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PHYTOCHEMICAL STUDIES AND IN-VITRO ANTI-OXIDANT ACTIVITY OF BREYNIA RETUSA DENNST

Y. Bhagyasri*, N. Siva Subramanian and M. Vaseem Akram

Assistant Professor (Department of Pharmacognosy) Gland Institute of Pharmaceutical Sciences Sy No.551, Shangri-La, Kothapet (V), Sivampet (M), Near Narsapur , Medak (Dist) Telangana, 502313.

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***Corresponding Author**
Y. Bhagyasri
Assistant Professor (Department of pharmacognosy) Gland Institute of Pharmaceutical Sciences Sy No.551, Shangri-La, Kothapet (V), Sivampet (M), Near Narsapur, Medak (Dist) Telangana, 502313.

ABSTRACT

To investigate the phytochemical screening and antioxidant potential of ethanolic and chloroform extract of *breynia retusa* leaf & stem. The study was done by using various *in- vitro* methods such as 1, 1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (N_2O_2) and Superoxide radical scavenging assays. Phytochemical screenings were performed by various standard procedures. Ethanolic and chloroform extract of 10-100µg/ml *Breynia retusa* leaf & stem showed high free radical scavenging activity as evidenced by the low IC_{50} values in DPPH (26µg/ml), in nitric oxide (31.2µg/ml) and in SOD (25.5µg/ml) radical scavenging assays at the concentration of 100µg/ml. The results of present comprehensive analysis demonstrated that ethanolic extract (100µg/ml) showed more significant anti-oxidant activity than chloroform extract of (100µg/ml) *Breynia retusa* leaf & stem. *Breynia retusa* could be used as a viable source of natural antioxidants and might be exploited for functional foods and neutraceutical applications.

KEYWORDS: *Breynia Retusa*, Phytochemical Screening, *In-Vitro* Antioxidant Activity.

INTRODUCTION

There is our days, an increasing interest in the measurement and use of plant antioxidants for scientific research as well as industrial (dietary, pharmaceutical and cosmetic) purposes. This is mainly due to their strong biological activity, exceeding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis.[1] Therefore, the need exits for safe, economic, powerful, and natural antioxidants to replace these synthetic

ones.[2] However, excessive accumulation of ROS can cause oxidative stress, which results in the damage of DNA, RNA, proteins and lipids inhibiting their normal functions. The abnormal functioning of these biomolecules can lead to an increased risk for cardiovascular disease, cancer, autism and other diseases.[3,4] Therefore, minimizing oxidative stress will promote our physical condition and prevent some degenerative diseases in which free radicals are involved.[5] The agents which can scavenge the free radicals and inhibit the harmful effects caused by the oxidants are called antioxidants. They can lessen the severity of destruction caused by the free radicals by neutralizing them ahead of the damages caused to lipids, proteins, enzymes, carbohydrates and DNA.[6] Antioxidants can be classified into two major classes i.e., enzymatic and non-enzymatic. The enzymatic antioxidants are produced endogenously and include superoxide dismutase, catalase, and glutathione peroxidase. The non-enzymatic antioxidants include tocopherols, carotenoids, ascorbic acid, flavonoids and tannins which are obtained from natural plant sources.[7,8] A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases.[9]

Plant Material

Breynia retusa belongs to family Euphorbiaceous. *Breynia retusa* is a shrub spreading branches. The *Breynia retusa* is Semi-evergreen plant and deciduous forests, and also in the plains. Flowering and fruiting in February-September. Pollinated by a wide variety of insects. Shrubs are 1-1.5m high. Leaves are simple, alternate 1.3-2.5× 0.7-1.3 cm, broadly elliptic or ovate, apex obtuse, black on drying. Flowers are unisexual solitary, axillary. The fruits are globose berries. *B. Retusa* leaf juice is used to cure body pain, skin inflammation, hyperglycaemia, diarrhoea and diuretic. The fruits have been used for dysentery and twigs used for toothache[10] Young leaves are cooked and used as poultice to hasten suppuration.[11,12] Literatures survey showed that *B. retusa* leaves have good total phenolic contents, antioxidant.[13] and anti-diabetic activity.[14,15] These studies can be taken as a strong platform to carry out antioxidant and anti-diabetic potentials in *B. retusa*. Hence, the present investigation on leaf and fruit was undertaken to study the nutritional composition, anti-nutritional, total phenolic content, antioxidant and anti-diabetic properties of *B. retusa*. It grows generally in China, Bhutan, Malaysia, Sri Lanka, and Thailand and in India. The folklore medicinal claim of this species was reported to have juice of leaf for pain, skin inflammation, hyperglycemia, diarrhoea and as diuretic. The stem juice is used to treat conjunctivitis. The species is also used to treatment of cirrhosis, flatulence, hyperacidity,

tetanus. The Chittagong hill living tribal used the juice of this whole plant for stomachache. Further fruits traditionally used for dysentery, roots for fits and meningitis, twigs for toothache.[16,17]

MATERIALS AND METHODS

Collection and Authentication

The leaves and stem of *Breynia retusa* were collected from Tirupati forest region, India in the month of March 2016 and it was identified and authenticated. The taxonomical identification and authentication of the plant was done by Dr. K. Madhava Chetty,Assistant professor, Department of Botany, Sri Venkateswara university, Tirupati, india. The vocher specimens (2016/2141) were preserved in laboratory, Department of Pharmacognosy, Gland institute of pharmaceutical sciences for further reference.

Preparation of Extracts

The 50 Gms of coarsely powdered plant material of *breynia retusa* leaves and stem of were defatted with petroleum ether and extracted successively with, chloroform, and ethanol using soxhlet apparatus. The extraction was carried out until the extractive becomes colorless. The extract was filtered through a cotton plug, followed by whattman filter paper (no-1). The extract was evaporated under reduced pressure using rotovac evaporator.

Phytochemical Screenning of Brle and Brse

The following chemical tests were performed to identify the phytochemical constituents present in chloroform & ethanolic extracts of *Breynia retusa* leaf & stem. The concentrated extracts were subjected to chemical tests as per the methods mentioned below for the identification of the various constituents as per the standard procedures given by Kokate (2005) and Khandelwal (1996).[18,19] The result were given in table no-1.

Test for Alkaloids

Dragendorff's Reagent

The extract was shaken with dilute HCl and filtered. To 2-3ml of filtrate, a few drops of Dragendroff's reagent were added. Orange brown precipitate observed indicates the presence of alkaloids.

Mayer's Test

The extract was shaken with dilute HCl and filtered. To 2-3ml of the filtrate, Mayer's reagent was added. Cream precipitate observed indicates the presence of alkaloids.

Hager's Test

The extract was shaken with dilute HCl and filtered. To the 2-3ml of filtrate, Hager's reagent was added. Yellow precipitate observed indicates the presence of alkaloids.

Wagner's Test

The extract was shaken with dilute HCl and filtered. To the 2-3ml of filtrate, Wagner's reagent was added. Reddish brown precipitate observed indicates the presence of alkaloids.

Test For Amino Acids

Ninhydrin test

Heat 3 ml of test solution and 3 drops of Nin hydrin solution in boiling water bath 10 min. Purple colour appears indicates the presence of amino acids.

Test for Glycosides

Baljets Test

A thick section showed yellow to orange colour with sodium picrate observed indicates the presence of glycosides.

Legal's Test

To the extract 1ml of pyridine and 1ml of sodium nitro prusside was added. Pink to red colour appeared. It indicates the presence of glycosides.

Liebermann's Test

3ml extract was mixed with 3ml acetic anhydride. Heated and cooled. Few drops of conc. sulphuric acid were added. Blue colour appeared. It indicates the presence of glycosides.

Borntragers Test

To 3ml of extract, dilute sulphuric acid was added. Boiled and filtered. To cold filtrate add equal volume of benzene or chloroform was added and shaken well. The organic solvent was separated. Ammonia was added. Ammonical layer turned to pink observed. It indicates the presence of glycosides.

Test for Tannins and Phenolic Compounds

Lead Acetate Solution

To 2-3ml of extract few drops of lead acetate solution was added. A white precipitate was formed. It indicates the presence of tannins & phenolic compound.

To 5ml of extract 5ml of 5% ferric chloride and 5ml dilute HCl were added and heated for 5min in boiling water bath. Equal volume of chloroform or benzene was added and shaken well. The organic solvent was separated and ammonia was added. Ammonical layer showed pinkish red colour. It indicates the presence of tannins & phenolic compound.

5% Ferric Chloride Solution

To 2-3ml of the extract few drops of 5% ferric chloride solution was added. A deep blue colour appeared. It indicates the presence of tannins & phenolic compound.

Test for Steroids

Salkowski Reaction

To 2ml of extract 2ml chloroform and 2ml conc. sulphuric acid was added. Shake well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence. It indicates the presence of steroids.

Liebermann's Test

3ml extract was mixed with 3ml acetic anhydride. Heated and cooled. Few drops of conc. sulphuric acid were added and blue colour appeared. It indicates the presence of steroids.

Test for Carbohydrates

Molisch's Test

To 2-3 ml of extract few drops of α-naphthol solution was added in alcohol, shaken and conc. sulphuric acid was added from sides of the test tube. Violet ring was formed at the junction of two liquids. It indicates the presence of carbohydrate.

Fehling's Test

About 50gms of extract was hydrolyzed with 10ml of dilute hydrochloric acid and neutralized with alkali. The mixture was heated with 1ml of Fehling's solution A and B and observed for precipitate. Formation of red precipitate indicated the presence of reducing sugars.

Benedict's test

To 0.5ml of filtrate 0.5ml of Benedict's reagent was added. The mixture was heated on water bath for 2 minutes and observed for precipitate. Formation of orange red precipitate indicated the presence of reducing sugars.

Test For Flavonoids

Shinoda test: To the extract add 5 ml of 95% ethanol, few drops of conc HCl and 0.5 g magnesium turnings. Pink colour indicates the presence of flavonoids.

Small quantity of residue and lead acetate solution. It shows the yellow colour ppt, presence of flavonoids.

Add incresing amount of naoh to the residue, yellow colorization which decolorizes after addition of acid, presence of flavonoids.

Test for Proteins

Xanthoprotein Test

3ml test solution was mixed with 1ml of conc. sulphuric acid. White precipitate was formed. It was boiled and precipitate turned to yellow. Ammonium hydroxide was added and then precipitate turned to orange.

Biuret Test

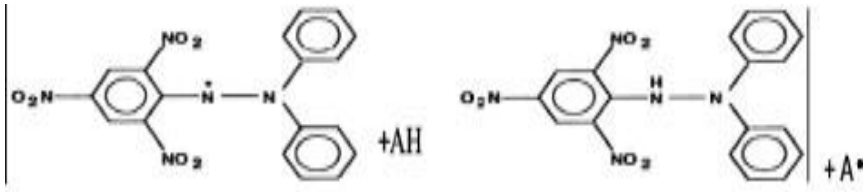
To 3ml test solution 4% ammonium hydroxide and few drops of 1% copper sulphate solution was added. Violet colour appeared.

In-Vitro Antioxidant Activity

Experimenral procedure

DPPH radical scavenging activity

The molecule 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity was measured according to the method. In brief, 3ml of reaction mixture containing 200 µl of DPPH (100 µM in methanol) and BRL and BRS extracts (at various concentrations; 10-100 µg/ml in methanol) was incubated at 37 ^{0} C for 30min and absorbance of the test mixture was read at 517nm using Beckman model DU-40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the following formula (Mondal *et al*.2006). The results were shown in table No-2&3.



Diphenylpicrylhydrazyl (free radical) Diphenylpicrylhydrazine (nonradical)

$$\% \text{ inhibition of DPPH radical} = ([A_{br} - A_{ar}]/A_{br}) \times 100$$

Where A_{br} is the absorbance before reaction and A_{ar} is the absorbance after reaction has taken place.

Nitric oxide scavenging activity

Nitric acid (NO) is generated from sodium nitroprusside solution in buffer saline and measured by Griess reagent. 1mL of 10 mM sodium nitro prusside was mixed with 1ml of BRL and BRS extract at different concentrations (10-100µg/ml) phosphate buffer saline (pH 7.4). The mixture was incubated at 25 °C. After 150 min.to 1mL of the incubated solution, 1ml of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthylethylenediamine dichloride (0.1% w/v)] was added. Absorbance was read at 546 nm and percentage. The amount of nitric oxide radical inhibition is calculated following this equation:

%inhibition of N_2O_2 radical = [A0-A1]/A0×100

Where A_0 is the absorbance before reaction and

A_1 is the absorbance after reaction has taken place with Griess reagent.

Superoxide radical scavenging activity (SO)

Superoxide scavenging activity of brl and brs extracts was measured according to the method of Robak and Gryglewski (1988) with some modification. All the solution was prepared in 100Mm phosphate buffer (pH 7.4). 1ml of nitroblue tetrazolium (NBT, 156 µM), 1ml of reduced nicotinamide-adenine dinucleotide (NADH, 468 Um) and 1ml of BRL and BRS extracts (to produce final concentrations of 10-100 µg/ml) were mixed. The reaction was started by adding 100 µl of phenazine methosulphate (PMS, 60 Um). The reaction mixture was incubated at 25 °C for 5 min followed by measurement of absorbance at 560 nm. The percentage inhibition was calculated from the above formula.

%inhibition of SO radical= [A0-A1]/A0×100

Where A_0 is the absorbance before reaction and

A_1 is the absorbance after reaction has taken place with reagent.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening On Extracts Of BRL and BRS

Table: 5.1.1 Preliminary Phytochemical Screening on Extracts of Brle and Brse.

S. No.	Test	BRL		BRS	
		Brlce	Brlae	Brsce	Brsae
1	Alkaloids	+	-	+	-
2	Glycosides	+	+	+	+
3	Carbohydrates	+	+	-	+
4	Proteins	-	-	-	+
5	Flavonoids	-	+	-	+
6	Resins	-	-	-	-
7	Tannins	-	+	-	+
8	Saponins	-	-	+	-
9	Steroids	+	-	+	-
10	Gums & Mucillage	-	-	-	+

In-Vitro Antioxidant Activity

Table: 5.2.1 Effect of BRLE Extracts In DPPH Radical Scavenging Capacity

S. No.	Concentration (µg/ml)	% Inhibition Of DPPH Radical		
		Standard (BHT)	BRLCE	BRLAE
1	10	80.47 ± 0.37	22.33 ± 1.28	25.71± 1.37
2	20	86.73± 0.39	33.72± 0.34	41.52± 2.31
3	40	89.74± 0.17	50.35± 0.41	64.95± 0.63
4	80	94.00± 0.55	68.47± 0.62	81.45± 0.65
5	100	97.15± 0.23	75.38± 1.35	91.17± 0.15
IC_{50} Value		5 µg/ml	40 µg/ml	26 µg/ml

Table: 5.2.2. Effect of BRSE Extracts in DPPH Radical Scavenging Capacity.

S. No.	Concentration (µg/ml)	% Inhibition Of DPPH Radical		
		Standard (BHT)	BRSCE	BRSAE
1	10	80.47 ± 0.37	24.69± 0.58	28.69± 0.43
2	20	86.73± 0.39	35.32± 0.64	40.64± 2.36
3	40	89.74± 0.17	48.46± 1.34	54.36± 1.38
4	80	94.00± 0.55	62.67± 1.32	68.59± 0.73
5	100	97.15± 0.23	72.53± 1.64	82.96 ± 0.53
IC_{50} Value		5 µg/ml	45 µg/ml	31 µg/ml

Table: 5.2.3. Effect of BRLE Extracts in Nitric Oxide Scavenging Capacity.

S. No.	Concentration (µg/ml)	% Inhibition Of Nitric Oxide Radical		
		Standard (BHT)	BRLCE	BRLAE
1	10	72.28± 0.69	24.32 ± 2.32	26.43± 2.46
2	20	77.57±0.54	36.36± 0.49	40.32± 2.36
3	40	82.23±1.77	52.84± 0.36	58.42± 1.34
4	80	84.99±0.49	68.46± 0.69	76.34± 0.32
5	100	90.11±0.61	76.56± 0.82	91.36± 0.17
IC_{50} Value		5.2 µg/ml	40 µg/ml	30 µg/ml

Table: 5.2.4. Effect of BRSE Extracts in Nitric Oxide Scavenging Capacity.

S. No.	Concentration (µg/ml)	% Inhibition Of Nitric Oxide Radical		
		Standard (BHT)	BRSCE	BRSAE
1	10	72.28± 0.69	23.46±0.34	26.42±0.52
2	20	77.57±0.54	38.34±0.46	45.48±1.34
3	40	82.23±1.77	50.32±0.58	56.48±1.48
4	80	84.99±0.49	58.68±1.04	66.43±0.43
5	100	90.11±0.61	69.72±0.46	84.42±0.68
IC_{50} Value		5 µg/ml	45 µg/ml	31.2µg/ml

Table: 5.2.5. Effect of BRLE Extracts in Super Oxide Scavenging Capacity.

S. No.	Concentration (µg/ml)	% Inhibition Of Super oxide Radical		
		Standard (BHT)	BRLCE	BRLAE
1	10	63.77±0.58	22.46±1.34	24.32±1.32
2	20	72.58±0.32	36.86±0.56	42.48±1.24
3	40	76.03±0.78	52.34±0.34	60.36±2.32
4	80	79.83±0.41	66.46±0.46	71.32±1.28
5	100	84.21±0.41	72.34±1.32	80.43±0.84
IC_{50} Value		5.4 µg/ml	35 µg/ml	25 µg/ml

Table: 5.2.6 .Effect of BRSE Extracts in Super Oxide Scavenging Capacity.

S. No.	Concentration (µg/ml)	% Inhibition Of Super oxide Radical		
		Standard (BHT)	BRSCE	BRSAE
1	10	63.77±0.58	24.36±0.36	29.38±1.28
2	20	72.58±0.32	37.43±0.63	45.42±0.39
3	40	76.03±0.78	54.64±0.69	60.24±1.36
4	80	79.83±0.41	67.32±0.84	71.32±0.56
5	100	84.21±0.41	71.68±0.92	79.26±0.56
IC_{50} Value		5.4 µg/ml	32 µg/ml	25.5µg/ml

DISCUSSION

The study showed the presences of Polyphenols are a large and diverse class of compounds, many of which occur naturally in a wide range of food and plants. The flavonoids are the largest and best studied group among polyphenols. A range of plant polyphenols is either being actively developed or already currently sold as dietary supplements and/or herbal,

derived medicines. The percentage scavenging and IC_{50} values were calculated for all models. The standard preliminary phytochemical investigation of various extract of *breynia retusa* showed the presence of primary and secondary metabolites like flavonoids, glycosides, phenolic compounds, carbohydrates, alkaloids, protein and amino acids. In-vitro antioxidant activity of BRLCE, BRLAE and BRSCE, BRSAE at various concentrations were also studied by DPPH, nitric oxide, super oxide radical method. IC_{50} Values of BRL and BRS extracts shows good anti-oxidant activity when compared to that of standard. Leaf extract shows the good anti-oxidant activity then comapared with stem extract. BRL & BRS ethanolic extracts shows significant antioxidant activity when compared to BRL & BRS chloroform extract.

CONCLUSION

The results obtained from the above studies in this work concluded that the results are reproducible in nature and these can be used for identification of species and its routine qualitatitive analyses. We conclude that, the results presented indicate that *Breynia retusa* extract attenuated oxidative stress via its antioxidant properties. However, further investigations on phenols, flavonoids, active principle, there *in vivo* antioxidant activity, and the different antioxidant mechanism are warranted.

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