Phytochemical Screening and in vitro Amylase Inhibitory Effect of the Leaves of Breynia retusa

K.G. Kripa, R. Sangeetha, P. Madhavi and P. Deepthi
Department of Biochemistry, School of Life Sciences, Vels University, Chennai-600 117, India

Abstract: This study was proposed based on the folklore claim and on the scarcity of scientific evidence from the literature for the medicinal uses of Breynia retusa. The aim of the present study was to analyze the phytochemical constituents of the leaves of B. retusa. The fractions obtained by successive fractionation using solvents of varying polarity were studied for the presence of primary and secondary metabolites and the total phenolic content of the different fractions were determined by HPLC. The results of the study support the traditional acclaim of the therapeutic uses of B. retusa. The potential of B. retusa to inhibit α-amylase, a prime enzyme involved in carbohydrate metabolism was analysed and it was observed that the ethyl acetate and methanolic extract of the leaves of B. retusa possessed in vitro amylase inhibitory activity.

Key words: Breynia retusa, phytochemical, phenolics, HPLC, α-amylase inhibition

INTRODUCTION

Consumption of medicinal herbs is tremendously increasing over the past decade as alternative approach to improve the quality of life and maintain a good health. Medicinal plants have been used for centuries as remedies for human diseases (Nostro et al., 2000).

World wide, several species of plants are currently being employed by human beings for many purposes (Karim et al., 2011; Sohail et al., 2011a, b; Sohail and Sohail, 2011). Many people, especially in the poorer, underdeveloped countries, rely on wild plants for food, construction materials, fuel wood, medicine and many other purposes. Traditionally, the people belonging to many local communities and tribes worldwide are extremely knowledgeable about plants and other natural resources and are hence dependent on plants for the maintenance of their health and to ameliorate ailments (Jothi et al., 2008).

The World Health Organization (WHO) has listed 20,000 medicinal plants globally and estimated that 80% of the world’s inhabitants rely mainly on traditional medicines for their health care. In India, about 2000 drugs used are of plant origin (Laloo et al., 2006). The majority of the Indian medicinal plants are yet to be scientifically evaluated for medicinal properties and their potential as a source of new drugs is being explored at large. The medicinal importance of a plant is due to the presence of active principles like alkaloids, glycosides, resins, tannins etc which are concentrated in parts of the plants like bark, leaves, roots, seeds etc.

Breynia retusa (Synonym-Phyllanthus retusus) belongs to the family Euphorbiaceae. Euphorbia is the largest genus in the family Euphorbiaceae and one of the sixth largest genera of flowering plants in the world, consisting of about 2000 species. Out of 81 species of Euphorbia occurring in India, about 40 species have been ethnombotanically studied (Kumar and Balakrishnan, 1996; Jothi et al., 2008). Many plants of this family have been used in traditional Chinese medicine for more than 2000 years as anti-tumour drugs. According to Schroeder et al. (1980), plants of this family have been used to treat cancer, tumours and warts from the time of Hippocrates (ca 400 BC). Ethnobotanical studies have revealed the folklore medicinal claim of Breynia sp. (Jothi et al., 2008; Verma et al., 2010). Macerated leaf juice is taken for body pain, skin inflammation, hyperglycemia, diarrhoea and as diuretic, bark as astringent and diuretic. Also the fruits have been used for dysentery, roots for fits and meningitis, twigs for toothache (Laloo et al., 2006; Franco and Narasimhan, 2009; Verma et al., 2010). The plant has been proved to possess herbicide potential against Parthenium hysterophorus (Arshad, 2010). A herbal drug consisting of extracts of Breynia retusa and Leptadenia reticulata has been used as a galactogogue. The juice of the stem is used in conjunctivitis and leaves as poultice to hasten suppuration (Pullaiah, 2006). Hence in the present investigation the phytochemical constituents and α-amylase inhibitory activity of B. retusa was analysed.

Corresponding Author: R. Sangeetha, Department of Biochemistry, School of Life Sciences, Vels University, Chennai-600 117, India

894
MATERIALS AND METHODS

Collection of plant materials: *Breynia retusa* leaves were collected from the wastelands and roadside location of the Chennai suburbs, Tamilnadu, India. The plant was identified by Dr. J. Jayaraman, Plant Anatomy Research Center, Tambaram, Chennai, Tamilnadu (Voucher number: PARC/ 2011/752).

Preparation of extracts: The leaves were air dried under shade, powdered mechanically and stored in airtight containers. Coarsely powdered material was subjected to cold maceration and extraction successively in solvents of increasing polarity such as petroleum ether, chloroform, ethylacetate and methanol for 72 h. Filtered contents were distilled, evaporated, air dried, freeze-dried and stored in air tight plastic containers. The respective extractive yields of the extracts were calculated.

Preliminary phytochemical screening: Plant extracts obtained were subjected to preliminary phytochemical analysis following standard methods. This is to screen the presence of the various active principles present in the plant.

**Test for alkaloids:** To the extract, add a few drops of acetic acid, followed by Dragendorff’s reagent and shaken well. Formation of orange-red precipitate indicated the presence of alkaloids.

**Test for anthraquinones:** Extract was macerated with ether and after filtration; aqueous ammonia or caustic soda was added. Pink, red or violet color in the aqueous layer shaking indicated the presence of anthraquinones.

**Test for quinones:** To the extract, sodium hydroxide was added and formation of blue color indicated the presence of quinones.

**Test for proteins:** To the extract few drops of Biuret reagent was added. Formation of blue color indicated the presence of proteins.

**Test for tannins:** The extract was mixed with basic lead acetate solution. Formation of orange precipitate indicated the presence of tannins.

**Analysis of primary metabolites:** The primary metabolites like carbohydrates, total proteins and lipid contents were quantified. Carbohydrates were quantified by the method of McReady *et al.* (1950), proteins by Lowry *et al.* (1951) and lipids by Zlatkis *et al.* (1953).

**Analysis of secondary metabolites:** Secondary metabolites like tannins, phenols and flavonoids were quantified in all extracts individually.

**Estimation of total phenols:** The total phenolic content of the purified fractions was determined using the Folin Ciocalteau method reported by Singleton and Rossi (1965). Briefly, to 0.1 mL of the extract, 0.5 mL of Folin Ciocalteau reagent and 5.0 mL of sodium carbonate were added. The reaction mixture was allowed to stand for 30 min and the absorbance was measured at 640 nm. Gallic acid was used as the standard. Extracts were analysed in triplicates.

**Estimation of total tannins:** Total tannins were estimated by the method of McDonald *et al.* (2001). 1 mg of each of the extracts were weighed and dissolved in 10 mL of methanol water (7:3). To this 0.5 mL folin’s phenol reagent (1:2) followed by 5 mL of 3.5 sodium carbonate was added and the color intensity was read at 640 nm after 5 min. Extracts were analysed in triplicates.

**Estimation of total flavonoids:** The total flavonoid content of the purified fractions was determined using the aluminum chloride method reported by Zhishen *et al.* (1999). To 1 mL of the extract added 4 mL of H₂O and
0.3 mL of NaNO₃ (5%). After 5 min, 0.3 mL of AlCl₃ (10%) was added followed by 2 mL of NaOH (1 M). The final volume was made up to 10 mL with H₂O and the solution was mixed well. The absorbance was read at 510 nm. Quercetin was used as the standard. Extracts were analysed in triplicates.

**Estimation of total phenols by HPLC:** The total phenolics in both the extracts were detected using a suitable analytical column with the stationary phase Octadecylsilyl silica and mobile phase [A-phosphoric acid water (0.5:99.5 v/v), B-acetonitrile]. Gallic acid, p-coumaric acid, ellagic acid, ferulic acid, mandelic acid and vanillic acid were used as reference compounds. Twenty microliter of the test solution and reference solutions were injected into the column. The detector used for analysis was a UV detector, set at 220 nm with a flow rate of 1.0 mL min⁻¹.

**In vitro assay of amylase inhibition:** In brief 100 μL of the test extract was allowed to react with 200 μL of α-amylase enzyme (Hi media Rm 638) and 100 μL of 2 mM of phosphate buffer (pH 6.9). After 20 min incubation, 100 μL of 1% starch solution was added. The same was performed for the control. The reaction mixture was replaced with buffer. After incubation for 5 min, 500 μL of dimethylsulfoxycarb acid reagent was added to both control and test. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540 nm using spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula:

\[
\text{Inhibition(%) = } \frac{100(\text{Control} - \text{test})}{\text{Control}}
\]

Suitable reagent blank and inhibitor controls were simultaneously carried out.

**Activity staining of amylase:** Activity staining of amylase was done according to the method of Scandalios (1974). The gel consisted of 1% agar in 0.4 M phosphate buffer of pH 7.5. The plant extracts (1 mg mL⁻¹), preincubated with the enzyme were loaded in to different wells. Untreated enzyme served as a positive control in a separate well. The buffer used in the gel was also used in the electrode compartments. A stabilized current of 100 V was passed through the gel for 2 h at 4°C. For visualization of the amylase bands the tray was immersed in 0.5% soluble starch and incubated at 37°C for 30 min. The excess starch was then washed and the gel was flooded with iodine potassium iodide solution for 1 min. Colorless bands against a deep blue background indicated amylase activity.

**RESULTS AND DISCUSSION**

The phytochemical constituents present in the Petroleum Ether (PEBR), Chloroform (CBR), Ethylacetate (EABR) and Methanol (MEBR) extracts were analysed. The preliminary phytochemical screening confirmed the presence of constituents like reducing sugars, phenolics, alkaloids, tannins, glycosides, flavonoids and saponins. The total phenol, phenolic and flavonoid content of all the four extracts were determined spectrophotometrically. This is because these secondary plant metabolites possess diverse biological activities and contribute to the medicinal properties of the plant. Many plant species of the Euphorbiaceae family have been reported to possess anti-cancer, anti-hepatitis, gastro-protective, anti-pyretic, anti-microbial and anti-arthritis factor (Verma et al., 2010).

The preliminary phytochemical screening of the extracts of B. retusa has indicated the presence of significant amounts of phenolic compounds, flavones, flavonoids and alkaloids in EABR and MEBR (Table 1). Quantitative analysis of primary and secondary metabolites again showed these extracts to have significantly greater quantities of proteins, phenols, tannins and vitamins C and E when compared to the PEBR and CBR extracts of the plant. EABR extracts was found to contain increased amounts of flavonoids and MEGR, carbohydrates and proteins (Table 2).

Flavonoids one of the most diverse and widespread group of natural compounds, are probably the most important natural phenolics. These compounds possess

| Table 1: Phytoconstituents in different fractions of B. retusa |
|----------------------|-----------------|-----------------|-----------------|-----------------|
| Phytoconstituents     | PEBR | CBR | EABR | MEBR |
| Phenolic compounds    | ++   | +++ | +++ | +   |
| Reducing sugars       | -    | -   | -   | -   |
| Flavones              | ++   | ++  | +++ | +++ |
| Glycosides            | -    | -   | ++  | ++  |
| Saponins              | -    | -   | ++  | ++  |
| Alkaloids             | ++   | ++  | ++  | ++  |
| Quinones              | -    | -   | -   | -   |
| Anthraquinones        | -    | -   | -   | -   |
| Proteins              | ++   | ++  | ++  | ++  |
| Tannins               | -    | -   | -   | -   |
| + : Negative; ++ : Positive; +++: Significant; +++: Highly significant |

| Table 2: Primary and Secondary metabolites of B. retusa |
|----------------------|-----------------|-----------------|-----------------|-----------------|
| Constituents         | PEBR | CBR | EABR | MEBR |
| Carbohydrate (mg%)   | 0.3±0.002 | 2.3±0.01 | 6.6±0.02 | 9.1±0.4 |
| Protein (mg%)         | 2.5±0.4 | 7.5±0.2 | 33.5±1.2 | 33.5±1.4 |
| Lipid (mg%)           | 4±0.1 | 20±0.85 | 17.8±0.91 | 13±0.9 |
| Phenols (mg mL⁻¹)     | 10±0.2 | 12±0.1 | 36±0.85 | 25±0.9 |
| Tannins (mg mL⁻¹)     | 0.005±0.0001 | 0.181±0.001 | 0.327±0.002 | 0.327±0.001 |
| Flavonoids (mg mL⁻¹)  | 6.6±0.1 | 3.33±0.1 | 10.0±0.12 | 6±0.11 |

All the results expressed are Mean±SD
a broad spectrum of chemical and biological activities including radical scavenging properties. Flavonoids and phenolic substances isolated from a wide range of vascular plants, act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellents and for light screening. HPLC analysis has indicated the total polyphenolic content in the extracts of *B. retusa* to be 6.48 mg (MBR) > 5.26 mg (PBR) > 4.244 mg (EABR) > 1.787 mg (CBR) (Fig. 1). The most abundant phenols present are gallic acid, ellagic acid, coumaric acid, ferulic acid and vanillic acid respectively (Table 3). Numerous studies have proved the relationship between the dietary intake of phenolics and amelioration of various ailments (Marinova et al., 2005).

The percentage inhibition of α-amylase by the extracts of *B. retusa* was studied in a concentration range of 10-640 μg mL⁻¹. The ethylacetate and methanol extracts proved to be efficient than petroleum ether and chloroform extracts. The IC₉₀ of ethyl acetate extract is 30 μg mL⁻¹ while that of methanol extract was 25 μg mL⁻¹. However, the IC₉₀ of petroleum ether and chloroform extracts were 80 and 100 μg mL⁻¹, respectively (Fig. 2).

The ethyl acetate and methanol extracts exhibited a maximum inhibition of 98% at 60 μg mL⁻¹ concentration. The percentage inhibition of all the extracts was not dose dependent beyond the concentration of 60 μg mL⁻¹. The inhibitory effect of all the extracts was also analysed on agar gel electrophoresis. The effect of the inhibitory at two different concentrations was studied. The concentrations chosen for ethyl acetate and methanol extracts were 10±IC₉₀ and that chosen for petroleum ether and chloroform extracts were 20±IC₉₀.

Complete inhibition of amylase was observed when the concentration of the extracts was above the IC₉₀ value. The activity of the extracts was compared with the enzyme control which exhibited a distinct achromatic band against a dark blue background on the agar gel. At concentrations below the IC₉₀ value, faint colorless bands

<table>
<thead>
<tr>
<th>Extract</th>
<th>Gallic acid (mg mL⁻¹)</th>
<th>Coumaric acid (mg mL⁻¹)</th>
<th>Ellagic acid (mg mL⁻¹)</th>
<th>Vanillic acid (mg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEBR</td>
<td>0.911</td>
<td>0.2001</td>
<td>2.053</td>
<td>0.91</td>
</tr>
<tr>
<td>CBR</td>
<td>0.331</td>
<td>0.1043</td>
<td>0.914</td>
<td>0.045</td>
</tr>
<tr>
<td>EABR</td>
<td>1.764</td>
<td>0.221</td>
<td>1.34</td>
<td>0.887</td>
</tr>
<tr>
<td>MEBR</td>
<td>1.918</td>
<td>0.346</td>
<td>2.908</td>
<td>1.054</td>
</tr>
</tbody>
</table>

![Fig. 1(a-d): HPLC profile of the total phenolic content of Breynia retusa. (a) PEBR; (b) CBR; (c) EABR and (d) MEBR](image-url)
could be observed with all the extracts and this indicates partial inhibition of α-amylase. With concentrations above the IC₅₀ value complete inhibition of α-amylase was observed with no colorless bands on the gel. This indicates complete inhibition of amylase activity and utilization of starch substrate (Fig. 3).

Enzyme inhibitors may be proteinacious or non-proteinacious in nature. Hence the inhibitory activity of the extracts was co-related with their protein and polyphenolic content. There was no correlation between the total phenol content and the extent of amylase inhibition by the extract.

A thorough study of literature shows the folklore claim of *B. retusa* in treatment of diabetes. However, there has been no experimental proof for the same. Hence this study is the first of its kind in establishing the antidiabetic effect of *B. retusa*.

Traditional medicament plays an important role in our day to day life in spite of overwhelming influence of modern medicine in treatment of various disorders like diabetes, viral infection, rheumatic disease, allergic condition, obesity, respiratory diseases, cardiovascular diseases, etc. Although numbers of poly herbal formulations are used in traditional system, only a few are accepted in modern medicine due to lack of accurate method for their standardization and evaluation. The findings of this study partially justify the traditional claim of the medicinal uses of *B. retusa*.

**REFERENCES**

