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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 14 (19): 894-899, 2011
ISSN 1028-8880 / DOI: 10.3923/pjbs.2011.894.899
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Phytochemical Screening and *in vitro* Amylase Inhibitory Effect of the Leaves of *Breynia retusa*

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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 analyse 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 ethnobotanically 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.

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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, freezed 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 phenolic compounds: To the extract, few drops of alcoholic ferric chloride solution were added. Bluish green or bluish black colour indicated the presence of phenol.

Test for reducing sugar: The extracts were mixed with Fehling's solution-I and solution-II. Formation of red colouration indicated the presence of sugars.

Test for flavones

- Shimoda test: To the extract, a few magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for five minutes. Red coloration showed the presence of flavones
- To the extract 10% NaOH solution was added. Dark yellow color indicated the presence of flavones

Test for glycosides: The extract was mixed with a little anthrone on a watch glass, one drop of concentrated sulphuric acid glycosides.

Test for saponins: The extract was shaken with water, copious formation indicated the presence of saponins.

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

Test for anthraquinones: Extract was mancerated 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 flavanoids 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 flavanoids: The total flavanoid content of the purified fractions was determined using the aluminium chloride method reported by Zhishen *et al.* (1999). To 1 mL of the extract added 4 mL of H_2O and

0.3 mL of $NaNO_2$ (5%). After 5 min, 0.3 mL of $AlCl_3$ (10%) was added followed by 2 mL of NaOH (1 M). The final volume was made upto 10 mL with H_2O 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 were 200 μL of the enzyme was replaced by buffer. After incubation for 5 min, 500 μL of dinitrosalicylic 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 iodide 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, flavones and saponins. The total tannin, phenolic and flavanoid 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-arthritic factors (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 metabolities 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 MEBR, 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 *Breynia 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	36.6±0.85	25±0.9
Tannins (mg mL^{-1})	0.0054±0.0001	0.181±0.001	0.327±0.002	0.327±0.001
Flavanoids (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 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, ferullic 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 IC_{50} of ethyl acetate extract is 30 μg mL^{-1} while that of methanol extract was 25 μg mL^{-1}. however, the IC_{50} of petroleum ether and chloroform extracts were 80 and 100 μg mL^{-1}, respectively (Fig. 2).

Table 3: HPLC determination of the polyphenolic acids in *Breynia retusa*

Extract	Gallic acid	Coumaric acid	Ellagic acid	Vanillic acid
	(mg mL^{-1})			
PEBR	0.911	0.2001	2.053	0.91
CBR	0.331	0.1043	0.914	0.045
EABR	1.764	0.221	1.34	0.887
MEBR	1.918	0.346	2.998	1.054

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 were 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±IC_{50} and that chosen for petroleum ether and chloroform extracts were 20±IC_{50}.

Complete inhibition of amylase was observed when the concentration of the extracts was above the IC_{50} 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 IC_{50} value, faint colorless bands

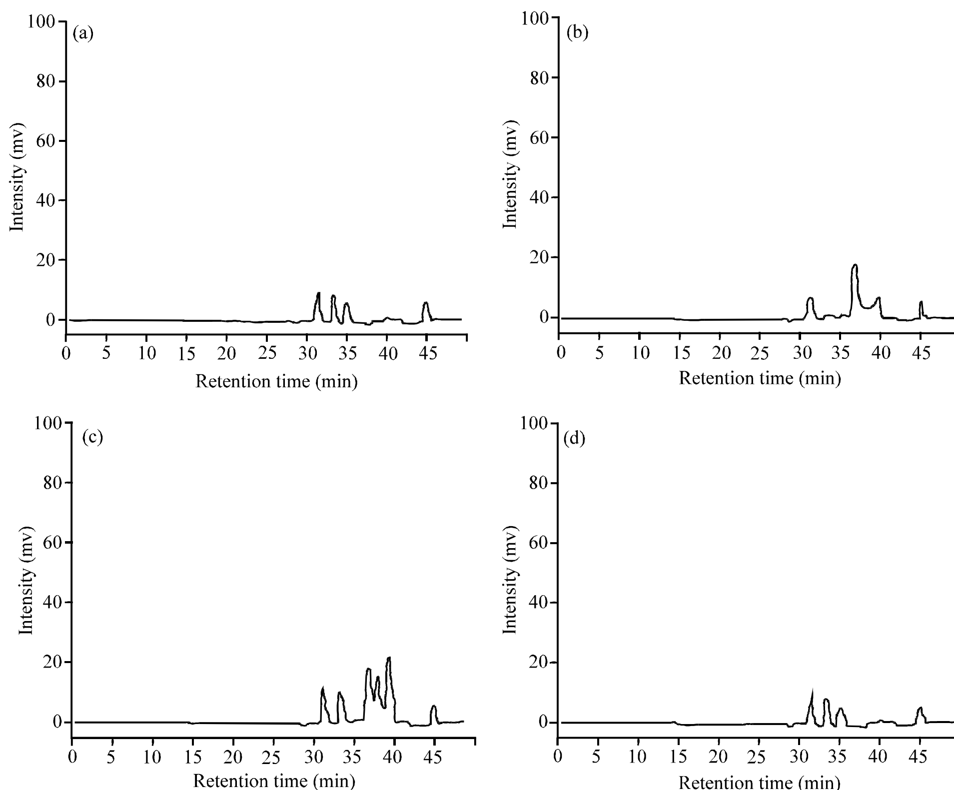

Fig. 1(a-d): HPLC profile of the total phenolic content of *Breynia retusa*. (a) PEBR; (b) CBR; (c) EABR and (d) MEBR

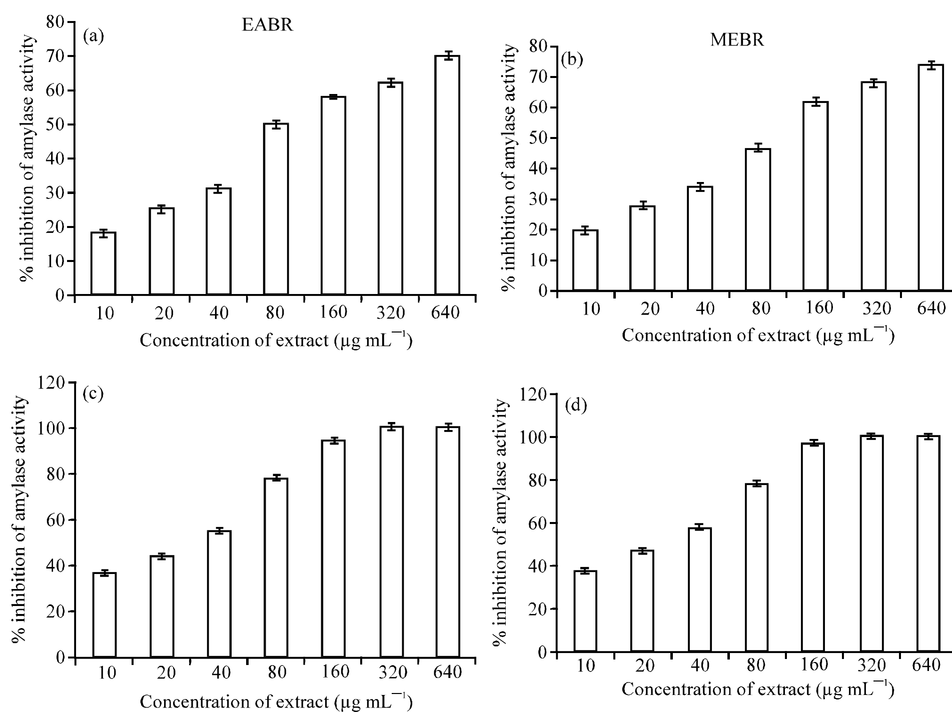

Fig. 2(a-d): Amylase inhibitory activity of *B. retusa*; (a) PEBR, (b) CBR, (c) EABR and (d) MEBR

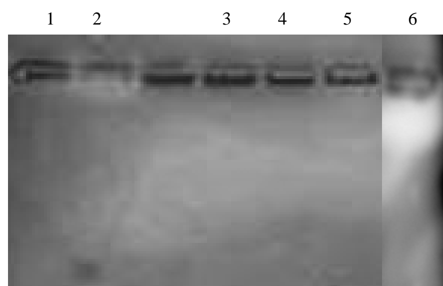

Fig. 3: Activity staining of amylase in agar gel electrophoresis. Lane 1: Negative control; Lane 2: EABR; Lane 3: CBR; Lane 4: PEBR; Lane 5: MEBR and Lane 6: Positive control

could be observed with all the extracts and this indicates partial inhibition of α-amylase. With concentrations above the IC_{50} 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 anti-diabetic 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*.

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