

Effect of solvents on total phenolics, antioxidant and antimicrobial properties of *Bridelia retusa* Spreng. stem bark

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Received 23 November 2010; Accepted 20 July 2011

Effect of different solvents such as water, ethanol (50%), methanol (50%) and acetone (70%) on the total polyphenol content, antioxidant and antimicrobial activities of stem bark of *B. retusa* Spreng. was studied. Phenolic content of extracts were determined using Folin-Ciocalteu assays and antioxidant activity was carried out by DPPH and reducing power assay. The results showed that different solvent with different polarity possess significant effect on polyphenolic contents and antioxidant activity. Acetone extract shown highest polyphenol content, 4.7-7.6 mg equivalent to Gallic acid. It also has shown highest antioxidant activity. MIC value <5mg/ml indicated that stem bark of this plant is a potential natural antimicrobial agent.

Keywords: Antibacterial, Antioxidant, *Bridelia retusa*, Polyphenolic content.

IPC code; Int. cl. (2011.01)—A61K 36/00

Introduction

The search of new antioxidants and phenolics from herbal source has taken very large attention in last decade. Antioxidant and antimicrobial properties are responsible for well being of human body hence, they are very much important for further characterization of plant material. Secondary metabolites from plants, mainly phenolics having antioxidants, antimicrobial, antitumour, antiviral, enzyme inhibiting and radical scavenging properties¹⁻⁴. Isolation of antioxidant compounds from plant is possible through extraction with different solvents and it depends on the nature of extracting solvents⁵. The extracts from plants contain different classes of phenols, which have the different solubility's in different solvents.

Bridelia retusa Spreng. (Family-Euphorbiaceae) is small to moderate tree, having grey bark, used to treat rheumatism, urinary infection, promote antifertility and wound healing, leaves and fruits are used as stomachic, anti-inflammatory and antifungal⁶⁻¹². These different pharmacological properties are due to the presence of different chemical constituents

as isoflavone¹³, decanoic acid, stigmasterol, dehydrostigmasterol, beta-sitosterol, tannins (40%) and triterpenes ketone. Fruit pulp contains gallic acid, ellagic acid and beta-sitosterol¹⁴. Literature on this plant revealed that there is no systematic approach on plant tannins therefore, screening of total phenolics, antioxidant and antimicrobial properties has been done during present study Polar solvents such as water, ethanol, methanol, hydro alcohol and acetone were used for extraction of polyphenols¹⁵.

Materials and Methods

Chemicals

All solvents used are of analytical grade. Gallic acid (Fluka, USA), Folin-Ciocalteu reagent (Qualigen, Mumbai), Ascorbic acid (Loba Chemie, Mumbai), DPPH (1, 1-diphenyl-2-picryl hydrazyl) from Sigma Aldrich, USA, Potassium ferricyanide (Loba Chemie, Mumbai) Trichloroacetic acid, Ferric chloride (Loba chemie, Mumbai), Iodonitrotetrazolium chloride (Sigma-Aldrich Co. Ltd., Poole, UK).

Plant material

The fresh bark of *B. retusa* was collected in the month of August (2007) from Ranipur (Toranmal) of Nandurbar District (MS), India. It was authenticated by Dr. D. A. Patil, Taxonomist, H.O.D. of Botany, Dr.

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P. R. Ghogrey College, Dhule (MS). A voucher specimen of the bark was deposited in Department of Pharmacognosy, at R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur for future reference (RAN-472).

Biological material

Four organisms were used in this study. All microorganism cultures were obtained from NCIM, Pune. The food-associated microorganisms were selected because they are frequently reported in foods. Nutrient broth (Hi Media), Sabouraud medium (Hi Media) was used for culturing microorganism.

Extraction procedure

The extraction of dried and finely powdered (50 g) bark of *B. retusa* was done by hot maceration with six different solvents such as water, methanol, methanol (50%) ethanol (50%), and acetone (70%) to compare the effect of extraction protocol, antioxidant activity and polyphenolic contents. The maceration was carried out in conical flasks by continuous shaking in incubator for 1h and boiled at 50°C for 1 h on water bath. The macerated mass was extracted two times with the same solvents. The combined filtered extracts of different solvents were dried, stored for further use for studying antioxidant, antimicrobial activity and polyphenolic contents.

Determination of total phenolic

The total phenolic content was determined by Folin-Ciocalteu colorimetric method¹⁶. A small quantity (2 ml) of extracted solution (100 µg/ml) and 1.5 ml of Folin-Ciocalteu reagent (20% v/v) was mixed thoroughly. After 4 min, 4ml Na₂CO₃ (7%) was added and volume was made up to 10ml by water. The mixture was allowed to stand for 90 min, protected from light at room temperature. The absorbance was measured at 760nm by using Shimadzu UV/Vis 2401 spectrophotometer. The phenolic content was calculated using calibration curve of Gallic acid (20-100 µg/ml). The result is expressed as mg of Gallic acid/g dry material.

In vitro antioxidant activity

Free radical scavenging activity by DPPH method

The DPPH radical scavenging activity of *B. retusa* sample was performed in flat bottom polystyrene 96-well microtiter plates using reported method¹⁷ with some modifications. Test extracts (100 µl) of different solvent of bark (20-100 µg/ml) and 100 µl of DPPH

(0.1 mm in methanol) were added to give a final volume of 200 µl. The resultant mixtures were shaken thoroughly and incubated at 37°C in the dark for 25 min. At the end of this period the absorbance of mixture was measured at 517 nm using Microtiter Plate Reader (Power Wave XS, Bio-tek, USA). The percent free radical scavenging activity was calculated by using following formula¹⁸.

$$\% \text{ Antiradical activity} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{Control}} \times 100$$

where A_{control} : Control absorbance (DPPH). A_{sample}: Sample absorbance

The antioxidant activity of the sample expressed as IC₅₀ value defined as concentration (in µg/ml) of sample that inhibits the formation of DPPH radicals by 50%.

Reducing power assay

The reducing power was determined according to the method described by Oliveira *et al* (2007) with some modifications. The various concentrations of extracts of *B. retusa* (20 to 100 µg/ml) in 1.0 ml of deionized water, mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10min. The upper layer of solution (2.5ml) was mixed with distilled water (2.5 ml) and a freshly prepared 0.5 ml ferric chloride solution. The absorbance of solution was measured at 700 nm. The blank was prepared by excluding extract. Ascorbic acid, standard used at different concentrations (20 to 100 µg/ml). Increased absorbance of the reaction mixture indicates increase in reducing power¹⁹.

Antimicrobial activity

The antimicrobial activity of the extracts was carried out by agar well diffusion method²⁰. Using 100 µl of suspension containing 10⁸ CFU/ml of bacteria spread on nutrient agar (NA) medium for bacteria and Sabouraud media for fungi. On the agar plate, 6 mm diameter well was creating using borer. The wells were impregnated with 50 µl of the different extracts in the concentration of 05, 10, 20 mg/ml in DMSO and Gentamycin (Gentamicin sulphate IP 40 mg/30 ml vial), Clotrimazole (Clotrimazole USP 150 mg/15 ml vial, i. e.10 mg/ ml) used as standard antibiotic for bacteria and fungi respectively. The bacterial inoculated plates, incubated at 27°C for 24 h and fungi at 27°C for 48 h. The antibacterial activity measured, as the diameter (mm) of clear zone of growth of

inhibition. Four well per plate and twenty-four plates used, and each test was run in triplicate.

Determination of MIC

The minimum inhibitory concentration (MIC) is the lowest concentration of the extract at which growth of microorganism is inhibited. MIC was determined by the broth micro dilution method in 96 well microtiter plate.²⁰ Double strength of sterile broth (50 μ l) was poured in well of plate. In the first well 100 μ l extract, 4.5 mg/ml was added. Then serial two-fold dilutions of extract was done up to 2 mg/ml by transferring 150 μ l of first well to the second well and so on. Similarly two fold serial dilutions of standard drugs Gentamycin and Ketoconazole were prepared from 20 - 0.625 μ g/ml. Then, to each well 50 μ l of bacterial (106 cfu/ml)/fungal (5×10^5 spores/ml) suspension was added. Micro titer plates incubated at 37°C for 24 h for bacteria and at 27°C for 48 h for fungi. The entire tests were performed in triplicates. Iodonitrotetrazolium chloride (INT) have been shown to be the best indicator for determining microbial growth, producing a stable pink color when there is microbial activity. Following addition of INT

(Sigma-Aldrich Co. Ltd., Poole, UK) and incubation, the MIC was determined as the lowest sample concentration at which no pink color appeared. For each compound, the assay was performing in triplicate to ensure reproducible results.

Results and Discussion

Effect of solvents on extraction of polyphenolic content

The yield of total phenolic contents and antioxidant assay of *B. retusa* extract is given in Table 1. The extractive value indicates 70% acetone gives the maximum yield (14% w/w) amongst the other extracts.

Total phenolic content in different extracts

The phenolic compounds in plant extract are more often associated with other molecules like proteins, polysaccharides, terpenes, chlorophyll and inorganic compounds. Hence, it requires suitable solvent for extraction of tannins. Table 1 and Figure 1 depict that the acetone extract of *B. retusa* contain higher amount of phenolics as compared to others. Results of the present study shown that among all the solvent; acetone -water and ethanol- water were better solvents

Table 1—Yield, total phenolic content and antioxidant assay of *B. retusa* extracts

Extract	Yield (%)	Total phenolic content (mg equivalent of gallic acid)	DPPH (IC ₅₀)	Reducing power assay*
Water (E1)	6.4	3.1-5.1	62.61	0.153±0.003
Ethanol (E2)	6.0	3.3-3.8	62.27	0.154±0.006
Methanol (E3)	4.5	1.6-1.7	61.94	0.154±0.006
Ethanol (50%) (E4)	10.2	4.0-5.4	62.61	0.153±0.004
Methanol (50%) (E5)	9.3	3.25-4.08	70.46	0.148±0.004
Acetone (70%) (E6)	14.0	4.7-7.6	61.93	0.156±0.005

* all values expressed in Mean \pm SEM.

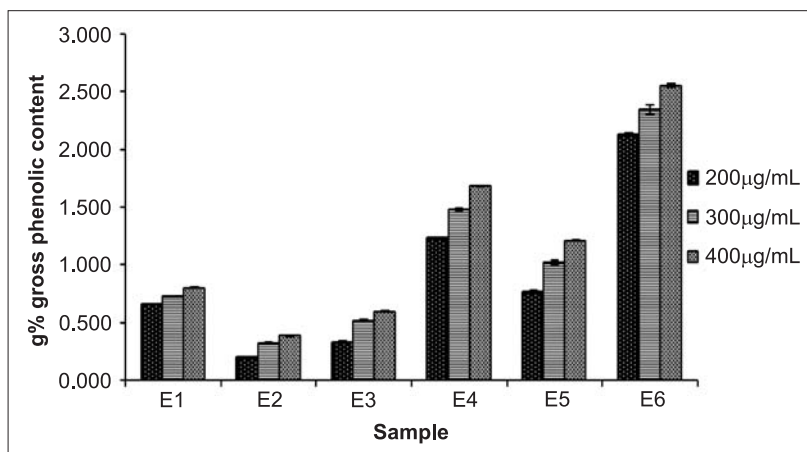


Fig.1—Comparison of phenolic content in different extracts of *B. retusa* bark

for effective extraction of tannins as compared to solvents like water, methanol and ethanol. This may be because phenolics are often extracted in higher amounts in more polar solvents. Acetone (70%) was efficient solvent for recovering tannins²¹⁻²². The gross phenolic content of acetone-water solvent was found to be higher i.e. 7.6% than others.

Free radical scavenging activity by DPPH method

Solvent used for polyphenolic extraction had significant effect on antioxidant activity. The free radical scavenging ability of *B. retusa* extracts evaluated by scavenging of DPPH radicals were produced by test solution. The model system of scavenging DPPH free radical is a simple method for evaluating the antioxidant activity of compounds. DPPH is a free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule²³. The reduction capability of DPPH radical was determined by the decrease in absorbance induced by plant antioxidants. The scavenging effect of different extracts of *B. retusa* and standards on the DPPH radical decreased in following order: ascorbic acid >70% acetone extract >water extract >ethanolic extract >50% ethanolic extract >methanolic extract >50% methanolic extract having IC₅₀ value, 58.78, 60.95, 61.28, 61.93, 61.94, 62.27, 62.61%, 62.61 and 70.46%, respectively. (Table 1 and Figure 2).

Reducing power assay

The presence of reductant substances in the samples prevents the reduction of the Fe⁺⁺⁺/ferricyanide complex to the ferrous form. The Fe⁺⁺ formation as indicated by Perl's Prussian blue at 700 nm. Greater the absorbance, greater will be the reducing

power. The reducing power of the different extracts and standard (ascorbic Acid) has been shown using the potassium ferricyanide reduction method. For the measurements of the reductive ability, the Fe⁺⁺⁺ - Fe⁺⁺ transformation were investigated.²³ The reducing power of different extracts of *B. retusa* increased with increasing in concentration. Out of the all extracts, acetone extract has potent activity very close to standard and showed higher activities than the control. The reducing power of different extracts and standard exhibited the following order: ascorbic acid >70 % acetone extract > water extract > ethanol extract >50% ethanol extract > methanol extract > 50% methanol extract as shown in Table 1 and Figure 3.

Correlation between antioxidant and phenolic content

Phenolic compounds possess a wide spectrum of biological effects including antioxidant and free radical scavenging²⁴. Results obtained from experimental data revealed that there might be correlation between total phenolic and antioxidant capacity of different extracts of *B. retusa*. However, some literature demonstrated that antioxidant was not solely dependent on phenolic content but it may be due to other phytoconstituents as tannins, triterpenoid or combine effect of them. Different types of phenolic compounds have different antioxidant activity, which mainly depends on their structure as extract contains different types of phenolic compounds which have different antioxidant capacities.

Antimicrobial activity

The complexing ability of tannins (polyphenol) is reactive with the cell wall of bacteria and the

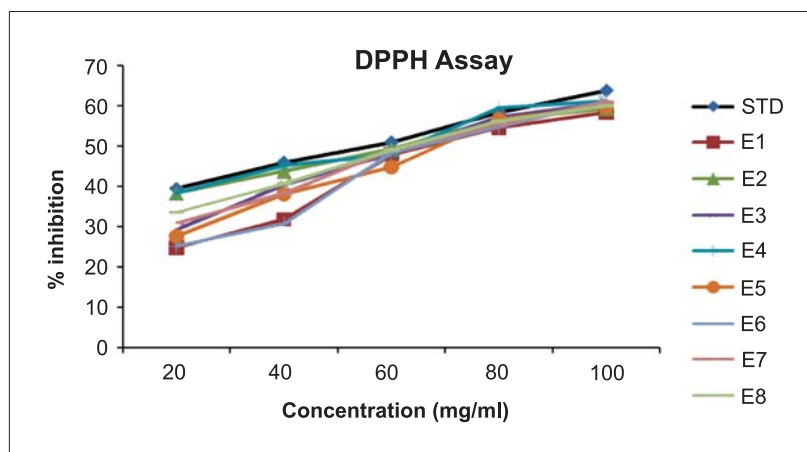


Fig. 2—Per cent inhibition vs concentration plot of different extracts of *B. retusa* bark by DPPH radical method

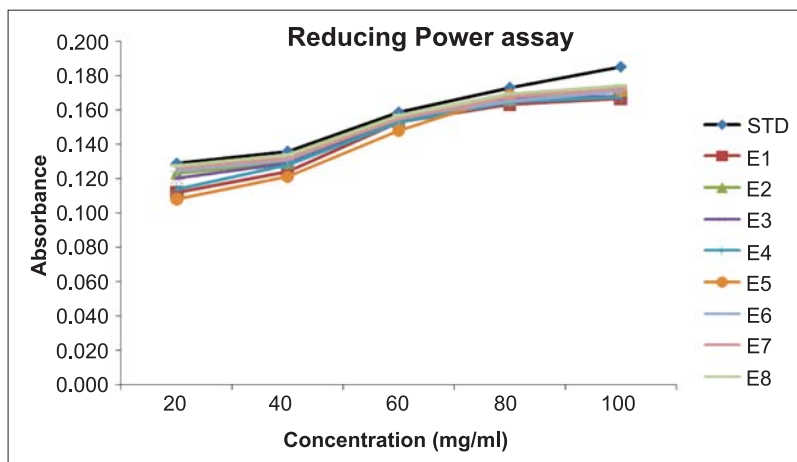


Fig. 3—Absorbance vs concentration plot of different extracts of *B. retusa* bark by reducing power assay

Table 2—Zone of inhibition of different extract of *B. retusa* bark

Extract/Standard (mg/ml)	Diameter of zone of inhibition in mm*			
	Gram +ve bacteria		Gram -ve bacteria	Fungus strains
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli.</i>	<i>C. albicans</i>
Water				
5	20.3±0.8	20±0.5	20.3±0.3	12±0.5
10	22±0.5	22±1.0	21±0.6	15±0.6
20	23.7±0.3	22±0.6	22.7±0.8	18±0.5
Methanol				
5	21±0.5	21.3±0.8	20.3±0.3	17±0.5
10	23±0.6	23±0.5	23.3±0.8	18±0.6
20	24±0.5	23±1.2	23.3±1.7	18.7±0.3
Ethanol				
5	20.3±1.2	15.3±0.8	22.3±1.5	15±0.6
10	22±0.5	19±0.6	20.7±1.2	18±0.5
20	23.3±0.3	20±0.5	24±0.6	16.7±0.7
50% Ethanol				
5	19±0.8	20.7±1.2	18±0.5	14±0.5
10	21.3±0.6	20±0.6	21±0.6	16±0.5
20	24±0.5	22.7±0.8	20.3±0.3	18±0.5
50% Methanol				
5	21±0.6	15±0.6	18.7±0.3	7.33±0.3
10	23±0.8	18±0.5	21.3±0.3	7.67±0.3
20	25.3±0.9	20.7±0.8	26±1.53	8.67±0.3
70% Acetone				
5	21±0.6	15±0.6	21.3±0.8	11.3±0.3
10	23.7±1.5	17±0.6	21±0.6	13.7±0.3
20	24.7±0.9	21.3±0.9	24±0.5	16±0.6
Standard (20µg/ml)	27.3±0.9	24.7±0.9	28.3±1.7	19±0.6

extracellular enzymes secreted. Tannins act as growth inhibitors towards many microorganisms including bacteria, yeasts and fungi by inhibiting the transport of nutrients into the cell and retard the growth of the organism. When tannins are complexed with

microbial proteins or polysaccharides, the interactions formed are often irreversible, and this characteristic confers bactericide and bacteriostatic properties. The all extracts exhibited prominent antimicrobial activity against all microorganism used in study (Table 2).

Table 3—Determination of MIC (mg/ml) of various extracts of *B. retusa* bark

Extracts (mg/ml)	Bacteria		Fungi	
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>A. niger</i>
Methanol	4	3.5	4	4
Aqueous	3	3	2.5	4
Ethanol	2.5	4.5	3.5	4
50% Ethanol	4	3.5	4	4
50% Methanol	3.5	4	4	4.5
70% Acetone	3	4	3.5	4.5
Standard	1.25	1.25	1.25	0.62

The strongest activity recorded against *Candida albicans* and lowest against *Bacillus subtilis*. MIC of all extract found to be less than 5 mg/ml (Table 3).

Conclusion

Extraction with different solvents affect yield of total polyphenol content, antioxidant and antimicrobial activity of *B. retusa*. Regardless of other solvent used, the most efficient solvent for polyphenolic extraction were acetone (70%) and ethanol (50%) while in water, absolute ethanol and methanol showed lowest polyphenolic content. Hence, acetone (70%) has proven to be most efficient solvent for extraction of phenolic constituents. Acetone (70%) also showed highest radical scavenging activity and reducing power. The existing data is not enough for explain mode of action for antioxidant but it may enrich the strength of comprehensive data of antioxidant activity of *B. retusa*. The antimicrobial activity shown by extracts may be because of presence of tannins or other constituents but all extracts were bacteriostatic against used microorganisms and have MIC less than 5 mg/ml.

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