Preliminary phytochemical screening and in vitro antibacterial activity of Bauhinia variegata Linn. against human pathogens

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Objective: To investigate the antimicrobial and phytochemical properties of hydromethanolic extracts of Bauhinia variegata Linn. (B. variegata) (leaf, stem bark and flower) to justify the traditional claim endowed upon this herbal drug as a rasayana in Ayurveda. This study thus can be further utilized to formulate the natural antioxidant which can be used as a dietary supplement to fight against several diseases such as cancer, ageing, artherosclerosis, etc.

Methods: The study showed that the number of different phytoconstituents present in the plant which makes it remarkable for its use by traditional practitioners. On the other set of experiment, the hydromethanolic extract of B. variegata (leaf, stem bark and flower) were evaluated against Gram-positive and Gram-negative by using disk diffusion assay.

Results: Phytochemical screening of all extracts showed the presence of alkaloids, steroids, phenolic compounds, tannins, saponins, carbohydrates, proteins, amino acids and organic acids. The antibacterial activity of all the extracts (leaf, stem bark and flower) of B. variegata was determined by agar well diffusion method at four different concentrations i.e., 1 000 mg/mL, 750 mg/mL, 500 mg/mL and 250 mg/mL using Gram-positive Bacillus subtilius, Staphylococcus aureus and Streptococcus epidermidis and Gram-negative Escherichia coli, Shigella flexineria, Pseudomonas aeruginosa bacteria.

Conclusions: These studies show that hydromethanolic extracts of B. variegata (leaf, stem bark and flower) inhibited the growth of microorganism’s in dose dependently. B. variegata leaf, stem bark and flower extracts have several phytochemical constituents who possess the antimicrobial activity. A tiny amount of data is presented, as the preliminary antimicrobial properties of the B. variegata here accessed, under the urgent necessity of new antibiotics in the market and in face of the increased resistance of infectious microorganisms to antimicrobials.

KEYWORDS
Phytochemical constituents, Antibacterial activity, Disc diffusion, Medicinal plants

1. Introduction

Nowadays, an increasing number of infectious agents are becoming more resistant to commercial antimicrobial compounds[1]. The necessity to develop new drugs requires varied strategies, the bioprospection of secondary metabolites produced by medicinal plants is one of them[2,3].

Antimicrobial agents are undeniably one of the most important therapeutic discoveries of the 20th century. However, with the ‘antibiotic era’ barely five decades old, mankind is now faced with the global problem of emerging resistance in virtually all pathogens[4]. During the last decades, there is an increasing interest to unlock the secrets of ancient herbal remedies. For
this purpose, various strategies have been developed e.g.,
biological screening, isolation as well as clinical trials for a
variety of plants[6].

In recent years, many possible sources of natural
antibiotics have been in use for several infectious diseases,
mostly bacterial and fungal. In view of this, the searches
for new anti–microbial agents from medicinal plants
are even more urgent in the countries like India where
infectious diseases of bacterial origin are not only rampant,
but the causative agents are also developing an increasing
resistance against many of the commonly used antibiotics[6].
Considering the high costs of the synthetic drugs and their
various side effects, the search for alternative products
from plants used in folklore medicine is further justified.
It is believed that plants which are rich in a wide variety
of secondary metabolites belonging to chemical classes
such as sterols, alkaloids, glycosides, saponins, flavonoids,
tannins, and carbohydrates are generally superior in their
antimicrobial activities[7]. For example, quinine (Cinchona)
and berberine (Berberis) are alkaloids obtained from
plants which are highly effective against microbes such as
Staphylococcus aureus (S. aureus), Escherichia coli (E. coli)[5].
Therefore, the determination of the compounds responsible
for any biological activity would facilitate the selection of the
plants for future investigations.

The potential for developing antimicrobials from higher
plants appears rewarding as it will lead to the development
of a phytomedicine to act against microbes. Plant–based
antimicrobials have enormous therapeutic potential as they
can serve the purpose with lesser side effects that are often
associated with synthetic antimicrobials. Continued further
exploration of plant–derived antimicrobials is needed today.
Further research is necessary to determine the identity of the
antibacterial compounds from within these plants and also
to determine their full spectrum of efficacy. However,
the present study of in vitro antimicrobial evaluation of some
plants forms a primary platform for further phytochemical
and pharmacological studies.

Bauhinia variegata Linn. (Leguminosae) (B. variegata),
is known as Kanchanar in Hindi, is a medium sized tree
abundant in Sub–Himalayan tract extending eastwards to
Assam, Eastern, Central and South India[8]. The various parts
of the plants viz., leaves, flower buds, flower, stem, stem
bark, seeds and roots are used in fever, as tonic, astringent,
diarrhoea, dysentery, hemorrhoids, piles, edema, laxative,
antihelmintic, antileprotic, in skin diseases, wound healing,
antigoitrogenic, antitumor, in obesity, stomatitis, antidote for
snake poisoning, dyspepsia, flatulence and as carminative[9].

The purpose of this study was to carry out preclinical
evaluation of some popular medicinal plant species, i.e.,
biological and phytochemical screening with particular
emphasis on those that seems to have very little scientific
information in the areas intended for the investigation.
This study facilitated the selection of plants with relatively
high level of potency and wide range of biological activities
suggesting that the strength of biological activities of a
natural product is dependent on the diversity and quantity
of such constituents. Therefore, simultaneous determination
of the compounds those are possibly responsible for any
biological activity would facilitate decision–making
process as in the selection of the plants for in–depth future
investigation.

The present study is therefore undertaken to study the
phytochemical and antibacterial screening of B. variegata
(leaf, stem bark, flower) which could be used as one of the
parameter for the standardization of the crude drug.

2. Material and methods

All the chemicals and solvents used in experiment were of
analytical grade.

2.1. Plant collection and identification

Fresh plant parts were obtained randomly from local
herbal botanical garden of Bhapal, Madhya Pradesh, India.
The taxonomic identities of the plant B. variegata were
confirmed by botanist Dr. S.S. Khan (Voucher Specimen
No: SP/101/LG0B/2006), Department of Botany, Safia Science
College, Bhopal, Madhya Pradesh, India. Fresh plant
materials were washed under running tap water, air dried
and then homogenized to fine powder and stored in airtight
bottles.

2.2. Preparation of extracts[10,11]

The dried plant material was pulverized into fine powder
using a grinder (mixer). About 50 g of powdered material was
extracted in separating funnel apparatus with 250 mL of 50%
methanol solvent. The extracts obtained with each solvent
were filtered through Whatman filter paper No. 1 and the
water bath mantle. The sticky greenish–brown substances
were obtained and stored in refrigerator for prior to use.

2.3. Protocol for measurement of preliminary phytochemical
screening[10,12-17]

Standard screening tests of methanolic extracts were
carried out for various plant constituents. The crude extracts
were screened for the presence or absence of secondary
metabolites such as alkaloids, steroidal compounds,
phenolic compounds, flavanoids, saponin, tannins using
standard procedures.

2.3.1. Test for carbohydrates and glycosides

2.3.1.1. Molish test

A total 2 mL of filtrate with two drops of alcoholic solution
of α-naphthol were added, the mixture was shaken well and
1 mL of concentrated sulphuric acid was added slowly along the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

2.3.1.2. Fehling test
One milliliter of filtrate was boiled on water bath with 1 mL each of Fehling solution A and B. Red precipitate in A indicates the presence of sugar.

2.3.2. Detection of glycosides
Borntrager’s test was used, 200 mg crude extract was mixed with 2 mL of dilute sulphuric acid and 2 mL of 5% aqueous ferric chloride solution, boiled for 5 min which led to oxidation to anthroquinones, indicating the presence of glycosides.

2.3.3. Test for proteins and amino acids

2.3.3.1. Biuret test
An aliquot of 2 mL of filtrate was treated with one drop of 2% copper sulphate solution. To this 1 mL of ethanol (90%) was added, followed by excess of potassium hydroxide pellets. Pink colour in ethanol layer indicated the presence of proteins.

2.3.3.2. Ninhydrin test
Two drops of ninhydrin solution were added to 1 mL of aqueous filtrate. A characteristic purple colour indicated the presence of amino acids.

2.3.4. Test for alkaloids

2.3.4.1. Mayer’s test
Crude extract was mixed with Mayer’s reagent (potassium mercuric iodide solution). Cream colour precipitate was formed, indicating the presence of alkaloids.

2.3.4.2. Dragendorff’s test
Crude extract was mixed with Dragendorff’s reagent (potassium bismuth iodide solution). Reddish brown precipitate was formed which suggested the presence of alkaloids.

2.3.5. Test for phytosterol
In chloroform test, 0.5 g of extract was treated with 10 mL chloroform and filtered. The filtrate was used to test the presence of phytosterols and triterpenoids. The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification has taken place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol. The residue was dissolved in few drops of dilute acetic acid, 3 mL of acetic anhydride followed by few drops of concentrated sulphuric acid. Appearance of bluish green color shows the presence of phytosterol.

2.3.6. Tests for steroidal compounds and triterpenoids

2.3.6.1. Salkowski’s test
Crude extract was mixed with few drops of acetic anhydride, boiled and cooled, conc. H₂SO₄, was then added from the sides of the test tube. A brown ring at the junction of two layers was formed. The upper layer turned green which showed the presence of steroids and formation of deep red colour indicated the presence of triterpenoids.

2.3.6.2. Lieberman’s test
Crude extract was mixed with chloroform and a few drops of conc. H₂SO₄, shaken well and allowed to stand for some time. Red color appeared at the lower layer indicated the presence of steroids and formation of yellow coloured layer indicated the presence of triterpenoids.

2.3.7. Tests for flavonoids

2.3.7.1. Tests for free flavonoids
A total 5 mL of ethyl acetate was added to a solution of 0.5 g of the extract in water. The mixture was shaken, allowed to settle, and inspected for the production of yellow colour in the organic layer, which is taken as positive for free flavonoids.

2.3.7.2. Lead acetate test
To a solution of 0.5 g extract in water, about 1 mL of 10% lead acetate solution was added. Production of yellow precipitate is considered as positive for flavonoids.

2.3.7.3. Reaction with sodium hydroxide
Dilute sodium hydroxide solution was added to a solution of 0.5 g of the extract in water. The mixture was inspected for the production of yellow colour which considered as positive test for flavonoids.

2.3.8. Tests for saponin
In the Froth test, 0.5 g extract were dissolved in 10 mL of distilled water for about 30 seconds. The test tube was stoppered and shaken vigorously for about 30 seconds. The test tube was allowed to stand in a vertical position and observed over 30 min. If a “honey comb” froth above the surface of liquid persists after 30 min, the sample is suspected to contain saponin.

2.3.9. Test for tannins

2.3.9.1. Ferric chloride test
A portion of the extracts were dissolved in water. The solution was clarified by filtration; 10% ferric chloride solution was added to the clear filtrate. This was observed for a change in colour to bluish black.
2.3.9.2. Formaldehyde test
To a solution of about 0.5 g extract in 5 mL water, three drops of formaldehyde and six drops of dilute hydrochloric acid were added. The resulting mixture was heated to boiling for 1 min and cooled. The precipitate formed (if any) was washed with hot water, warm alcohol, and warm 5% potassium hydroxide successively. A bulky precipitate, which leaves a coloured residue after washing, indicated the presence of phlobatannins.

2.3.9.3. Test for phlobatannins
Deposition of a red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

2.3.9.4. Modified iron complex test
To a solution of 0.5 g of the plant extract in 5 mm of water a drop of 33% acetic acid and 1 g sodium potassium tartarate was added. The mixture was warmed and filtered to remove any precipitate. A 0.25% solution of ferric ammonium citrate was added to the filtrate until no further intensification of colour is obtained and then boiled. Purple or blackish precipitates, which are insoluble dilute ammonia, denotes the presence of in hot water, alcohol, or dilute ammonia, denotes the presence of pyrogallol tannin.

2.3.10. Test for phenolic compound
Ferric ferrocynide reagent for phenolics test: 10% iron chloride (FeCl₃) (aq) was mixed with iron cyanide (FeCN₆) (1 g/100 mL or 0.1 g/10 mL), 0.1 g of ferric chloride and 0.1 g of potassium ferricyanide (K₃Fe(CN)₆) was freshly prepared by dissolving in 10 mL of distilled water. Equal portions of 1 and 2 was mixed, sprayed to the plates and heated at 110 °C. Change of colour to blue (instant) indicates the presence of phenolics.

2.3.11. Tests for fixed oils and fats

2.3.11.1. Stain test
The small quantity of crude extract was pressed between two filter papers; the stain on 1st filter paper indicated the presence of fixed oils.

2.3.11.2. Saponification test
In small quantity of crude extract few drop of 0.5 N of alcoholic potassium hydroxide were added to which a drop of phenolphthalein was added separately and heated in a water bath for 1 h. The formation of soap indicated the presence of fixed oils and fats.

2.4. Determination of antibacterial activity

2.4.1. Bacterial strains
The hydromethanolic extracts of leaves, stem bark and flower of B. variegata of 1000 mg/mL, 750 mg/mL, 500 mg/mL and 250 mg/mL concentrations were tested against gram positive Bacillus subtilis (B. subtilis) (ATCC 11778), S. aureus (ATCC 25923), Streptococcus epidermidis (S. epidermidis) (ATCC 24676) and Gram-negative E. coli (ATCC 25922), Shigella flexneri (S. flexneri) (ATCC 11435), Pseudomonas aeruginosa (P. aeruginosa) (ATCC 17440) for their antimicrobial activity. All the bacterial strains were obtained from National Chemical Laboratory, Pune, India. The bacteria were grown in the nutrient broth at 37 °C and maintained on nutrient agar slants at 4 °C.

2.4.2. Preparation of inoculums(Muller Hinton media)
One single colony of each type of microorganism (from the nutrient agar stock culture) was taken with a sterile loop, and was transferred into 10 mL sterile nutrient broth. The broth cultures were incubated in a shaking incubator at 37 °C for 16–20 h.

2.4.3. Antibacterial susceptibility test: disc diffusion assay
The antimicrobial activity of crude methanolic extracts of plants were initially assessed against the three tested microorganisms using the agar diffusion method as recommended by the Clinical Laboratory Institute. Nutrient agar medium was prepared by suspending nutrient agar stock culture (Oxoid). These were subsequently applied to the inoculated plates and then incubated 24 h at 37 °C. Antibacterial activity was indicated when clear inhibition zones were noted around the discs.

In small quantity of crude extract few drop of 0.5 N of alcoholic potassium hydroxide were added to which a drop of phenolphthalein was added separately and heated in a water bath for 1 h. The formation of soap indicated the presence of fixed oils and fats.

3. Results

The phytochemical tests revealed the presence of flavonoids, saponins, alkaloids, fatty acid, tannins glycosides in methanolic extract of B. variegata (Table 1).
Table 1

Phytochemical screening of solvent extracts of *B. variegata* L. (leaf, stem bark and flower).

| S. No. | Name of tests          | Tests/reagents | BVL | BVB | BVF |
|--------|------------------------|----------------|-----|-----|-----|
| 1      | Carbohydrates          | Fehlings test  | +   | +   | +   |
|        |                        | Molish test    | +   | -   | -   |
| 2      | Glycosides             | Borntrager’s   | +   | +   | +   |
| 3      | Alkaloids              | Dragendorff’s test | − | +   | +   |
|        |                        | Mayer’s test   | −   | +   | −   |
| 4      | Phytosterol            | chloroform     | −   | −   | −   |
| 5      | Steroidal compounds    | Salkowski’s test | − | −   | −   |
|        |                        | Lieberman’s test | − | −   | −   |
| 6      | Saponins               | Froth test     | +   | +   | +   |
| 7      | Tannins                | Ferric chloride test | + | +   | +   |
|        |                        | Formaldehyde test | + | +   | +   |
|        |                        | Test for phlobatanins | + | +   | +   |
| 8      | Fixed oils and fats    | Spot test      | +   | +   | +   |
| 9      | Flavonoids             | Test for free flavanos | + | +   | +   |
|        |                        | Lead acetate test | + | +   | +   |
|        |                        | Sodium hydroside | + | +   | +   |
| 10     | Phenolic compound      | Ferric chloride test | + | +   | +   |
| 11     | Protein and amino acid | Biuret test   | +   | +   | +   |
|        |                        | Ninhydrin test | +   | +   | +   |

+: presence, −: absence, BVL: *B. variegata* leaf, BVB: *B. variegata* stem bark, BVF: *B. variegata* flower

The antibacterial activity of *B. variegata* extract was assayed *in vitro* by agar disc diffusion method against Gram-positive and negative bacteria. Tables 2–6 summarize the microbial growth inhibition of hydromethanolic extracts of the screened different parts of *B. variegata*.

Table 5

Antibacterial of standard antibiotics to Gram–positive bacteria in different concentrations of antibiotic (5 μg/disc).

| Name of the organisms | Zone of inhibition in mm |
|-----------------------|--------------------------|
|                        | TE | OF | AZ | PC |
| *B. subtilis*         | 14 | 16 | 18 | 14 |
| *S. epidermidis*      | 16 | 18 | 17 | 17 |
| *S. aureus*           | 15 | 16 | 16 | 14 |

Table 6

Antibacterial of standard antibiotics to Gram–negative bacteria in different concentrations of antibiotic (5 μg/disc).

| Name of the organisms | Zone of inhibition in mm |
|-----------------------|--------------------------|
|                        | FU | GM | CX | NF |
| *E. coli*             | 12 | 16 | 8  | 16 |
| *P. aeruginosa*       | 14 | 13 | 18 | 20 |
| *S. flexneri*         | 18 | 18 | 12 | 21 |

3.1 Phytochemical screening

The phytochemical tests revealed the presence of flavonoids, saponins, alkaloids, fatty acid, tannins glycosides in methanol extract. The results of phytochemical screening are given in Table 1.

3.2 Antibacterial activity

The Tables 2–6 shows the zone of inhibition (in mm) values of *B. variegata* extracts of all tested microorganisms. All the
extracts of *B. variegata* showed considerable antibacterial activity at all the four concentrations 1000 mg/mL, 750 mg/mL, 500 mg/mL, 250 mg/mL. Tables 2–6 shows susceptibility pattern of the hydromethanolic extracts of *B. variegata* and standard antibiotic discs against some Gram–positive and Gram–negative bacteria studied. The results of antibacterial activity are reported in below:

### 4. Discussion

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay. Bacterial resistance to antibiotics has become a serious problem of public health that concerns almost all antibacterial agents and that manifests in all fields of their application. Novel antimicrobial compounds against new bacterial targets and drug resistance mechanisms are urgently needed. Plant derived antibacterials are always a source of novel therapeutics.

Medicinal herbs possess curative properties due to the presence of various complex chemical substance of different composition, which are found as secondary plant metabolites in one or more parts of these plants[19]. There is continuous and urgent need for discovery of new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because of alarming increase in the incidence of new and re-emerging infectious diseases[20].

Our earlier report on preliminary phytochemical studies of the partitioned portions showed the presence of anthraquinones derivatives, cardenolides and cardiac glycosides, flavonoids, resins, saponins and tannins. These are compounds that are known to have various sort of curative effects against most pathogenic organisms as reported by many researchers[21,22].

These principles have been known for many years to exhibit biological activity, such as effects on the central nervous system, and antibacterial, antitumour, and anthehelminctic activity have reported oils, alkaloids and anthraquinones associated with plants to have medicinal value[23]. Others are tritepenoids, which include: cardiac glycosides, sterols, saponins and tritepenes. Mode of action of compounds present in the extracts indicates that the extracts from these plants have the potential of solving the problem of multi–drug resistance.

Maximum activity was conferred against gram positive and negative bacteria for *B. variegata* stem bark and flower extracts as compared leave extract respectively. In the present era, plant and herb resources are abundant, but these resources are dwindling fast due to the onward march of civilization. Although a significant number of studies have been used to obtain purified plant chemical, very few screening programmers have been initiated on crude plant materials. It has also been widely observed and accepted that the medicinal value of plants lies in the bioactive phytocomponents present in the plants[24].

The phytochemical tests revealed the presence of flavonoids, saponins, alkaloids, fatty acid, tannins glycosides in methanol extract. These phytochemical constituents are good source of antimicrobial and antioxidant activity[25].

In conclusion, *B. variegata* extracts possess a broad spectrum of activity against a panel of bacteria responsible for the most common bacterial diseases. The preliminary results obtained in these experiments pave the road to explore the potential development of new compounds to be launched in the pharmaceutical market filling a tremendous gap, as day by day new multiresistant microorganisms emerges.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### Comments

#### Background

In recent years, many possible sources of natural antibiotics have been identified and in use for several infectious diseases, mostly bacterial and fungal, but as the diseases are growing and infectious agent changing the need of newer and natural antimicrobial agents has become ardently necessary. In view of this, the searches for new antimicrobial and phytochemical agents from medicinal plants are needed. It is believed that plants which are rich in a wide variety of secondary metabolites belonging to chemical classes are generally superior in their antimicrobial activities.

#### Research frontiers

The purpose of this study was to carry out preclinical evaluation of popular medicinal plant species, *i.e.*, biological and phytochemical screening with particular emphasis on those compounds of which very little scientific information is available on locale specific species, in the areas intended for the investigation. This study facilitated the selection of plants with relatively high level of potency of a natural product.
Related reports

Medicinal herbs possess curative properties due to the presence of various complex chemical substances of different composition, which are found as secondary plant metabolites in one or more parts of plants. In this context these plant compounds that are known to have various sorts of curative effects against most pathogenic organisms as reported by many researchers (Geidam et al., 2007).

Innovations & breakthroughs

The present study is the continuation of a program aimed at investigation of antimicrobial and phytochemical properties of *B. variegata* extract to justify the traditional claim endowed upon this herbal drug as a rasayana in Ayurveda. Data regarding the antimicrobial and phytochemical properties of the plant is very significant and has been reported to have high positive results in all groups.

Applications

The results of the present study further strengthen the claim as per Ayurved and suggest the antimicrobial and phytochemical properties of plant. A drug development programme should be undertaken to develop modern drugs with the compounds isolated from plant. Proper clinical applications may also be performed, so that the natural drug may be developed and thus can be used for the welfare of the mankind.

Peer review

It is a good study in which the author evaluated the antimicrobial and phytochemical properties of the well-known traditional plant that is found to have good antimicrobial activity. The result is very interesting and is significant in terms of qualitative authentication of the age-old claim. The result and such documentation are very important for natural drug development as well.

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