by the positive control
A – Amount of glucose produced by the addition of Extracts
B – Glucose production value in blank

Measurement of total phenol and flavonoid contents

Determination of total phenolic content was made using Folin-Ciocalteau’s phenol reagent[29]. Test sample (1ml), 0.5ml of Folin-Ciocalteau’s phenol reagent (2N), and 2ml of Na2CO3 (5%) were mixed and the reaction mixture was allowed to proceed for 5 min at room temperature, before dilution with 5ml of deionized water. Each sample was mixed thoroughly and placed in dark for 1 h and the absorbance was measured at 725nm with a UV-Vis spectrophotometer. Gallic acid equivalent (mg/g) was determined from a standard concentration curve.

The flavonoid content was determined according to the aluminum chloride colorimetric method [30] with some modifications. Quercetin was used as a standard to make the calibration curve. The sample solution (0.5ml) was mixed with 1.5ml of 95% ethanol, 0.1ml of 10% aluminum chloride hexahydrate, 0.1ml of 1M potassium acetate, and 2.8ml of distilled water. After incubation at room temperature for 40 min the absorbance of the reaction mixture was measured at 415 nm. The same amount (0.1ml) of distilled water substituted for the amount of 10% aluminum chloride as the blank and a seven point standard curve (0-500µg/ml) was obtained. All tests were performed in independent triplicates ([n]=3) and the datas were expressed as mean ±SD.

Reducing power assay

Various concentrations of Ethyl acetate extract of stem (1ml) were mixed with 2.5 ml of 1% sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Then the mixture was incubated at 50°C for 30 min. After 2.5 ml of 10% TCA were added to the mixture was centrifuged at 3,000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml deionized water with 0.5 ml of 0.1% of ferric chloride and the absorbance was measured at 700 nm. The assays were carried out in triplicate and the results were expressed as mean ± SD. Ascorbic acid was used as a standard[31].

Lipid peroxidation inhibition assay

This assay was carried out using rat liver homogenate as substrate. Male rat of Wister strain was sacrificed by cervical dislocation and liver was immediately excised, and a homogenate (5.1 w/v) was prepared using phosphate buffered saline (PBS) in cold condition. It was centrifuged at 200 g for 10 min. The supernatant was collected and finally suspended in PBS so as to contain 10 mg protein in 1 ml suspension to perform in vitro experiment. Protein content was estimated by using diagnostic kit (Span Diagnostics, India). Various concentrations of the ethyl acetate stem extracts dissolved in 1 ml of PBS were mixed with 3 ml of homogenate. Lipid peroxidation was initiated by adding 100 µl of H2O2 (10 mM), gently

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mixed and incubated at 37°C for 30 min. After 30 min the thiobarbituric acid-reactive substances (TBARS) were estimated by standard method[27] and reduced glutathione was estimated according to the standard method[28]. A control is run without the addition of extract and the percent inhibition of lipid peroxidation was calculated by the following formula.

\[
\text{Percentage of inhibition (\%) =} \frac{Abs \ control - Abs \ sample}{Abs \ control} \times 100
\]

Abs control - absorbance of the control reaction (Containing all reagents except the test compound), Abs sample - absorbance of the test compound. BHT was used as positive controls and all tests were carried out in triplicates.

**MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay**

Muscle cell line (L6) were grown in DMEM supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin, and 5 mg/ml amphotericin B) at 37°C in a humidified atmosphere composed of 5% CO₂ in a 24 well plate with 3.5x10⁵ L6 myotubes/well. The medium was replaced at every third day. All experiments were performed in plastic tissue culture flasks, dishes, or in microplates. For differentiation, the L6 myotubes were transferred to DMEM having 2% FBS for 4–6 days post-confluence. The extent of differentiation was established by observing the formation of elongated and multinucleate myotubes. These differentiated cells were used for further studies.

Cell viability was evaluated by measuring the mitochondrial dependent reduction of colourless MTT to a coloured blue formazan. The cells were seeded at 5x10⁴ cells/ml density and incubated plant extracts (50-1000 µg/ml) for 24-h and 48-h. Then the medium was changed and the cells were incubated with MTT (0.5 mg/ml in PBS) for 4h at 37°C. The formazan was dissolved in HCl in isopropanol and the absorbance at 595 nm was measured with spectrophotometer. The number of viable cells is directly proportional to the formation of formazan. Percentage Growth inhibition (%) =100 - [M (Mean of individual test group) / Mean OD of the control group X 100]

**Statistical Analysis**

All the experiments were repeated at least three times. Results were reported as mean ± SE.

**RESULTS AND DISCUSSION**

Owing to the presence of complicated phyto-ingredient diversities in plants, the *in vitro* activity guided extraction has been effectively applied to screen the biological activities. These activities may contribute important indications for investigating the characteristics of active component[29]. In this context different plant parts (Leaf, Stem & Root) of TT were subjected to activity guided extraction based on polarity to identify the potent bioactive fraction for claim for the maximum antioxidant activity.

High amount of flavonoids and moderate amount of phenols. Total phenol and flavonoid content of the EA-Stem extract of TT was 40 mg/gm (Gallic acid/gm) and 64 mg/gm respectively compared with Acarbose (2008) observation in some Iranian medicinal plants of fabaceae family[30]. The ethyl acetate extract of stem and root extract of TT has strong antioxidant activity (IC₅₀ value 33.54±4.27 µg/ml) compared to other extracts (leaf & root) shown in Table 1 & Fig. 1. The percentage inhibition of Tephrosia purpurea showed IC₅₀ value of 523.8 µg/ml[31]. Our result shows that the Ethyl acetate stem extract of TT has significant potential to scavenge free radicals at minimum dosage which was comparable to the positive control a-tocopherol (IC₅₀ value 31.79±3.81 µg/ml).

**Table 1. a-Glucosidase inhibitory activity of TT extracts**

<table>
<thead>
<tr>
<th>Zinzicoria</th>
<th>a-Glucosidase inhibitory activity R², Value(µg/ml)</th>
<th>DPPH Assay IC₅₀ Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet-Leaf</td>
<td>-</td>
<td>555.5±4.52</td>
</tr>
<tr>
<td>Pet-Stem</td>
<td>-</td>
<td>477.8±3.65</td>
</tr>
<tr>
<td>Pet-Root</td>
<td>-</td>
<td>596.2±3.32</td>
</tr>
<tr>
<td>C-Leaf</td>
<td>-</td>
<td>508.8±4.31</td>
</tr>
<tr>
<td>C-Stem</td>
<td>-</td>
<td>499.5±4.71</td>
</tr>
<tr>
<td>C-Root</td>
<td>1506.02±6.69</td>
<td>566.1±2.72</td>
</tr>
<tr>
<td>EA-Leaf</td>
<td>621.89±2.94</td>
<td>693.5±9.55</td>
</tr>
<tr>
<td>EA-Stem</td>
<td>94.31±6.65</td>
<td>33.54±4.27</td>
</tr>
<tr>
<td>EA-Root</td>
<td>1143.36±4.79</td>
<td>472.0±4.37</td>
</tr>
<tr>
<td>E-Leaf</td>
<td>543.37±2.4</td>
<td>452.7±2.41</td>
</tr>
<tr>
<td>E-Stem</td>
<td>1160.63±3.59</td>
<td>455.4±2.04</td>
</tr>
<tr>
<td>E-Root</td>
<td>2446.18±6.64</td>
<td>542.0±1.73</td>
</tr>
<tr>
<td>Acarbose</td>
<td>38.89±3.52</td>
<td>31.79±3.81</td>
</tr>
</tbody>
</table>

*Pet-Petroleum ether; C-Chloroform; EA-Ethyl acetate; E-Ethanol; Results are expressed as Mean ± SE; n=3

**Figure 1. Radical scavenging activity of TT compared with a-Tocopherol**

**Figure 2. a-Glucosidase inhibitory activity of T. tinctoria extracts compared with Acarbose**

**Total Phenol and Flavonoids**

The phenol and flavonoid content of the EA-Stem extract of TT was analysed, since most of the flavonoids have antioxidant activity and they ascribed to various properties like anticancer, anti-diabetic, anti-inflammatory and prevention of cardiovascular diseases[32-34]. The ethyl acetate extract of TT was found to have maximum antioxidant activity which may be due to the presence of high amount of flavonoids and moderate amount of phenols. Total phenol and flavonoid content of the EA-Stem extract of TT was 40 mg/gm (Gallic acid/gm) and 64 mg/gm (Quercetin/gm) respectively. The high content of flavonoid in the extracts substantiates the claim for the maximum antioxidant activity.

**Reducing power assay**

Reducing power assay is based on the principle that substances, which has reduction potential, react with potassium ferricyanide (Fe⁺³) to form potassium ferrocyanide (Fe⁺²), which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700nm. The reducing capacity of the compound may serve as a significant indicator of its potential antioxidant activity[35]. So the hydrogen donating ability of the EA-Stem extract of TT...
Lipid peroxidation inhibition assay

The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perperyl complex or through OH radicals by Fenton reaction[45-46], thereby initiating a cascade of oxidative reactions. Generation of free radicals eventually causes depletion of antioxidants and glutathione (GSH) and increases TBARS. Hence the estimation of the reduced glutathione may serve as a better marker of antioxidant status[47]. Fig. 4 shows the lipid peroxidation assay of EA-Stem & BHT (IC50 value 6.4±1.5 µg/ml & 38.19±2.14 µg/ml respectively). The results obtained in the present study may be credited to several reasons viz., the inhibition of free-radical-perperyl complex formation; scavenging of OH or peroxide radicals or by changing the ratio of Fe3+/Fe2+; reducing the rate of conversions of ferrous to ferric by chelating of the iron itself[48].

MTT Assay

The MTT assay is used to assess the viability and the proliferation of cells[49]. It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since these agents would stimulate or inhibit cell viability and growth. The yellow colour MTT is reduced to purple formazan by the enzyme reductase which present in the living cells. The absorbance of the EA-Stem extract of TT treated with the L6 Muscle cell line at the different concentrations (50-500 µg/ml) compared with control (untreated L6 cells) shown in Table 2. The results confirm that there was “nil or low” toxicity effect of EA-Stem extract at higher concentrations.

Table 2: Absorbance of EA-Stem treated L6 cell line in MTT Assay

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Percentage of viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>97.2±2.8</td>
</tr>
<tr>
<td>100</td>
<td>93.2±6.9</td>
</tr>
<tr>
<td>250</td>
<td>77.9±1.6</td>
</tr>
<tr>
<td>500</td>
<td>73.5±6.8</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E, n=3

CONCLUSION

Our present investigation reveals the antioxidant and antidiabetic potentiality in the partial fractions of stem extracts (EA) using in vitro model. Further “investigations are in progress to isolate the active principle and study the complete mechanism in both in vitro and in vivo model.”

ACKNOWLEDGEMENT

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