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In vitro Antimicrobial Properties and Phytochemical Evaluation of Mature Seed Extracts of *Wrightia tinctoria* R. Br.

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The numerous side effects associated with the use of allopathic drugs have led to renewed level of interest in Ayurvedic medicines. *Wrightia tinctoria* (Roxb.) R. Br., belonging to family Apocynaceae, is a deciduous flowering plant native to India and Burma. Its leaves, bark and seeds have been known to possess certain medicinal and curative properties. The present investigation focuses on *in vitro* antimicrobial properties and phytochemical analysis of aqueous and methanolic extracts of two different coloured mature seed varieties of *Wrightia tinctoria.* The phytochemical screening revealed the presence of carbohydrates, reducing sugars, alkaloids, sterols, glycosides, phenolics, tannins, flavonoids and amino acids. Greater effectivity was observed against gram positive bacterial pathogens such as *Staphylococcus aureus* ATCC 25923, *S. aureus, S. citreus* and *B. cereus* than the gram negative strains. The methanolic seed extracts were largely inhibitory against pathogenic yeasts like *Trichophyton rubrum, Candida albicans, C. parapsilosis* and *Cryptococcus.* The results indicated that the methanolic extract of the brown variety seeds is pharmacologically more active than that of the beige variety seeds. The aqueous extracts of both the seed varieties were moderately effective against *S. aureus* ATCC 25923 and *S. citreus,* with no effect against the fungal strains.

Key words: *Wrightia tinctoria*, antimicrobial, phytochemical analysis, methanolic extracts.

The scientific concept of medicine to alleviate illnesses and to attribute overall positive health, popularly known as 'Ayurveda', appeared and developed in the Indian subcontinent about four thousand years ago[1]. It offers a vast literature in Sanskrit covering all aspects of diseases, pharmacy and therapeutics[2]. The Indian subcontinent is blessed with an abundance of valuable medicinal plants that are used in traditional medical treatments[3]. Medicinal plants contain inherent bioactive ingredients used to cure diseases or relieve pain[4]. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic and/or analgesic effects of the phytochemicals in them[5, 6]. There has been an ever-increasing demand for the phytopharmaceutical products of Ayurveda in Western countries because of the side effects associated with the use of allopathic drugs[1].

In India, among 20,000 recorded medicinal plants about 7,000 - 7,500 have been used by the traditional communities for curing different diseases[7,8]. *Wrightia tinctoria* (Roxb.) R. Br., belonging to family Apocynaceae, is a small deciduous flowering plant with a light grey, scaly smooth bark, generally up to 1.8 m tall and often under 60 cm girth, sometimes up to 7.5 m high, distributed all over India and Burma[9]. It is commonly known as sweet Indrajao, Pala indigo plant or

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dyers's oleander. The raw leaves when chewed have been reported to relieve toothache. The bark and seeds have been found effective against psoriasis and non-specific dermatitis. The bark is also used as antidysenteric, antidiarrhoeal and antihaemorrhagic[10] and in the treatment of abdominal pain and skin diseases[11]. The immature seed pods of the plant contains wrightial, a triterpenoid chemical, along with cycloartenone, cycloeucalenol, β-amyrin and β-sitosterol[12].

Although there are many reports suggesting the usage and antimicrobial properties of the leaves and bark, very few reports are available on the antimicrobial activities of the mature seeds of this plant. Therefore, the present study focuses on *in vitro* antimicrobial activities of the seed extracts of *W. tinctoria* and its qualitative screening for detection of active phytochemical ingredients.

MATERIALS AND METHODS

Source of plant material

Seeds of two varieties of *W. tinctoria* were purchased from Amruta Kesari Depot, Bangalore, India.

Preparation of extracts

The seeds were finely powdered using an electric blender. Five grams of each powdered material was subjected to extraction with methanol and water separately. The extracts were centrifuged at 5000 rpm for 30 min at 4°C and evaporated to dryness under controlled temperature (35-40°C). The residue was reconstituted with 25 ml of methanol and water, respectively. The extracts were stored in air tight containers under refrigeration. These extracts were used for phytochemical analysis and antimicrobial studies.

Source of microorganisms

The test bacterial pathogens included *Staphylococcus aureus* ATCC 25923, *S. aureus, S. citreus, Bacillus cereus, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Serratia marcescens* and *Klebsiella pneumoniae.* The test fungal pathogens comprised of *Aspergillus niger, A. flavus, Trichoderma viride, Penicillium, Trichophyton rubrum, T. mentagrophytes, Candida albicans, C. parapsilosis* and *Cryptococcus. Staphylococcus aureus* ATCC 25923 and all the dermatophytic fungi were hospital isolates obtained from Kempegowda Institute of Medical Sciences, Bangalore. The remaining bacterial and fungal pathogens were obtained from the Department of Biotechnology, The Oxford College of Science, Bangalore.

Assay of antibacterial activity

Antibacterial activity of the extracts was studied by agar well diffusion method[13]. Test cultures of bacterial pathogens were prepared by transferring a loop full of bacteria from nutrient agar slants into Mueller Hinton broth and incubated at 37°C. Lawn cultures of the test pathogens were prepared by swabbing sterile Mueller Hinton agar plates with 24 hrs old bacterial broth. Wells were punched with a sterile cork borer (6 mm internal diameter) and 35 µl of the extracts was added to each well. Controls were maintained with respective solvents. Ampicillin and streptomycin (50 mg/ml) were used as standard antibiotics for gram positive and gram negative bacteria, respectively. Following incubation at 37°C for 24 hrs, diameters of the inhibitory zones were measured to the nearest millimeter.

Assay of antifungal activity

Antifungal activity of the extracts was studied by agar well diffusion method. Suspensions of fungal pathogens were prepared by transferring a loop full of fungi from Sabouraud Dextrose agar slants into Sabouraud Dextrose broth. Lawn cultures of the test pathogens were prepared by swabbing sterile Sabouraud Dextrose agar plates with the fungal suspensions. Wells were punched with a sterile cork borer (6 mm internal diameter) and 35 µl of the extract was added to each well. Controls were maintained with respective solvents. Fluconazole (20 mg/ml) was used as the standard antifungal. Following incubation at 27°C for 48 hrs, diameters of the inhibitory zones were measured to the nearest millimeter.

Phytochemical analysis of the seed extracts

Qualitative screening for the presence of various phytochemical compounds was performed using methanolic extracts. Presence of carbohydrates and reducing sugars was determined by Molish's test, Benedict's test and Fehling's test[14]. Presence of glycosides was detected by Borntrager's test[15]. Alkaloids in the extract was evaluated by Mayer's test. The presence of phytosterols was indicated by Salkowski's test. Deoxy sugars were detected by Killer Kiliani's test. The saponins were analyzed by Froth's test[16]. The

occurrence of phenolic compounds and tannins were confirmed by ferric chloride test and gelatin test, respectively[17]. The presence of flavonoids was investigated by lead acetate test. The occurrence of amino acids in the extracts was assessed by Ninhydrin's test[18], while the possibility of gums was studied by conducting borax test.

RESULTS AND DISCUSSION

The Ayurvedic system of medicine has witnessed worldwide acceptance and is nowadays practised not only in the Indian subcontinent but also in the developed countries of Europe, United States and Japan[1]. Presently, the main focus of pharmaceutical research is on the use of various medicinally important plants mentioned in Indian Ayurvedic medicine. Medicinal plants have always been the sources of biologically active compounds used for the treatment of various infectious diseases[7]. In this context, detailed research on the phytochemistry and pharmacology of traditionally valued plant products is essential and may lead to the discovery of new medicine of therapeutic importance.

Wrightia tinctoria R. Br. is a deciduous plant of pharmacological significance whose leaves, bark and seeds are used in traditional healing. It is found in arid, semi-arid and moist regions with a wide range of soil types. The bark is considered for antidiarrhoeal, aphrodisiac, antihelminthic, febrifuge, stomachic, toothache, tonic and dog bite[19, 20, 21]. It is also employed in seminal weakness and flatulence, in piles and skin diseases[21, 22]. The extract of fresh leaves is used in jaundice and is very effective in relieving toothache and psoriasis[23, 24]. Though several studies have been conducted on the leaves and bark of the plant, reports on the antimicrobial properties and phytochemical evaluation of the seed extracts are very few. The seeds of *Wrightia tinctoria* are linear, with pointed ends, light yellowish-grey, crowned with a tuft of white silky hairs. They are bitter, astringent and antihelminthic. The present investigation involves the study of two seed varieties of *Wrightia tinctoria*; one is dark brown while the other is pale yellow or beige in colour (Figure 1 and Figure 2). The dark variety is bitter whereas the beige variety is tasteless[25].

Antibacterial activity of the seed extracts

Aqueous and methanolic extracts of the seeds were prepared and tested against several gram positive and gram negative bacterial pathogens. The methanolic extracts of both the seed varieties were more effective against gram positive bacteria (*S. aureus* ATCC 25923, *S. aureus, S. citreus* and *B. cereus*) than the gram negative ones. The methanolic seed extracts of brown and beige varieties showed maximum inhibition against *Staphylococcus citreus* (21 mm and 27 mm, respectively). The methanolic brown seed extract was moderately effective against *E. coli*, *P. mirabilis* and *S. marcescens*, whereas, the methanolic beige seed extract inhibited only *E. coli* (10 mm). The aqueous seed extracts of brown and beige varieties inhibited *S. aureus* ATCC 25923 and *S. citreus*, respectively. This inhibitory effect of *Wrightia tinctoria* seed extracts, particularly against *Staphylococcus* sp., clearly emphasizes the fact that this plant is effective in psoriasis, nonspecific dermatitis, scalp and inflammatory skin disorders which are often caused by *S. aureus*. The significant results of the antibacterial activities of the seed extracts have been clearly presented in Table 1. In some previous studies, the methanolic bark extract of *Wrightia tinctoria* has been found to inhibit *Staphylococcus aureus, Bacillus sp., Micrococcus luteus, E. coli, Salmonella typhi, Pseudomonas aeruginosa*[26, 27]. Similar to this study, our results also indicated that the seed extracts did not inhibit *K. pneumoniae.* Other studies have reported inhibitory effects of leaf extracts against *S. aureus, B. cereus, P. aeruginosa, S. typhi* and *Streptococcus pyogenes*[28, 29, 30].

Antifungal activity of the seed extracts

The results of the antifungal study revealed that the methanolic mature seed extracts of *Wrightia tinctoria* effectively inhibited pathogenic yeasts and dermatophytes. Methanolic extracts of the brown and beige seeds showed significant zones of inhibition against *T. rubrum* (16 mm and 15 mm), *C. albicans* (11 mm and 10 mm) and *Cryptococcus* sp. (14 mm and 12 mm), respectively. Among the phytopathogenic molds the methanolic extracts inhibited only *Penicillium* sp., whereas, the aqueous extracts did not exhibit any antifungal properties. The significant results of the antifungal activities of the seed extracts have been presented in Table 2. Earlier studies have also

Table 1. *In vitro* antibacterial activities of the methanolic and aqueous extracts of *Wrightia tinctoria* seeds showing diameters of the inhibitory zones (in mm)

Bacterial pathogens	A1	A2	B1	B2	Antibiotic
S. aureus ATCC 25923	16 ± 0.17*	8 ± 0.21	6 ± 0.18	-	30 ± 0.20[a]
S. aureus	17 ± 0.14	-	11 ± 0.22	-	48 ± 0.13[a]
S. citreus	21 ± 0.13	-	27 ± 0.16	22 ± 0.08	40 ± 0.24[a]
B. cereus	10 ± 0.09	-	15 ± 0.11	-	18 ± 0.19[a]
E. coli	11 ± 0.22	-	10 ± 0.31	-	20 ± 0.11[s]
P. mirabilis	12 ± 0.16	-	-	-	20 ± 0.11[s]
P. aeruginosa	-	-	-	-	22 ± 0.29[s]
S. marcescens	7 ± 0.24	-	-	-	24 ± 0.13[s]
K. pneumoniae	-	-	-	-	18 ± 0.21[s]

A1, methanolic extract of brown seeds; A2, aqueous extract of brown seeds; B1, methanolic extract of beige seeds; B2, aqueous extract of beige seeds; *, mean of triplicate ± standard deviation; [a], ampicillin; [s], streptomycin; -, no zone

Table 2. *In vitro* antifungal activities of the methanolic and aqueous extracts of *Wrightia tinctoria* seeds showing diameters of the inhibitory zones (in mm).

Fungal pathogens	A1	A2	B1	B2	Fluconazole
A. niger	-	-	-	-	14 ± 0.34*
A. flavus	-	-	-	-	20 ± 0.26
T. viride	-	-	-	-	10 ± 0.41
Penicillium	8 ± 0.17	-	9 ± 0.42	-	19 ± 0.29
T. rubrum	16 ± 0.51	-	15 ± 0.28	-	18 ± 0.38
T. mentagrophytes	-	-	-	-	20 ± 0.15
C. albicans	11 ± 0.10	-	10 ± 0.34	-	20 ± 0.23
C. parapsilosis	10 ± 0.26	-	8 ± 0.21	-	35 ± 0.17
Cryptococcus	14 ± 0.57	-	12 ± 0.46	-	27 ± 0.13

A1, methanolic extract of brown seeds; A2, aqueous extract of brown seeds; B1, methanolic extract of beige seeds; B2, aqueous extract of beige seeds; *, mean of triplicate ± standard deviation; -, no zone

Table 3. Screening for the presence of various phytochemical compounds in the methanolic extracts of *Wrightia tinctoria* seeds

Phytochemical Tests	Compounds Detected	A1	B1
Molish's test	Carbohydrates	++	++
Benedict's test	Reducing sugar	+	+
Fehling's test	Reducing sugar	+	+
Mayer's test	Alkaloids	+	++
Salkowski's test	Sterols	+	+
Killer Kiliani's test	Deoxy sugars	++	+
Borntrager's test	Glycosides	+	+
Froth's test	Saponins	-	-
Ferric chloride test	Phenolic compounds	-	++
Gelatin test	Tannins	+	++
Lead acetate test	Flavonoids	+	++
Ninhydrin's test	Amino acids	+	++
Borax test	Gums	-	-

+, positive; ++, strongly positive; -, negative

reported the antifungal effects of *Wrightia tinctoria* woody stem extracts against *C. albicans, A. niger, T. viride*[27, 29]. These findings suggest that this plant extracts may be used in topical formulations against dermatophyte infections.

Phytochemical evaluation of the seed extracts

Medicinal plants are the sources of various phytopharmaceutical compounds. Different phytochemical tests were conducted with the methanolic extracts of mature seeds of *Wrightia tinctoria* since better antimicrobial properties have been found to be associated with the methanolic extracts than the aqueous extracts. Our study reveals the presence of carbohydrates, reducing sugars, deoxy sugars, alkaloids, sterols, glycosides, phenolic compounds, tannins, flavonoids and amino acids, as presented in Table 3. It was also interesting to note that the brown seeds showed higher phytochemical content than the beige seeds. The presence of carbohydrates,

Fig. 1. Mature whole seeds of *Wrightia tinctoria.* A, brown variety seeds; B, beige variety seeds

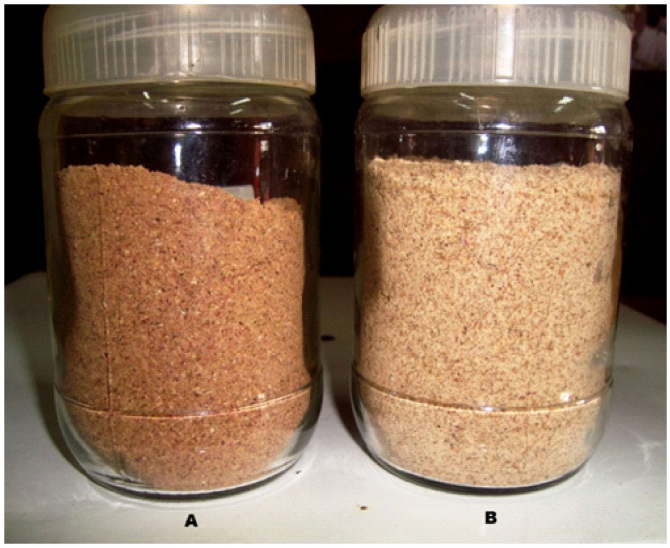

Fig. 2. Powdered seeds of *Wrightia tinctoria.* A, brown variety seed powder; B, beige variety seed powder

proteins, steroids, terpenoids, reducing sugars, flavonoids, phenols, alkaloids and tannins in the leaf, woody stem and bark extracts of *Wrightia tinctoria* has also been reported earlier [26, 28-31]. This occurrence of various phytochemicals in the mature seeds may be due to the plants natural defense mechanism to protect the seeds from microbial invasion in the environment and to keep them safe from rodents and nematodes. The antimicrobial action of the methanolic extracts of mature seeds may also be attributed to the presence of numerous bioactive compounds in them.

CONCLUSION

The methanolic extracts of mature seeds of *Wrightia tinctoria* have been found to possess good antibacterial and antifungal properties against gram positive bacterial pathogens and dermatophytic yeasts, respectively. The extracts are also rich in various phytochemical contents. Further studies are necessary to evaluate the safety of the mature seeds for pharmaceutical applications.

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