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Antimicrobial activity of *Abelmoschus moschatus* leaf extracts

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Abstract

Hexane, ethyl acetate, methanol and aqueous extracts prepared from the leaves of *Abelmoschus moschatus* were evaluated for their antimicrobial activity against a number of pathogens by using disc diffusion assay method. Clear zones of inhibition were reported for *Staphylococcus aureus, Bacillus megaterium, Shigella flexneri, Proteus mirabilis, Proteus vulgaris* and *Cornebacterium diphtheriae.* After performing a bioassay guided fractionation of the eight hexane fractions, it was revealed that the fraction exhibiting major antibacterial activity against *C. diphtheriae* contained terpenoid oil.

Key words: Antibiotic, bioactivity guided fractionation, crude extracts, disc diffusion assay.

Introduction

It is well known that infectious diseases are responsible for a high proportion of health problems, especially in developing countries. Microorganisms have developed resistance to many antibiotics due to their frequent use. The situation has created immense clinical problems for infectious disease treatment. More scientists are in search for new antimicrobial substances derived from plants. Historically, plants provide us with a good source of anti-infective agents. Emetine, quinine, berberine etc. remain highly effective drugs in the fight against microbial infections (1). In the traditional systems of medicine, plants are used in the form of crude extracts, infusions and powders to treat common infections without scientific evidence of efficacy (2). Therefore it is of great interest to screen these plants to validate their use in traditional medicine and to reveal active principles through isolation and characterization. Once their therapeutic action(s) is/are established, they may serve as an important source for designing new and more effective drugs for chemotherapy.

In the present study the antimicrobial properties of *A. moschatus* have been evaluated. *A. moschatus* is a known medicinal plant used for the cure of bacterial diseases in the Indian traditional system of medicine (3-4). The plant is used for the treatment of gonorrhea, leucoderma, diabetes, cramps, poor circulation, and aching joints (5). The microbes used in the present study for evaluation of antimicrobial activity of *A. moschatus* were *S. aureus (*causative agent of hospital acquired surgical wound infections*), B. megaterium* (causative agent of diseases like meningitis, endocarditis, conjunctivitis and acute gastroenteritis*), S. flexneri* (common pathogen for diarrhea*), Proteus species (*urinary tract and wound infections*), C. diphtheria* etc. The seeds of the plant are known for musk like essential oil. However, these are used as antiseptic, antispasmodic, opthalmic, diuretic, deodorant, and to sweeten the breath (6-

7). Major reports of isolation and identification of secondary metabolites come from the seeds of *A. moschatus* that include: ambrettolic acid, β-sitosterol, β-sitosterol-β-D-glycoside, farnesol, and furfural (8). A brief overview of the traditional medicinal use of *A. moschatus* indicates its effectiveness in the treatment of various bacteriological and fungal pathogenesis. *In vitro* activity of extracts prepared from the leaves were evaluated against a number of pathogenic microorganisms to validate and provide a scientific basis to support these claims.

Materials and methods

Plant material

A. moschatus was grown in the Green House of the School of Biotechnology, Devi Ahilya University, Indore. The plants were used for their supply of the leaves.

Bacterial strains

The test microorganisms included a number of clinical isolates listed in Table 1 procured from Choithram Hospital and Research centre, Indore.

Preparation of crude extracts

Leaves of *A. moschatus* were washed thoroughly under tap water and shade dried at room temperature (25-30^{0}C) until a constant weight was obtained. The dried leaves were pulverized with a mechanical grinder. Seventy grams of pulverized powder was defatted by soaking in 100 ml of hexane. The soaking sample was kept for 48 h on a rotary shaker at room temperature. Hexane was decanted every 48 h and fresh hexane was added. This was repeated until the sample was colorless. The hexane extracts were pooled and concentrated under vacuum until dry product was collected. The residue of the leaf powder left after hexane extraction was further extracted with solvents of increasing polarity such as ethyl acetate, methanol and water. The ethyl acetate, methanol, and aqueous extracts obtained were concentrated under vacuum and stored at 4^{0}C. The percent yield of each extract was calculated (9).

Disc diffusion assay for microbial sensitivity testing

Agar disc diffusion assay for screening the anti-bacterial potential of hexane, ethyl

Table 1. List of test pathogens along with the (+) ve controls used in the disc diffusion assay

Microorganism	Zone (in mm)	Amount (μg/disc)	(+)ve control (antibiotic)
Escherichia coli	17	10	Ampicillin
Escherichia coli	18	10	Ampicillin
Bacillus megaterium	17	50	Chloramphenicol
Bacillus subtilis	18	50	Chloramphenicol
Proteus mirabilis	16	50	Chloramphenicol
Proteus vulgaris	19	15	Erythromycin
Klebsiella pneumoniae	26	50	Chloramphenicol
Cornebacterium diphtheriae	24	50	Chloramphenicol
Candida albicans	24	50	Chloramphenicol
Pseudomonas aeruginosa	28	50	Chloramphenicol
Shigella flexneri	13	50	Chloramphenicol
Salmonella typhii	17	50	Chloramphenicol

acetate, methanol, and aqueous extracts of *A. moschatus* leaf powder was carried out as described by Murray et al. (10). The dried extracts were dissolved in their respective solvents to a final concentration of 0.1, 1 and 2 % and sterilized by filtration through a 0.45 µm membrane syringe filter. The sterile discs (6 mm in diameter) were soaked in their respective extracts for complete saturation. Afterwards, discs were completely dried to ensure complete evaporation of the solvent. The discs were stored under aseptic conditions. A 5µl of each bacterial strain was inoculated in 5 ml of Mueller Hinton broth (Meat infusion 2.0 g/l; casein hydrolyzate 17.5 g/l; starch 1.5 g/l) in a test tube and incubated at 37^0C for 24 h. Thereafter, Mueller Hinton Agar No. 2 (Mueller Hinton broth with agar-agar 13.0 g/l) was prepared and poured in sterile petri plates. The test strain 200 µl (inoculum size 10^8 cells/ml) was evenly spread on agar petri plates with a sterile glass spreader. Sterile discs impregnated with the respective extracts were transferred aseptically to the agar plates containing the inoculum. Seven discs were placed per plate. Each test was repeated three times with a positive control (antibiotic respective for each microorganism) and a negative control (solvent disc). After incubation at 37^0C for 24 h, the plates were examined for development of zones of inhibition. Microbial growth was determined by measuring the diameter of zone of inhibition in millimeter by the antibiotic zone scale.

Bioactivity guided fractionation of active crude extracts

Based on the results of bioassay and Thin Layer Chromatography (TLC) profile, the hexane extract of *A. moschatus* leaf powder was further fractionated by silica gel adsorption column chromatography. Silica gel is a 3–D polymer of tetrahedron groups of silicon oxide ($SiO_2.H_2O$) consisting of exposed silanol groups that act as active centers for formation of H–bonds with compounds being chromatographed. A thick wall glass column size 45 x 4.5 cm was packed with a suspension of silica gel (mesh size 60 -120; Merck) in hexane with a bed height of 20 –30 cm and 40-60 cm head space. A 100 g of silica / g of the crude extract was used for column packing. After equilibration, crude active hexane fraction was absorbed on minimum amount of silica and loaded on top of the column. Solvents of increasing polarity (mixture of hexane – ethyl acetate) were passed through the column for elution of the sample. A linear flow rate of 2 ml/ min was maintained and fractions of 30 ml were collected. The 30 ml fraction samples were then subjected to TLC. The identical fractions were pooled and concentrated to dryness under vacuum. The different fractions obtained through fractionation of *A. moschatus* hexane extract were subjected to disc diffusion assay for identification of bioactive fractions.

Results and Discussion

The percent yield of hexane, ethyl acetate, methanol and aqueous extracts of *A. moschatus* were found to be 4.4 %, 4.2 %, 14 % and 32.66 % respectively. TLC profile of the crude extracts indicated positive tests with Liebermann-Burchard reagent (LB reagent), Noller's reagent and tetranitromethane revealing presence of terpenoids. The samples were also positive for alkaloids and saponins as tested by standard phytochemical methods (11). These compounds have been isolated from numerous plants and are known to exhibit a broad spectrum of anti-microbial activities (12-15).

The results of the antimicrobial activities of the hexane, ethyl acetate, methanol and aqueous extracts of *A. moschatus* leaf powder extracts by the disc diffusion assay method indicated that they were active against a number of tested pathogenic microorganisms (Table 2). There was significant antimicrobial activity of the hexane extract against *C. diphtheriae,* the causative agent of diphtheria. A 2 %

Table 2. Anti-Bacterial disc diffusion assay of *A. moschatus* leaf extracts

Organisms	Hexane Extract (%)			Ethyl acetate extract (%)			Methanol extract (%)			Aqueous extract (%)		
	0.1	1	2	0.1	1	2	0.1	1	2	0.1	1	2
E. coli	-	T	-	-	T	T	-	-	-	-	-	T
S. aureus	-	T	T	-	T	-	-	10±0.2	10±0.2	-	-	-
B. megaterium	-	-	-	-	T	12± 0.5	-	-	-	-	-	-
B. subtilis	-	T	T	-	-	-	-	-	-	-	-	-
P. mirabilis	T	T	14±0.2	-	-	-	-	-	-	-	-	-
P. vulgaris	T	10±0.2	16±0.2	T	T	T	T	16±0.2	16±0.2	-	-	T
K. pneumoneae	-	-	-	-	-	-	T	T	T	T	T	T
C. diptheriae	-	-	19± 0.2	-	-	T	-	15±0.2	15±0.4	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-
S. flexneri	-	-	16±0.2	-	-	-	-	-	-	-	-	-
S.typhii	-	-	-	-	-		-	-	-	-	T	T

All the zones are in 'mm' and each value represents an average of three replications. T- Activity in traces (zone of inhibition < 10 mm); ± is the standard error.

concentration of the hexane extract showed a zone of inhibition of the size 19 mm. Hexane extract also inhibited the growth of *Proteus* species (*P. mirabilis* and *P. vulgaris*) and *Shigella flexneri*. The *P. mirabilis* has the ability to produce high levels of urease making the urine more alkaline. If left untreated, the increased alkalinity can lead to the formation of crystals of struvite, and calcium carbonate. *P. vulgaris* is known to cause urinary tract and wound infections. *S. flexneri* is common pathogen for diarrhea. The ethyl acetate extract at a concentration of 2 % exhibited activity only against *B. megaterium.* This bacterium has ability to form tough, protective endospore allowing the organism to tolerate extreme environmental conditions. It is the causative agent of diseases like meningitis, endocarditis, conjunctivitis and acute gastroenteritis in immuno-compromised patients.

Methanol extract exhibited significant activity against a number of bacteria tested, probably due to the presence of high amounts of flavonoids and alkaloids that are also known to possess antibacterial activity (16). A 1 % concentration of methanol extract inhibited growth of *S. aureus, P. vulgaris* and *C. diphtheria* (Figure 1). The *S. aureus is* a major cause of hospital acquired surgical wound infections. It is a leading cause of various ailments such as soft tissue infections, pneumonia, meningitis, boils, arthritis, osteomyelitis (chronic bone infection),

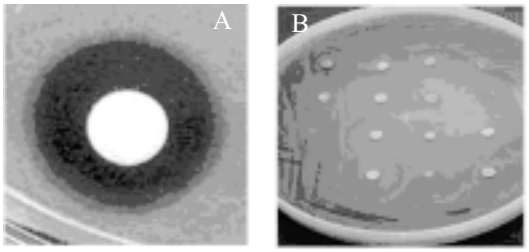

Fig.1 : Zone in inhibition obtained in anti-bacterial disc diffusion assay of A. moschatus extracts. A- Methanol extract against P. valgaris. B-Methanol extract against S. aureus

toxic shock syndrome (TSS) and scalded skin syndrome. Besides, it rapidly develops resistance to many antimicrobial agents (17). Both hexane and ethyl acetate extracts exhibited equal amount of activity against *P. vulgaris* (zone of inhibition 16 mm). Aqueous extract of *A. moschatus* did not exhibit activity against any of the tested microorganisms. Neither of the extracts was active against *Candida albicans, Pseudomonas aeruginosa* and *Salmonella typhii*. The microorganisms were least sensitive to the aqueous crude extracts due to negligible secondary metabolites in it.

Column chromatography of the bioactive hexane fraction of *A. moschatus* yielded 8 different fractions. Results of the bioassays of these fractions concluded that major activity was present in fractions 2-7 eluted by hexane containing 10-70 % ethyl acetate (Table 3). Negligible activity was demonstrated by fractions 1 and 8. The fraction 5 was found responsible for major inhibition of the growth of *C. diphtheriae*. The fractions 2 to 7 exhibited activity against *Proteus* species. The Fraction 7 exhibited strongest activity against *P. mirabilis* (zone of inhibition 28 mm) whereas all other fractions were equally effective against *P. vulgaris*. The results are very promising since *Proteus* species are responsible for 29% of all human urinary tract infections and hospital acquired wound infections. The *S. flexneri* active fractions were 3, 5 and 6. Shiga toxin produced by *Shigella* species is the cause of shigellosis (Bacillary dysentery). However, nearly 3% people with a certain genetic predisposition, namely HLA-B27 infected with *S. flexneri* subsequently develop Reiter's syndrome (pains in their joints, irritation of the eyes, and painful urination) which can last for years and can lead to chronic arthritis which is difficult to treat. The present findings may be exploited for a new drug against *Shigella* species.

The active column fractions resolved into a series of spots when TLC plates were observed under UV light and after spray detection. The fractions 1 to 5 were thick non volatile oils and developed blue green colored spots on spraying with LB reagent indicating presence of terpenoids. Noller's reagent also showed positive reaction with these fractions developing a range

Table 3. Anti-Bacterial disc diffusion assay of *A. moschatus* hexane extract and column fractions

Organism	Crude Hexane extract (%)			Hexane column Fractions (1%)							
	0.1	1	2	1	2	3	4	5	6	7	8
P. mirabilis	T	T	14±0.2	T	12±0.2	16±0.2	12±0.4	18±0.5	14±0.4	28±0.2	T
P. vulgaris	T	10±0.2	16±0.2	T	10±0.4	12±0.2	12±0.2	12±0.4	10±0.2	12±0.2	T
C. diphtheriae	-	-	19± 0.2	-	-	-	21±0.2	-	-	-	-
S. flexneri	-	-	16±0.2	-	T	16±0.2	T	12±0.4	12±0.4	-	T

All the zones are in 'mm' and each value represents an average of three replications. T- Activity in traces (zone of inhibition< 10 mm); ± is the standard error.

of colors from red, magenta to purple further confirming that all these oils are mixtures of terpenes mainly sesquiterpenes (18). Terpenes (oils) from various plants such as *Mentha piperita*, *Mentha spicata*, *Thymus vulgaris*, *Origanum vulgare*, *Origanum applii*, *Aloysia triphylla*, *Ocimum gratissimum*, *Ocimum basilicum* are shown to exhibit considerable inhibitory effects against *Pseudomonas aeruginosa, S. aureus* and many other pathogens (19-23). Efforts are in progress for complete characterization of the oils.

Increasing number of plants are being screened for their anti-microbial activity day by day. According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. *A. moschatus* shows anti-microbial activity against a wide variety of pathogens not a common characteristic as usually a single plant species does not show activity against not so many microbes tested (24-25).

Conclusion

The present study revealed the role of essential oil from leaf extracts of *A. moschatus* as a strong anti-bacterial agent against *Proteus* sp and *C. diphtheriae* under the laboratory conditions. It may be considered as a fruitful approach towards the search of new drugs. The overall results of the antimicrobial activity of the leaf extracts of *A. moschatus* justified the traditional uses of the plant and suggested that the indigenous traditional medicines could be used as a guide in the continuing search of new natural products with potential medicinal properties.

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